Modulation of the neuroinflammatory response after spinal cord injury

Citation for published version (APA):

van Neerven, S. G. A. (2011). *Modulation of the neuroinflammatory response after spinal cord injury*. [Doctoral Thesis, Maastricht University]. Maastricht University. <https://doi.org/10.26481/dis.20110420sn>

Document status and date:

Published: 01/01/2011

DOI:

[10.26481/dis.20110420sn](https://doi.org/10.26481/dis.20110420sn)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Modulation of the neuroinflammatory response after spinal cord injury

Sabien G.A. van Neerven

©Sabien G.A. van Neerven, Maastricht 2010

ISBN 978-90-9026008-2

Typesetting and cover design: Michal Slawinski

Printed in Poland

Modulation of the neuroinflammatory response after spinal cord injury

Proefschrift

Ter verkrijging van de graad van doctor aan de
Universiteit Maastricht

op gezag van Rector Magnificus

Prof. Mr. G. P. M. F. Mols

volgens het besluit van het College van Decanen

in het openbaar te verdedigen op

woensdag 20 April 2011 om 14.00 uur

door

Sabien. G.A. van Neerven

Geboren op 22 februari 1983 te Heerlen

Promotors

Prof. Dr. H.W.M. Steinbusch

Prof. Dr. M. van Kleef

Co-promotors

Dr. R. Deumens

Dr. J. Mey

Assessment Committee

Prof. Dr. M. de Baets

Prof. Dr. E. Hermans

Prof. Dr. C. Heijnen

Dr. G. Kenis

Prof. Dr. M.A.E. Marcus

*There has to be evil,
so that good can prove its purity above it.*

Buddha

The printing of this thesis was supported by
Noldus Information Technology

Noldus
Information Technology

Contents

Chapter 1	9 General introduction
Chapter 2	35 RAR/RXR and PPAR/RXR signalling in neurological and psychiatric diseases
Chapter 3	83 RAR/RXR and PPAR/RXR signalling in spinal cord injury
Chapter 4	109 Inflammatory chemokine release of astrocytes <i>in vitro</i> is reduced by all- <i>trans</i> retinoic acid
Chapter 5	141 Inflammatory cytokine release of astrocytes <i>in vitro</i> is reduced by all- <i>trans</i> retinoic acid
Chapter 6	171 Systemic but not local administration of retinoic acid reduces early transcript levels of pro-inflammatory cytokines after experimental spinal cord injury
Chapter 7	185 Liver X receptor activation does not attenuate early pro-inflammatory cytokine gene expression after experimental spinal cord injury
Chapter 8	199 Repetitive intrathecal VEGF165 treatment has limited therapeutic effects after spinal cord injury in the rat
Chapter 9	221 Discussion
	245 Summary
	249 Samenvatting
	253 Acknowledgments/Dankwoord
	261 Curriculum Vitae
	263 List of publications

General introduction

General introduction

1.1. Traumatic spinal cord injury

Traumatic impact to the spinal cord, which is commonly caused by falls, accidents or acts of violence, leads to the immediate destruction of spinal tissue. Spinal cord injury (SCI) patients not only experience the devastating consequences of immobility and sensory loss, approximately 50% of the SCI-patients develops central neuropathic pain (CNP), persistently occurring in remitting and relapsing episodes (Finnerup *et al.* 2001; Siddall *et al.* 2003; Cardenas *et al.* 2004). CNP severely impairs the quality of life of SCI-patients and can become so debilitating that depression and even suicide occurs (Cairns 1996; Felix 2007). SCI-patients that suffer from CNP experience spontaneous pain, which includes pain that is independent of a peripheral stimulus as well as evoked pain, where the perception of pain is induced by a peripheral stimulus. Evoked pain or hypersensitivity in CNP patients, which includes a condition known as “hyperalgesia”, where a painful stimulus is experienced as more painful, and “allodynia”, where a normally non-noxious stimulus is experienced as painful. CNP patients are reported to experience pain at 3 different levels relative to the site of injury, known as at-level pain, below-level pain and above-level pain (Siddall *et al.* 2003; Siddall & McClelland 1999; Siddall *et al.* 1999). CNP significantly impairs daily life and is, as peripheral neuropathic pain conditions, mostly resistant to currently used analgesics.

Approximately 12,000, 1,500, 15,200, 5,500 and 1,250 new cases of SCI are reported each year in the USA, Canada, Western Europe, Japan and Australia and New Zealand respectively (www.proneuron.com). Since SCI mostly affects young adult people, life-long treatment and medical care is needed for these patients causing a high social-economic burden in addition to the emotional and financial discomfort for affected individuals.

Up to date, there exists still no effective treatment for SCI, which makes this condition one of the most challenging research topics. The only recognized treatment of SCI patients involves stabilization of the spine and rehabilitation therapy. In some countries, the corticosteroid methylprednisolone is used in early treatment of SCI, but up to this day it remains controversial whether treatment with methylprednisolone improves long term functional outcome after SCI. Moreover, methylprednisolone treatment has been associated with adverse side effects. In view of SCI management it is therefore of vital importance to explore novel therapeutic approaches which render improved SCI outcome without adverse side effects. In order to gain insights into interesting therapeutic targets or medicinal agents, more insights into the pathophysiological processes following traumatic impact to the spinal cord are needed. This input mostly comes from studies using animal models.

1.2. Animal models of spinal cord injuries

SCI in humans can be categorized by the characteristics of the lesion, such as open and closed injuries or injuries with or without bony lesions. According to these criteria, SCI can be classified into 4 types including contusion/cyst, cord, maceration/massive compression, cord laceration, and solid cord injury (Bunge *et al.* 1993; Bunge *et al.* 1997; Silver & Miller 2004). Contusion injury is the most frequently reported type of SCI in humans. This clinical situation can be mimicked and studied in animal models, where the injury, as in humans, is characterized by a closed injury (intact meninges) with cyst formation and a rim of spared white matter that results in motor deficits and pain behaviour. Contusion injury can be inflicted several ways, including weight drop and is most frequently studied in rats and mice.

Different methods of behavioural testing have been developed to study motor performance and pain behaviour after SCI. Motor performance is often assessed by using the Basso Beattie and Bresnahan (BBB) locomotor scale, in which the animals are observed in an open field and scored for their motor performance on a 21-point scale. Within this scale, animals that score 21 points have excellent and coordinated motor performance, whereas animals which score 0 exhibit complete paralysis. Information about gross and subtle aspects of gait can be obtained by use of the Catwalk method (Fig. 1). In this behavioural test, rodents are trained to cross a glass runway which is internally illuminated by a luminescent tube. As soon as the glass plate is touched, the paw prints of the animals are highlighted. The light source is captured within the glass runway, but as soon as the glass plate is touched by the paws of the animals, these areas light up. As such the paw prints are visualized during gait. The illumination is captured with a camera,

Fig. 1. Catwalk™ gait analysis system provided by Noldus Information Technology BV: Catwalk method can be used to provide gross and detailed information about gait in rodents. (a) Overview of the Catwalk gait analysis system. (b) Above picture demonstrates the computer software walking pattern of the animal, including analysis of gait. Below picture demonstrates camera view of a rat crossing the glass plate. (c) Side view of the Catwalk gait analysis system, note the glass plate (yellow arrow) which is illuminated by a luminescence tube.

positioned underneath the glass runway and provides a detailed picture of the footprints during the run. As such, the walking pattern that is created, can give detailed information about motor performance.

Since many patients develop chronic pain conditions after SCI, much attention has been paid to the assessment of pain behaviour in animals. Pain behaviour in rodents is typically studied by evoking paw withdrawal responses to thermal and mechanical stimuli to study hypersensitivity to mechanical stimuli, or to thermal stimuli. Especially mechanical hypersensitivity (allodynia) is a severe problem in patients with chronic neuropathic pain, since even slight touch of the skin such as occurs when putting on clothes is experienced as painful in these patients. Mechanical hypersensitivity can be determined by the use of von Frey hairs (Von Frey test). These hairs are placed perpendicular onto the plantar surface of the animals paw. Subsequently the filaments are slightly bent to elicit a fixed force, depending on the strength and diameter of the filament. Typically a positive withdrawal response is characterized by a brisk withdrawal of the paw, postural changes, licking of the paw and sometimes vocalisations. The withdrawal threshold can be determined as the force that is needed to elicit a withdrawal of the paw of an animal. Animals that suffer from mechanical hypersensitivity show reduced withdrawal thresholds to mechanical stimuli. Meanwhile automated von Frey tests are introduced (Fig. 2a), where a gradually increasing force is applied to the animals paw until withdrawal occurs, providing the paw withdrawal threshold. Thermal hypersensitivity can be studied by the application of a heat source with defined intensity to the paw (Fig. 2b). This behavioural test described by Hargreaves and co-workers, determines withdrawal latencies to this thermal stimulus. Animals that are hypersensitive withdraw their paw after a shortened delay and accordingly, withdrawal latencies are lower when compared to normal animals.

Fig. 2. Behavioural test to assess hypersensitivity in rodents provided by Ugo Basil: (a) Automated von Frey to assess mechanical hypersensitivity. The filament on top of the measuring unit (yellow arrow) is placed perpendicular on the plantar surface of the rats paw. Increasing force is applied until withdrawal occurs. (b) Behavioural system to assess thermal hypersensitivity described by Hargreaves and co-workers. The measuring unit is placed underneath the animal (yellow arrow) from which an infrared light source is projected onto the plantar surface of the paw until withdrawal occurs.

In the studies described in this thesis, moderate contusion injury of the rat spinal cord was inflicted by weight drop via the NYU impactor of a 10 grams weight from 12.5 mm height, causing an injury that was characterized by cyst formation, complete grey matter loss, few spared white matter, and intact dura mater. With this type of contusion injury, animals develop motor deficits that can be scored with the BBB test demonstrating a typically behavioural pattern that exist of almost complete paralysis in the first few days after SCI (BBB score 0-3), followed by a gradual recovery to weight supported stepping (BBB score 11/12) and sometimes coordinated stepping (BBB > 11). Kloos and co-workers demonstrated that contusion injury of this intensity leads in approximately 40% of the animals to mechanical hypersensitivity. Additionally gradual increase of injury led to an augmented percentage of animals developing pain after SCI (Kloos *et al.* 2005).

1.3. Pathophysiology after traumatic spinal cord injury

The immediate tissue damage due to the physical impact on the spinal cord consists of necrosis of local cells (neurons and glia), injured neurites (axons and dendrites) and damage to blood vessels. As a consequence of cell death and hemorrhage, a wide variety of molecules are released into the spinal cord parenchyma, including adenosine triphosphate (ATP), the neurotransmitter glutamate, heat shock proteins (HSP) and fibrinogen from the blood (Schachtrup 2010), many of which trigger an inflammatory reaction resulting in secondary injury. Oxidative stress, excitotoxicity and cell death resulting from excessive levels of glutamate following impaired blood circulation, lead to the production of reactive oxygen species, lipid peroxidation, disturbances in intracellular Ca^{2+} homeostasis, damage to cell membranes, imbalance of electrolytes, formation of edema, production of prostaglandins and vasoactive substances and induction of apoptosis, processes which all potentiate the inflammatory reaction that follows SCI.

1.3.1. Activation of the immune system

SCI triggers the activation of the innate as well as the adaptive immune system. Innate immune cells present in the CNS (microglia or astrocytes) or derived from the blood (monocytes/macrophages and polymorphonuclear leukocytes such as neutrophils) respond very fast to the injury, yet their response is unspecific since the molecules, (reactive oxygen species like superoxides) released by these cells have general, but not specific toxic effects and cause collateral damage to surrounding tissue. At later time points this a-specific response is followed by the specific response of the adaptive immune system, which includes activation of lymphocytes and antibody production against e.g. myelin molecules.

Microglia which are classically known as the immune competent cells of the CNS, respond very quickly to the initial injury by changing their morphological phenotype. In normal,

physiological conditions, microglia are characterized by a ramified morphology with numerous long and slender processes that are used to survey the environment (Nimmerjahn *et al.* 2005; Davalos *et al.* 2008; Davalos *et al.* 2005). When activated, microglia adopt a morphological phenotype in which their processes are retracted, become amoeboid, hypertrophic, and cells become phagocytic. Moreover, microglial activation is characterized by proliferation. Upon activation, microglia produce a wide variety of inflammatory mediators which makes them essential players in the inflammatory reaction after SCI. Microglia activation peaks at approximately 12 h after SCI in rats and remains present up to weeks after injury (Popovich & Hickey 2001; Donnelly & Popovich 2008).

Astrocytes which are star-shaped glia cells found in the CNS, and under normal physiological conditions known for their supportive function. They provide structural and metabolic support to neurons, regulate neurotransmitter uptake and release, ion concentrations in the extracellular space, and are involved in the modulation of synapses and the vascular system. After a pathological event, astrocytes change their morphological phenotype and protein expression pattern characterized by increased expression of the intermediate filament GFAP and hypertrophied cell bodies. Upon activation, astrocytes produce inflammatory mediators and extracellular matrix molecules that are inhibitory to re-growing axons and form scar tissue. Increased GFAP staining and hypertrophy of cell bodies, can be detected as early as 3 days after injury (Popovich *et al.* 1997). However others already demonstrated astrocyte chemokine and cytokine production within hours after SCI, suggesting that astrocytes release inflammatory mediators before enhanced GFAP expression is detectable (Pineau *et al. in press*; Pineau & Lacroix 2007). Astrocyte activation is essential for the formation of the glial scar after SCI. This glial scar consists of a dense rim of predominately hypertrophied astrocytes that surround the lesion site. Besides the physical barrier the glial scar creates, the production of matrix molecules by astrocytes, such as chondroitin sulphate proteoglycans (CSPGs), inhibit re-growth of axons into the lesion area (Silver and Miller, 2004). In this way, the glial scar seals off the healthy spinal cord parenchyma from affected and inflamed tissue.

Neutrophils and macrophages are recruited from the blood. Neutrophils infiltrate the spinal cord tissue from a few hours after injury, peaking at 24 h and are gone after 3–4 days. Macrophages are recruited from the blood at later time points after SCI, starting at 2–3 days, peaking 7 days after injury (Popovich & Hickey 2001; Donnelly & Popovich 2008) and remaining in the spinal cord tissue up to weeks after SCI. Neutrophils and macrophages are mainly involved in the clean-up of cell debris, but via the production of ROS, proteases and cytokines, these cells can also induce further neuronal and glial cell damage.

Lymphocytes, are also recruited from the blood, but belong to the adaptive immune system. Lymphocytes infiltrate the spinal cord tissue from 24 h up to 7 days after injury and

return gradually back to normal levels at 4 to 5 weeks (Donnelly & Popovich 2008; Popovich *et al.* 1996b). Lymphocytes can be subdivided in B cells and T cells, of which B-cells are associated with antibody production against e.g. myelin whereas T-cells respond to tissue damage and myelin breakdown by production of cytotoxic chemicals, such as perforin and proteases. Although the inflammatory response after SCI is largely associated with detrimental effects on spinal tissue it has also been suggested to have beneficial effects. This dual character requires further explanation in the following section

1.3.2. Spread of inflammation into rostro-caudal direction

Spread of inflammation into rostro-caudal direction is a crucial aspect that determines long term outcome after SCI. Severity of injury is related to the spread of inflammation, which occurs in rostral and caudal direction. Additionally glial activation rostral and caudal to the lesion epicenter has been suggested to be involved in the development of above and below level pain after SCI. At the lesion epicenter, microglial activation was found at 12 h after SCI. At 12 h also in the white matter tracts of the fasciculus gracilis rostral to T8 and at the corticospinal tracts caudal to T8 microglia activation was visible. When compared to 12 h after SCI, at 3 days a stronger microglia reactivity was found rostral and caudal to the lesion (Popovich *et al.* 1997), which was also detectable at lumbar levels 5 weeks after SCI (Detloff *et al.* 2008). At the lesion site, predominantly astrocyte activation was observed, but remarkably at 7 to 28 days after SCI, astrocytes cluster around regions where reactive microglia were detected in rostral and caudal funiculi. At day 33 after SCI, at lumbar levels astrocyte demonstrated reactive morphology and distribution was uniform in white and grey matter (Hains & Waxman 2006). Additionally activated astrocytes surrounding the lesion site play an important role in the formation of the glial scar after SCI, as will be explained in more detail in the next paragraph.

1.4. Beneficial vs detrimental effects of immune system activation

Whether activation of the immune system is detrimental or beneficial after SCI is a question that is not easy to answer, since the inflammatory reaction has two faces: activated immune cells often have dual functions that can either induce repair mechanisms or exacerbate tissue destruction. The beneficial or detrimental effect of neuroinflammation obviously depends on the inflammatory mediators released during particular phases of inflammation following SCI.

1.4.1. Detrimental effects of inflammation

Cytokines are small molecules released by immune competent cells that activate other immune cells. Under physiological conditions, cytokines function as communication signals between cells, yet after a pathological event cytokine levels are elevated and potentiate the inflammatory reaction by activating other immune competent cells. Pro-inflammatory cytokines like IL-1 β , IL-6 and TNF α transcript levels peak already within 6 h after SCI (Streit *et al.* 1998; Donnelly & Popovich 2008; Pineau & Lacroix 2007). High levels of TNF α have been shown to be toxic to neurons and glia cells by inducing apoptotic pathways (Guicciardi & Gores 2009; Lee *et al.* 2000a; Hermann *et al.* 2001) whereas IL-1 β exacerbates ischemia induced neuronal damage (Hailer *et al.* 2005) and can be neurotoxic (Fogal & Hewett 2008; Fogal *et al.* 2007). Additionally TNF α has been shown to potentiate glutamate-mediated cell death after SCI (Hermann *et al.* 2001). Therefore inhibition of these early cytokines has been associated with neuroprotection after SCI (Genovese *et al.* 2006; Tuna *et al.* 2001).

Chemokines are small cytokines that chemotactically attract other immune cells, which are released quickly after SCI. Glial cells have been shown to produce chemokines that attract neutrophils and macrophages. mRNA production of the neutrophil attracting chemokine IP-10 (CXCL10), and macrophage chemo-attractants MCP-1 (CCL2) and MIP-1 α (CCL3) was already demonstrated as early as 1 h after spinal cord contusion injury (Lee *et al.* 2000b), peaking at 6 h and 24 h respectively (McTigue *et al.* 1998). Protein levels of MCP-1, and neutrophil chemoattractants MIP-2 (CXCL2) and KC (CXCL1) were already detected at 3 h and peaked at 12 h after spinal cord injury in mice (Pineau *et al. in press*). Inhibition of chemokines by infusion of a chemokine receptor antagonist has been shown to be neuroprotective after SCI, as evidenced by reduced infiltration of blood-born cells accompanied by increased axonal sparing and decreased myelin breakdown (Ghirnikar *et al.* 2001)

Reactive oxygen species (ROS) are under normal physiological conditions produced as a by-product of the respiratory chain in the mitochondria of cells. ROS are highly reactive oxygen containing molecules that cause damage to membranes, proteins, RNA and DNA. Internal antioxidant systems in the cell are able to scavenge these harmful molecules. After SCI, ROS are produced in the tissue itself when blood flow and oxygen supply is impaired (anoxia), as well as during reperfusion, when blood flow is restored. Also immune cells with phagocytic activity like microglia, macrophages and neutrophils produce ROS to destroy infected or damaged cells. Normally these ROS are scavenged by free radical scavengers and anti-oxidants, such as superoxide dismutase and vitamin C, but after SCI, the capacity of these systems are exceeded and ROS react with membrane molecules, DNA and RNA, causing lipid peroxidation and damage to the cell (Azbill *et al.* 1997).

ROS production starts within a few hours after injury, peaks at 24 h and returns gradually to normal levels at 3 – 4 weeks. ROS are potent activators of the intracellular second messenger pathways involving the transcription factor NF- κ B, which is a potent regulator of the expression of several inflammation-related genes including chemokines, cytokines, enzymes for prostaglandin synthesis (COX2) and nitric oxide production (NOS). NF- κ B activation has also been associated with the activation of apoptotic pathways (La Rosa *et al.* 2004).

Prostaglandins are produced from arachidonic acid (AA) which is cleaved from membrane lipids a process which compromises the first step in the prostaglandin synthesis pathway. Production of AA occurs after SCI as a result of the imbalance in Ca^{2+} signaling in cells that are under physiological stress. Elevated intracellular Ca^{2+} concentrations induce phospholipase A activation which results in the cleavage of membrane lipids into AA. Under inflammatory conditions, AA is then further converted into prostaglandins by the enzyme cyclooxygenase 2 (COX2) (Phillis *et al.* 2006). As cell damage and physiological stress occurs quickly after SCI, prostaglandins are rapidly released by neurons, glial cells and endothelial cells (Adachi *et al.* 2005; Resnick *et al.* 1998). They have been shown to induce neurotoxicity (Kawano *et al.* 2006), are vasoactive and are able to sensitize nociceptive neurons in the spinal cord which causes pain (Zeilhofer 2007; Zhao *et al.* 2007a). Inhibition of COX2 activation has been shown to reduce secondary injury after SCI (Resnick *et al.* 1998; Kwon *et al.* *in press*; Hains *et al.* 2001)

1.4.2. Beneficial effects of inflammation

Growth factors play an important role in neuronal differentiation, survival and path-finding. Early after SCI, they are predominantly produced by glial cells, whereas at later time points also macrophages and lymphocytes release a wide variety of growth factors including TGF β , NGF, BDNF and GDNF (Donnelly & Popovich 2008; McTigue *et al.* 2000; Lagord *et al.* 2002; Ikeda *et al.* 2001; Brown *et al.* 2004; Hashimoto *et al.* 2005). Elevated levels of BDNF, GDNF, NGF, and TGF β could already be observed in the first hours after SCI, increasing and peaking up to days after SCI (Hashimoto *et al.* 2005; Donnelly & Popovich 2008; Brown *et al.* 2004; Ikeda *et al.* 2001; McTigue *et al.* 2000). Endogenous production and exogenous application of growth factors after CNS injury has been associated with re-growth of axons, axonal sprouting, remodeling of the neurovascular system and tissue preservation (Houweling *et al.* 1998a; Houweling *et al.* 1998b; Grill *et al.* 1997; Vavrek *et al.* 2006; Sasaki *et al.* 2009; Brock *et al.* *in press*; Namiki *et al.* 2000). Recently the vascular endothelial cell growth factor (VEGF) gained scientific interest after CNS injury. This growth factor was originally described as a proliferation-inducing agent for endothelial cells in the periphery, however recently new roles of VEGF in the CNS were described; The VEGF gene contains a hypoxia response element (Semenza 2000). Astrocytes *in vitro* were shown to respond to VEGF treatment by proliferation (Mani *et al.* 2005, Rosenstein & Krum 2004). Furthermore VEGF and its re-

ceptors were up-regulated in neurons and astrocytes after brain injury (Kovacs *et al.* 1996; Marti *et al.* 2000). After SCI, VEGF was mainly detected in macrophages and microglia at the border of the lesion (Bartholdi *et al.* 1997). This typical expression pattern, suggest a role in the inflammatory reaction that may include repair mechanisms and neuroprotection after injury (Rosenstein & Krum 2004). Indeed, elevated VEGF levels either induced by exogenous application or induced by endogenous elevation via a viral construct, had neuroprotective effects and improved motor outcome after SCI (Widenfalk *et al.* 2003; Liu *et al.* 2010 in press). Endogenous VEGF may be produced by astrocytes and may stimulate angiogenesis, blood brain barrier repair, increase astrocyte proliferation in an autocrine fashion and induce the expression of other beneficial growth factors (Krum *et al.* 2008).

Blood spinal cord barrier repair: Repair of the blood spinal cord barrier (BSCB) is an important step to re-establish normal physiological balance in the injured spinal cord. After SCI the BSCB is maximally permeable at 24 h after SCI, followed by gradual re-establishment up to 14 days and closure at 3 weeks (Popovich *et al.* 1996a, Noble & Wrathall 1989, Whetstone *et al.* 2003). Whereas the mechanical impact onto the spinal cord disrupts the BSCB in the first place, at later time points vasoactive molecules released by immune cells play an important role in maintaining permeability. The cytokines IL-1 β and TNF α which are released early after SCI were shown to induce BSCB permeability (Schnell *et al.* 1999; Nish-ioku *et al.* in press). Other vasoactive substances including ROS, NO, elastase and histamine produced by astrocytes and leukocytes (Butt 1995; Nag *et al.* 2000; Sarker *et al.* 1998; Unterberg *et al.* 1988) may contribute to BBB permeability. Endogenous restructuring BSCB has been associated with restoring ion balance, reduction of infiltration of blood immune cells, improved tissue homeostasis and reduced oedema (Sharma 2005).

Glial scar formation: As in peripheral tissues, CNS injury is followed by formation of scar tissue. When the dura mater is still intact, like in a contusion injury, the scar tissue exists primarily of activated astrocytes (Silver & Miller 2004). After SCI when the BSCB is breached, astrocytes become hypertrophic, proliferate and start expressing intermediate filaments such as GFAP (Schachtrup *et al.* in press), a process known as reactive astrogliosis (Popovich *et al.* 1997). Indeed, areas of intensive glial scarring are also associated with increased BSCB permeability and macrophage activity (Silver & Miller 2004). The opinions about the functions of the glial scar are quite opposite: On the one hand glial scar formation is supposed to be detrimental to outcome, since it forms a physical barrier and production of extra cellular matrix molecules which impede re-growth of axons across the lesion site. On the other hand, beneficial effects of the glial scar have been claimed, since components of the scar are able to promote BSCB repair and inhibition of glial scar formation may induce spread of inflammation into rostro-caudal direction which may be associated with decreased tissue sparing and worsened functional outcome (Faulkner *et al.* 2004; Okada *et al.* 2006; Bush *et al.* 1999).

1.5. Long term effects of SCI inflammation: loss of motor and sensory function and chronic pain conditions

Loss of motor and sensory function after SCI is caused in first place by the direct damage to neurons and descending/ascending tracts. In the second phase after SCI, inflammation results in further demyelination and damage to axons. Tissue debris and its clearance by immune cells ultimately lead to cavitations. Re-growing attempts by severed axons eventually fail rendering a permanent status of impaired function. Demyelination starts immediately after the lesion, reaches a maximum at approximately 2 days, and then slowly diminishes at 7 days, to increase again after 4 weeks after the injury (Totou & Keirstead 2005). As monocytes and lymphocytes were shown to respond to myelin breakdown, their temporal activation and infiltration pattern into the spinal cord parenchyma coincides with the second wave of demyelination observed at 7 days after injury (Donnelly & Popovich 2008).

Development of CNP is a second major problem after SCI. Experimental models have shown that glial activation, via the release of neuroactive mediators (such as pro-inflammatory cytokines and PGE2) play an essential role in the sensitization of neuronal nociceptive networks in the dorsal horn of the spinal cord (Hains & Waxman 2006). In animal models increased tissue damage after SCI was correlated with augmented severity of pain after SCI (Kloos *et al.* 2005). The spread of inflammation in rostral-caudal direction has been presented as an important phenomenon in the development of neuropathic pain at levels other than the lesion site (above-level pain, below-level pain), a phenomenon that is frequently seen in spinal cord injured patients (Finnerup *et al.* 2001; Siddall *et al.* 2003).

Several lines of evidence support this concept: 1) Early inhibition (starting immediately after SCI and lasting for 1 week) as well as late treatment (single injection at 28 days after SCI) of SCI-induced glial activation using intrathecal propentofylline partially reduced established mechanical allodynia and thermal hypersensitivity in rats (Gwak & Hulsebosch 2009). Moreover inhibition of microglial activation immediately after SCI by minocycline was sufficient to reduce established SCI-induced pain symptoms in animals (Hains & Waxman 2006; Marchand *et al.* 2009; Tan *et al.* 2009). 2) Patch-clamp recordings of dorsal horn neurons demonstrated that inflammatory mediators such as cytokines and chemokines are able to modulate the frequency of spontaneous IPSPs and EPSPs. Additionally TNF α and IL-1 β enhanced excitatory AMPA- and NMDA-induced currents, whereas inhibitory GABA- and Glycin-induced currents were suppressed by IL-1 β and IL-6. Furthermore the chemokine CCL2 was able to reduce GABA-induced currents in spinal cord neurons. These data indicate that inflammatory mediators are able to modulate neuronal functioning directly (Gosselin *et al.* 2005; Kawasaki *et al.* 2008). 3) Intrathecal administration of cytokines, chemokines or prostaglandins to naïve rats has been shown to induce pain

behavior (Saito *et al.* 1995; Thacker *et al.* 2009; Zhao *et al.* 2007b; Dansereau *et al.* 2008), whereas inhibition of spinal cytokines or chemokines leads to a reduction of pain behavior in different pain models, including SCI (Milligan *et al.* 2005; Marchand *et al.* 2009; Sweitzer *et al.* 2001; Gao *et al.* 2009).

As cytokines levels peak early after SCI (Streit *et al.* 1998; Pineau & Lacroix 2007; Donnelly & Popovich 2008) the role of cytokines in pain after SCI was pointed out by a study where blocking of TNF α (starting immediately after SCI and lasting for 1 week) prevented the SCI-induced mechanical allodynia, whereas delayed treatment (starting at 14 dpo and lasting for 1 week) did not reverse established mechanical allodynia (Marchand *et al.* 2009).

These data point out that cytokine release after SCI may play an important role in the development chronic pain conditions and that inhibition of these early inflammatory mediators can be of therapeutical benefit to reduce chronic pain after SCI.

1.6. Ways to interfere with SCI-induced inflammatory reaction

As previous described the cellular and molecular processes that occur during the inflammatory reaction following SCI have their own specific temporal expression patterns. Despite the complexity of the inflammatory reaction and the many processes that occur simultaneously, intervention with detrimental events such as early cytokine production, gliosis, pro-inflammatory signaling and extravasation of blood borne cells, may be promising strategies to attenuate secondary injury after SCI. Inhibition of these early detrimental events has been attempted in SCI-patients via the use of corticosteroids. The reason why there is a need for alternatives will be discussed into more detail in the following section.

1.6.1. Interference with pro-inflammatory signaling

Stabilization of the spine when needed and rehabilitation therapy belongs to the standard care after SCI. Besides these invasive methods, in some countries glucocorticoid treatment with methylprednisolone (MP) is also used. According to the National Acute Spinal Cord Injury Study 2 (NASCIS 2) protocol, MP is typically administered within 8 h after SCI in a high bolus injection of 30 mg/kg, followed by 23 h infusion of 5.4 mg/kg/h (Bracken *et al.* 1990). MP is the only recognized therapeutically pharmacological treatment of SCI injury patients, yet its use has been shown to be controversial. Increased incidence of severe adverse side effects, such as pulmonary embolism, increased risk of wound infection, severe sepsis, gastrointestinal hemorrhage, or pneumonia have been reported in MP-treated patients (Bracken *et al.* 1990; Gerndt *et al.* 1997; Qian *et al.* 2005, Suberviola *et al.* 2008; Bracken *et al.* 1997), whereas data on improved long term functional outcome are not conclusive (Bracken *et al.* 1984; Bracken 1990).

MP binds to the glucocorticoid receptor, which belongs to the family of ligand activated nuclear hormone receptors (NHR) including androgen, estrogen and progesterone receptors, but also thyroid hormone receptor (THR), liver X receptor (LXR), proliferator activated receptor (PPAR) and the retinoid receptors (RAR and RXR). Via the classical activation pathway, these receptors upon ligand binding translocate into the cell nucleus and become a transcriptional active unit. This ligand-receptor complex binds to specific sequences in the DNA in the promoter of target genes, where it starts gene transcription, a mechanism known as transactivation. However, the anti-inflammatory effects of MP are suggested to be mediated by a different mechanism which is known as transrepression. This mechanism involves interference with pro-inflammatory transcription factor signaling (such as NF- κ B) which is upstream of several inflammatory mediators including COX2, chemokines and cytokines. In contrary, the adverse side effects are thought to be mediated by glucocorticoid target gene induction mainly concerning genes that are involved in metabolic processes.

In the cerebrospinal fluid of SCI patients that received MP, several cytokines and chemokines were found to be reduced when compared to non-treated patients (Tsai *et al.* 2008). Experimentally, data on MP use reported only small improvements of motor outcome (Weaver *et al.* 2005) and tissue preservation (Chvatal *et al.* 2008; Merola *et al.* 2002; Takami *et al.* 2002) after SCI, whereas treatment had no effect on at-level or below-level mechanical allodynia (Weaver *et al.* 2005). Together with the minor functional improvement in humans and risk of adverse side effects, these studies point out the importance of exploring new possible anti-inflammatory therapies that are more effective and have less adverse side effects.

Other members of the family of NHRs are now explored for their anti-inflammatory properties in experimental models, including SCI. For instance, Tamoxifen an estrogen receptor antagonist has been shown to be neuroprotective after experimental stroke (Kimelberg 2008). Immediately after SCI, Tamoxifen treatment reduced cytokine levels, apoptosis and myelin loss, which was accompanied with improved motor function, suggesting a detrimental role for estrogens after SCI (Tian *et al.* 2009). In contrary, also neuroprotective effects of estrogen treatment have been reported after SCI including improved motor outcome and tissue sparing (Sribnick *et al. in press*; Sribnick *et al.* 2005; Chen *et al. in press*; Cuzzocrea *et al.* 2008; Ritz & Hausmann 2008; Sribnick *et al.* 2010 *in press*). Immediate treatment with progesterone reduced neuronal loss and induced the differentiation of immature oligodendrocytes suggesting a role in re-myelinating processes (Labombarda *et al.* 2010 *in press*; Labombarda *et al.* 2009; Gonzalez *et al.* 2009).

Other members of the NHR family, the PPARs, have been shown to be anti-inflammatory in a wide variety of inflammatory models, including SCI. The PPAR γ agonists pioglitazone and rosiglitazone, drugs that were originally designed for the treatment of diabetes mellitus, reduced lesion size, attenuated inflammatory mediated cytokine expression, reduced hypersensitivity and improved motor performance after SCI (McTigue

et al. 2007; Park *et al.* 2007). Also the LXR ligand T0901317 was able to reduce cytokine levels after SCI, which was accompanied with improved motor performance and increased tissue preservation (Paterniti *et al.* *in press*). These studies suggest that targeting nuclear hormone receptors may be also neuroprotective and inflammatory reducing in human SCI. As such, PPAR agonists have already been clinically tested in other diseases with inflammatory pathology, such as Alzheimer's disease, rheumatoid arthritis, and ischemia reperfusion injury (<http://clinicaltrials.gov>), but not in SCI yet.

1.7. Routes of application: adverse side effects vs therapeutical benefit

Besides understanding the cellular and molecular events following SCI, which is important for correct timing of drug application. Knowledge of the drugs properties, such as risk of side effects, is of vital importance for adequate treatment. The route of administration of drugs largely determines the adverse side effects. Systemic side effects can be avoided by local application, rather than systemic administration. Hence after experimental SCI, local drug delivery is often applied via catheters which deliver drugs into the intrathecal space. Systemically drugs are often applied via intraperitoneal, intravenous or subcutaneous injections and involve uptake and transport via the blood stream and passage of the liver. The route of application also determines the effectiveness of a drug. It is therefore necessary to test whether the route of application of a drug differentially influences the effectiveness.

1.8. Understanding the working mechanisms of anti-inflammatory substances

Knowledge of drugs' properties also includes understanding of working mechanisms of such drugs. For instance, the anti-inflammatory effect of methylprednisolone is supposed to occur via affecting transcription by interference with pro-inflammatory transcription factors, thereby reducing transcript levels of inflammatory mediators. In order to achieve synergistic effects, drugs that interfere with transcription may be combined with drugs that have post-transcriptional effects. Also combinations of drugs that interfere with inflammatory events in a temporal fashion may provide synergistic effects. Knowledge of molecular mechanisms of action of the drugs applied as well of the inflammatory events that occur after SCI is therefore required

All together this overview demonstrates that there are many possible strategies to modulate inflammation after SCI. Until now, only MP is used in clinical setting to attenuate inflammation after SCI in humans. Since the inflammatory reaction is a complex cascade of beneficial and detrimental events following their very own expression pattern, it may be important to combine strategies. Combinations of therapies can be made by timing, like for instance drugs that affect different processes in time, or to combine

therapies based on different mechanisms of action, such as drugs that work on the level of transcriptional inhibition combined with drugs that work on protein level. Additionally the route of drug administration provides further possibilities to optimize treatment. Local application may improve efficacy of a drug, as well reduce the risk of adverse side effects, which often occur with systemic applications. Eventually these strategies should improve functional outcome and reduce chronic pain after SCI.

Research questions

The aim of this thesis was to study novel therapeutic approaches to modulate the inflammatory reaction following experimental SCI leading to improved motor function and reduced pain.

As a first strategy, treatments to reduce detrimental effects of the inflammatory reaction were analyzed. As early pro-inflammatory cytokine production after SCI is essential for the initiation and maintenance of the inflammatory reaction, the cytokine transcript reducing effect of the nuclear hormone receptor ligands retinoic acid (RA) and T0901317 (T09) was investigated.

As a second strategy, boosting of beneficial effects of the inflammatory reaction was studied, via exogenous application of the growth factor VEGF after SCI.

Chapters 2 and 3 include reviews on two members of the nuclear hormone receptor family, PPAR and RAR, and their anti-inflammatory effect in different animal models of neurological disorders and neuroinflammation, including SCI. The research question addressed in these two literature overviews can therefore be formulated as:

1. Does activation of nuclear hormone receptors PPAR or RAR have anti-inflammatory effects in models of neuroinflammation?

Chapters 4 and 5 involve *in vitro* studies of primary astrocyte cultures stimulated with the bacterial product lipopolysaccharides (LPS) which elicits an inflammatory response. These chapters demonstrate that the nuclear hormone receptor ligand RA, was able to reduce transcript as well as protein levels of cytokines and chemokines in these LPS-challenged primary astrocytes

As such the following research question has been addressed in these two chapters:

2. Is RA able to attenuate LPS induced cytokine and chemokine expression in primary astrocyte cultures?

Chapter 6 includes the *in vivo* application of RA. As the route of application of a drug, largely determines the efficacy as well as the risk of adverse side effect. Two different routes of RA application after SCI were investigated, where transcript levels of early cytokines was used as a measure. In this study the following research question was formulated:

3. Does the route of administration of RA differentially affect mRNA transcription of pro-inflammatory cytokines at 6 h after experimental SCI?

Chapter 7 describes the potential cytokine reducing effect of another member of the nuclear hormone receptor family, the LXR. Therefore the LXR ligand T09 was investigated for its early cytokine transcript reducing effect after SCI, to answer the following research question:

4. Does LXR activation reduce pro-inflammatory cytokine gene transcription, peaking at 6 h after experimental SCI?

Chapter 8 includes the study describing the effect of VEGF treatment on motor outcome and pain behavior after SCI. In the previous chapters modulation of the inflammatory reaction after SCI was achieved via inhibition of pro-inflammatory cytokine transcription, whereas in this study stimulation of beneficial effects was performed via the application of VEGF,

5. Does early repetitive VEGF treatment after SCI result in decreased hypersensitivity, increased motor function and improved tissue preservation?

References

- Adachi, K., Yimin, Y., Satake, K., Matsuyama, Y., Ishiguro, N., Sawada, M., Hirata, Y. and Kiuchi, K. (2005) Localization of cyclooxygenase-2 induced following traumatic spinal cord injury. *Neurosci Res*, **51**, 73–80
- Azbill, R. D., Mu, X., Bruce-Keller, A. J., Mattson, M. P. and Springer, J. E. (1997) Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. *Brain Res*, **765**, 283–290
- Bartholdi, D., Rubin, B. P. and Schwab, M. E. (1997) VEGF mRNA induction correlates with changes in the vascular architecture upon spinal cord damage in the rat. *Eur J Neurosci*, **9**, 2549–2560
- Bracken, M. B. (1990) Methylprednisolone in the management of acute spinal cord injuries. *Med J Aust*, **153**, 368
- Bracken, M. B., Collins, W. F., Freeman, D. F. *et al.* (1984) Efficacy of methylprednisolone in acute spinal cord injury. *JAMA*, **251**, 45–52
- Bracken, M. B., Shepard, M. J., Collins, W. F. *et al.* (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med*, **322**, 1405–1411
- Bracken, M. B., Shepard, M. J., Holford, T. R. *et al.* (1997) Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA*, **277**, 1597–1604
- Brock, J. h., Rosenzweig, E. S., Blesch, A., Moseanko, R., Havton, L. A., Edgerton, V. R. and Tuszynski, M. h. (in press) Local and remote growth factor effects after primate spinal cord injury. *J Neurosci*, **30**, 9728–9737
- Brown, A., Ricci, M. J. and Weaver, L. C. (2004) NGF message and protein distribution in the injured rat spinal cord. *Exp Neurol*, **188**, 115–127
- Bunge, R. P., Puckett, W. R., Becerra, J. L., Marcillo, A. and Quencer, R. M. (1993) Observations on the pathology of human spinal cord injury. A review and classification of 22 new cases with details from a case of chronic cord compression with extensive focal demyelination. *Adv Neurol*, **59**, 75–89
- Bunge, R. P., Puckett, W. R. and Hiester, E. D. (1997) Observations on the pathology of several types of human spinal cord injury, with emphasis on the astrocyte response to penetrating injuries. *Adv Neurol*, **72**, 305–315
- Bush, T. G., Puvanachandra, N., Horner, C. h., Polito, A., Ostefeld, T., Svendsen, C. N., Mucke, L., Johnson, M. h. and Sofroniew, M. V. (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron*, **23**, 297–308
- Butt, A. M. (1995) Effect of inflammatory agents on electrical resistance across the blood-brain barrier in pial microvessels of anaesthetized rats. *Brain Res*, **696**, 145–150
- Cardenas, D. D., Hoffman, J. M., Kirshblum, S. and McKinley, W. (2004) Etiology and incidence of rehospitalization after traumatic spinal cord injury: a multicenter analysis. *Arch Phys Med Rehabil*, **85**, 1757–1763

- Chen, S. h., Yeh, C. h., Lin, M. Y., Kang, C. Y., Chu, C. C., Chang, F. M. and Wang, J. J. (*in press*) Premarin improves outcomes of spinal cord injury in male rats through stimulating both angiogenesis and neurogenesis. *Crit Care Med*, **38**, 2043–2051
- Chvatal, S. A., Kim, Y. T., Bratt-Leal, A. M., Lee, h. and Bellamkonda, R. V. (2008) Spatial distribution and acute anti-inflammatory effects of Methylprednisolone after sustained local delivery to the contused spinal cord. *Biomaterials*, **29**, 1967–1975
- Cuzzocrea, S., Genovese, T., Mazzon, E. *et al.* (2008) Effect of 17beta-estradiol on signal transduction pathways and secondary damage in experimental spinal cord trauma. *Shock*, **29**, 362–371
- Dansereau, M. A., Gosselin, R. D., Pohl, M. *et al.* (2008) Spinal CCL2 pronociceptive action is no longer effective in CCR2 receptor antagonist-treated rats. *J Neurochem*, **106**, 757–769
- Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L. and Gan, W. B. (2005) ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat Neurosci*, **8**, 752–758
- Davalos, D., Lee, J. K., Smith, W. B., Brinkman, B., Ellisman, M. h., Zheng, B. and Akassoglou, K. (2008) Stable *in vivo* imaging of densely populated glia, axons and blood vessels in the mouse spinal cord using two-photon microscopy. *J Neurosci Methods*, **169**, 1–7
- Detloff, M. R., Fisher, L. C., McGaughy, V., Longbrake, E. E., Popovich, P. G. and Basso, D. M. (2008) Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp Neurol*, **212**, 337–347
- Donnelly, D. J. and Popovich, P. G. (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol*, **209**, 378–388
- Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B. and Sofroniew, M. V. (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*, **24**, 2143–2155
- Finnerup, N. B., Johannesen, I. L., Sindrup, S. h., Bach, F. W. and Jensen, T. S. (2001) Pain and dysesthesia in patients with spinal cord injury: A postal survey. *Spinal Cord*, **39**, 256–262
- Fogal, B. and Hewett, S. J. (2008) Interleukin-1beta: a bridge between inflammation and excitotoxicity? *J Neurochem*, **106**, 1–23
- Fogal, B., Li, J., Lobner, D., McCullough, L. D. and Hewett, S. J. (2007) System x(c)- activity and astrocytes are necessary for interleukin-1 beta-mediated hypoxic neuronal injury. *J Neurosci*, **27**, 10094–10105
- Gao, Y. J., Zhang, L., Samad, O. A. *et al.* (2009) JNK-induced MCP-1 production in spinal cord astrocytes contributes to central sensitization and neuropathic pain. *J Neurosci*, **29**, 4096–4108
- Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Muia, C., Bramanti, P. and Cuzzocrea, S. (2006) Immunomodulatory effects of etanercept in an experimental model of spinal cord injury. *J Pharmacol Exp Ther*, **316**, 1006–1016
- Gerndt, S. J., Rodriguez, J. L., Pawlik, J. W., Taheri, P. A., Wahl, W. L., Micheals, A. J. and Papadopoulos, S. M. (1997) Consequences of high-dose steroid therapy for acute spinal cord injury. *J Trauma*, **42**, 279–284
- Ghirnikar, R. S., Lee, Y. L. and Eng, L. F. (2001) Chemokine antagonist infusion promotes axonal sparing after spinal cord contusion injury in rat. *J Neurosci Res*, **64**, 582–589
- Gonzalez, S. L., Lopez-Costa, J. J., Labombarda, F., Deniselle, M. C., Guennoun, R., Schumacher, M. and De Nicola, A. F. (2009) Progesterone effects on neuronal ultrastructure and expression of microtubule-associated protein 2 (MAP2) in rats with acute spinal cord injury. *Cell Mol Neurobiol*, **29**, 27–39

- Gosselin, R. D., Varela, C., Banisadr, G., Mechighel, P., Rostene, W., Kitabgi, P. and Melik-Parsadaniantz, S. (2005) Constitutive expression of CCR2 chemokine receptor and inhibition by MCP-1/CCL2 of GABA-induced currents in spinal cord neurones. *J Neurochem*, **95**, 1023–1034
- Grill, R. J., Blesch, A. and Tuszynski, M. h. (1997) Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells. *Exp Neurol*, **148**, 444–452
- Guicciardi, M. E. and Gores, G. J. (2009) Life and death by death receptors. *FASEB J*, **23**, 1625–1637
- Gwak, Y. S. and Hulsebosch, C. E. (2009) Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. *Neuroscience*, **161**, 895–903
- Hailer, N. P., Vogt, C., Korf, h. W. and Dehghani, F. (2005) Interleukin-1beta exacerbates and interleukin-1 receptor antagonist attenuates neuronal injury and microglial activation after excitotoxic damage in organotypic hippocampal slice cultures. *Eur J Neurosci*, **21**, 2347–2360
- Hains, B. C. and Waxman, S. G. (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci*, **26**, 4308–4317
- Hains, B. C., Yucra, J. A. and Hulsebosch, C. E. (2001) Reduction of pathological and behavioral deficits following spinal cord contusion injury with the selective cyclooxygenase-2 inhibitor NS-398. *J Neurotrauma*, **18**, 409–423
- Hashimoto, M., Nitta, A., Fukumitsu, h., Nomoto, h., Shen, L. and Furukawa, S. (2005) Inflammation-induced GDNF improves locomotor function after spinal cord injury. *Neuroreport*, **16**, 99–102
- Hermann, G. E., Rogers, R. C., Bresnahan, J. C. and Beattie, M. S. (2001) Tumor necrosis factor-alpha induces cFOS and strongly potentiates glutamate-mediated cell death in the rat spinal cord. *Neurobiol Dis*, **8**, 590–599
- Houweling, D. A., Lankhorst, A. J., Gispen, W. h., Bar, P. R. and Joosten, E. A. (1998a) Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery. *Exp Neurol*, **153**, 49–59
- Houweling, D. A., van Asseldonk, J. T., Lankhorst, A. J., Hamers, F. P., Martin, D., Bar, P. R. and Joosten, E. A. (1998b) Local application of collagen containing brain-derived neurotrophic factor decreases the loss of function after spinal cord injury in the adult rat. *Neurosci Lett*, **251**, 193–196
- Ikeda, O., Murakami, M., Ino, h., Yamazaki, M., Nemoto, T., Koda, M., Nakayama, C. and Moriya, h. (2001) Acute up-regulation of brain-derived neurotrophic factor expression resulting from experimentally induced injury in the rat spinal cord. *Acta Neuropathol*, **102**, 239–245
- Kawano, T., Anrather, J., Zhou, P. et al. (2006) Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nat Med*, **12**, 225–229
- Kawasaki, Y., Zhang, L., Cheng, J. K. and Ji, R. R. (2008) Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord. *J Neurosci*, **28**, 5189–5194
- Kloos, A. D., Fisher, L. C., Detloff, M. R., Hassenzahl, D. L. and Basso, D. M. (2005) Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. *Exp Neurol*, **191**, 251–265

- Kovacs, Z., Ikezaki, K., Samoto, K., Inamura, T. and Fukui, M. (1996) VEGF and flt. Expression time kinetics in rat brain infarct. *Stroke*, **27**, 1865–1872; discussion 1872–1863
- Krum, J. M., Mani, N. and Rosenstein, J. M. (2008) Roles of the endogenous VEGF receptors flt-1 and flk-1 in astroglial and vascular remodeling after brain injury. *Exp Neurol*, **212**, 108–117
- Kwon, B. K., Okon, E., Hillyer, J., Mann, C., Baptiste, D., Weaver, L. C., Fehlings, M. G. and Tetzlaff, W. (in press) A Systematic Review of Non-Invasive Pharmacologic Neuroprotective Treatments for Acute Spinal Cord Injury. *J Neurotrauma*
- La Rosa, G., Cardali, S., Genovese, T., Conti, A., Di Paola, R., La Torre, D., Cacciola, F. and Cuzzocrea, S. (2004) Inhibition of the nuclear factor-kappaB activation with pyrrolidine dithiocarbamate attenuating inflammation and oxidative stress after experimental spinal cord trauma in rats. *J Neurosurg Spine*, **1**, 311–321
- Labombarda, F., Gonzalez Deniselle, M. C., De Nicola, A. F. and Gonzalez, S. L. (2010 in press) Progesterone and the spinal cord: good friends in bad times. *Neuroimmunomodulation*, **17**, 146–149
- Labombarda, F., Gonzalez, S. L., Lima, A., Roig, P., Guennoun, R., Schumacher, M. and de Nicola, A. F. (2009) Effects of progesterone on oligodendrocyte progenitors, oligodendrocyte transcription factors, and myelin proteins following spinal cord injury. *Glia*, **57**, 884–897
- Lagord, C., Berry, M. and Logan, A. (2002) Expression of TGFbeta2 but not TGFbeta1 correlates with the deposition of scar tissue in the lesioned spinal cord. *Mol Cell Neurosci*, **20**, 69–92
- Lee, Y. B., Yune, T. Y., Baik, S. Y. et al. (2000a) Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury. *Exp Neurol*, **166**, 190–195
- Lee, Y. L., Shih, K., Bao, P., Ghirnikar, R. S. and Eng, L. F. (2000b) Cytokine chemokine expression in contused rat spinal cord. *Neurochem Int*, **36**, 417–425
- Liu, Y., Figley, S., Spratt, S. K., Lee, G., Ando, D., Surosky, R. and Fehlings, M. G. (2010 in press) An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. *Neurobiol Dis*, **37**, 384–393
- Mani, N., Khaibullina, A., Krum, J. M. and Rosenstein, J. M. (2005) Astrocyte growth effects of vascular endothelial growth factor (VEGF) application to perinatal neocortical explants: receptor mediation and signal transduction pathways. *Exp Neurol*, **192**, 394–406
- Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A. K., Bradbury, E. J. and McMahon, S. B. (2009) Effects of Etanercept and minocycline in a rat model of spinal cord injury. *Eur J Pain*, **13**, 673–681
- Marti, h. J., Bernaudin, M., Bellail, A., Schoch, h., Euler, M., Petit, E. and Risau, W. (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *Am J Pathol*, **156**, 965–976
- McTigue, D. M., Popovich, P. G., Morgan, T. E. and Stokes, B. T. (2000) Localization of transforming growth factor-beta1 and receptor mRNA after experimental spinal cord injury. *Exp Neurol*, **163**, 220–230
- McTigue, D. M., Tani, M., Krivacic, K., Chernosky, A., Kelner, G. S., Maciejewski, D., Maki, R., Ransohoff, R. M. and Stokes, B. T. (1998) Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury. *J Neurosci Res*, **53**, 368–376
- McTigue, D. M., Tripathi, R., Wei, P. and Lash, A. T. (2007) The PPAR gamma agonist Pioglitazone improves anatomical and locomotor recovery after rodent spinal cord injury. *Exp Neurol*, **205**, 396–406

- Merola, A., O'Brien, M. F., Castro, B. A., Smith, D. A., Eule, J. M., Lowe, T. G., Dwyer, A. P., Maher, T. R. and Espat, N. J. (2002) Histologic characterization of acute spinal cord injury treated with intravenous methylprednisolone. *J Orthop Trauma*, **16**, 155–161
- Milligan, E., Zapata, V., Schoeniger, D., Chacur, M., Green, P., Poole, S., Martin, D., Maier, S. F. and Watkins, L. R. (2005) An initial investigation of spinal mechanisms underlying pain enhancement induced by fractalkine, a neuronally released chemokine. *Eur J Neurosci*, **22**, 2775–2782
- Nag, S., Picard, P. and Stewart, D. J. (2000) Increased immunolocalization of nitric oxide synthases during blood-brain barrier breakdown and cerebral edema. *Acta Neurochir Suppl*, **76**, 65–68
- Namiki, J., Kojima, A. and Tator, C. h. (2000) Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats. *J Neurotrauma*, **17**, 1219–1231
- Nimmerjahn, A., Kirchhoff, F. and Helmchen, F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma *in vivo*. *Science*, **308**, 1314–1318
- Nishioku, T., Matsumoto, J., Dohgu, S., Sumi, N., Miyao, K., Takata, F., Shuto, h., Yamauchi, A. and Kataoka, Y. (*in press*) Tumor necrosis factor-alpha mediates the blood-brain barrier dysfunction induced by activated microglia in mouse brain microvascular endothelial cells. *J Pharmacol Sci*, **112**, 251–254
- Noble, L. J. and Wrathall, J. R. (1989) Distribution and time course of protein extravasation in the rat spinal cord after contusive injury. *Brain Res*, **482**, 57–66
- Okada, S., Nakamura, M., Katoh, h. *et al.* (2006) Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*, **12**, 829–834
- Park, S. W., Yi, J. h., Miranpuri, G., Satriotomo, I., Bowen, K., Resnick, D. K. and Vemuganti, R. (2007) Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther*, **320**, 1002–1012
- Paterniti, I., Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Galuppo, M., Bramanti, P. and Cuzzocrea, S. (*in press*) Liver X receptor agonist treatment regulates inflammatory response after spinal cord trauma. *J Neurochem*, **112**, 611–624
- Phillis, J. W., Horrocks, L. A. and Farooqui, A. A. (2006) Cyclooxygenases, lipoxygenases, and epoxygenases in CNS: their role and involvement in neurological disorders. *Brain Res Rev*, **52**, 201–243
- Pineau, I. and Lacroix, S. (2007) Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J Comp Neurol*, **500**, 267–285
- Pineau, I., Sun, L., Bastien, D. and Lacroix, S. (*in press*) Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion. *Brain Behav Immun*, **24**, 540–553
- Popovich, P. G. and Hickey, W. F. (2001) Bone marrow chimeric rats reveal the unique distribution of resident and recruited macrophages in the contused rat spinal cord. *J Neuropathol Exp Neurol*, **60**, 676–685
- Popovich, P. G., Horner, P. J., Mullin, B. B. and Stokes, B. T. (1996a) A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury. *Exp Neurol*, **142**, 258–275

- Popovich, P. G., Stokes, B. T. and Whitacre, C. C. (1996b) Concept of autoimmunity following spinal cord injury: possible roles for T lymphocytes in the traumatized central nervous system. *J Neurosci Res*, **45**, 349–363
- Popovich, P. G., Wei, P. and Stokes, B. T. (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J Comp Neurol*, **377**, 443–464
- Qian, T., Guo, X., Levi, A. D., Vanni, S., Shebert, R. T. and Sipski, M. L. (2005) High-dose methylprednisolone may cause myopathy in acute spinal cord injury patients. *Spinal Cord*, **43**, 199–203
- Resnick, D. K., Graham, S. h., Dixon, C. E. and Marion, D. W. (1998) Role of cyclooxygenase 2 in acute spinal cord injury. *J Neurotrauma*, **15**, 1005–1013
- Ritz, M. F. and Hausmann, O. N. (2008) Effect of 17beta-estradiol on functional outcome, release of cytokines, astrocyte reactivity and inflammatory spreading after spinal cord injury in male rats. *Brain Res*, **1203**, 177–188
- Rosenstein, J. M. and Krum, J. M. (2004) New roles for VEGF in nervous tissue—beyond blood vessels. *Exp Neurol*, **187**, 246–253
- Saito, Y., Kaneko, M., Kirihaara, Y., Kosaka, Y. and Collins, J. G. (1995) Intrathecal prostaglandin E1 produces a long-lasting allodynic state. *Pain*, **63**, 303–311
- Sarker, M. h., Easton, A. S. and Fraser, P. A. (1998) Regulation of cerebral microvascular permeability by histamine in the anaesthetized rat. *J Physiol*, **507 (Pt 3)**, 909–918
- Sasaki, M., Radtke, C., Tan, A. M., Zhao, P., Hamada, h., Houkin, K., Honmou, O. and Kocsis, J. D. (2009) BDNF-hypersecreting human mesenchymal stem cells promote functional recovery, axonal sprouting, and protection of corticospinal neurons after spinal cord injury. *J Neurosci*, **29**, 14932–14941
- Schachtrup, C., Ryu, J. K., Helmrick, M. J., Vagena, E., Galanakis, D. K., Degen, J. L., Margolis, R. U. and Akassoglou, K. (*in press*) Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J Neurosci*, **30**, 5843–5854
- Schnell, L., Fearn, S., Klassen, h., Schwab, M. E. and Perry, V. h. (1999) Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord. *Eur J Neurosci*, **11**, 3648–3658
- Semenza, G. L. (2000) HIF-1: using two hands to flip the angiogenic switch. *Cancer Metastasis Rev*, **19**, 59–65
- Sharma, h. S. (2005) Pathophysiology of blood-spinal cord barrier in traumatic injury and repair. *Curr Pharm Des*, **11**, 1353–1389
- Siddall, P. J. and McClelland, J. (1999) Non-painful sensory phenomena after spinal cord injury. *J Neurol Neurosurg Psychiatry*, **66**, 617–622
- Siddall, P. J., McClelland, J. M., Rutkowski, S. B. and Cousins, M. J. (2003) A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury. *Pain*, **103**, 249–257
- Siddall, P. J., Taylor, D. A., McClelland, J. M., Rutkowski, S. B. and Cousins, M. J. (1999) Pain report and the relationship of pain to physical factors in the first 6 months following spinal cord injury. *Pain*, **81**, 187–197
- Silver, J. and Miller, J. h. (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci*, **5**, 146–156

- Sribnick, E. A., Samantaray, S., Das, A., Smith, J., Matzelle, D. D., Ray, S. K. and Banik, N. L. (2010 *in press*) Postinjury estrogen treatment of chronic spinal cord injury improves locomotor function in rats. *J Neurosci Res*, **88**, 1738–1750
- Sribnick, E. A., Samantaray, S., Das, A., Smith, J., Matzelle, D. D., Ray, S. K. and Banik, N. L. (*in press*) Postinjury estrogen treatment of chronic spinal cord injury improves locomotor function in rats. *J Neurosci Res*, **88**, 1738–1750
- Sribnick, E. A., Wingrave, J. M., Matzelle, D. D., Wilford, G. G., Ray, S. K. and Banik, N. L. (2005) Estrogen attenuated markers of inflammation and decreased lesion volume in acute spinal cord injury in rats. *J Neurosci Res*, **82**, 283–293
- Streit, W. J., Semple-Rowland, S. L., Hurley, S. D., Miller, R. C., Popovich, P. G. and Stokes, B. T. (1998) Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. *Exp Neurol*, **152**, 74–87
- Suberviola, B., Gonzalez-Castro, A., Llorca, J., Ortiz-Melon, F. and minambres, E. (2008) Early complications of high-dose methylprednisolone in acute spinal cord injury patients. *Injury*, **39**, 748–752
- Sweitzer, S., Martin, D. and DeLeo, J. A. (2001) Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience*, **103**, 529–539
- Takami, T., Oudega, M., Bethea, J. R., Wood, P. M., Kleitman, N. and Bunge, M. B. (2002) Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome. *J Neurotrauma*, **19**, 653–666
- Tan, A. M., Zhao, P., Waxman, S. G. and Hains, B. C. (2009) Early microglial inhibition preemptively mitigates chronic pain development after experimental spinal cord injury. *J Rehabil Res Dev*, **46**, 123–133
- Thacker, M. A., Clark, A. K., Bishop, T., Grist, J., Yip, P. K., Moon, L. D., Thompson, S. W., Marchand, F. and McMahon, S. B. (2009) CCL2 is a key mediator of microglia activation in neuropathic pain states. *Eur J Pain*, **13**, 263–272
- Tian, D. S., Liu, J. L., Xie, M. J., Zhan, Y., Qu, W. S., Yu, Z. Y., Tang, Z. P., Pan, D. J. and Wang, W. (2009) Tamoxifen attenuates inflammatory-mediated damage and improves functional outcome after spinal cord injury in rats. *J Neurochem*, **109**, 1658–1667
- Totoiu, M. O. and Keirstead, h. S. (2005) Spinal cord injury is accompanied by chronic progressive demyelination. *J Comp Neurol*, **486**, 373–383
- Tsai, M. C., Wei, C. P., Lee, D. Y., Tseng, Y. T., Tsai, M. D., Shih, Y. L., Lee, Y. h., Chang, S. F. and Leu, S. J. (2008) Inflammatory mediators of cerebrospinal fluid from patients with spinal cord injury. *Surg Neurol*, **70 Suppl 1**, S1:19–24; discussion S11:24
- Tuna, M., Polat, S., Erman, T., Ildan, F., Gocer, A. I., Tuna, N., Tamer, L., Kaya, M. and Cetinalp, E. (2001) Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5'-triphosphatase and superoxide dismutase activation, and ultrastructural changes. *J Neurosurg*, **95**, 64–73
- Unterberg, A., Wahl, M. and Baethmann, A. (1988) Effects of free radicals on permeability and vasomotor response of cerebral vessels. *Acta Neuropathol*, **76**, 238–244

- Vavrek, R., Girgis, J., Tetzlaff, W., Hiebert, G. W. and Fouad, K. (2006) BDNF promotes connections of corticospinal neurons onto spared descending interneurons in spinal cord injured rats. *Brain*, **129**, 1534–1545
- Weaver, L. C., Gris, D., Saville, L. R., Oatway, M. A., Chen, Y., Marsh, D. R., Hamilton, E. F. and Dekaban, G. A. (2005) Methylprednisolone causes minimal improvement after spinal cord injury in rats, contrasting with benefits of an anti-integrin treatment. *J Neurotrauma*, **22**, 1375–1387
- Whetstone, W. D., Hsu, J. Y., Eisenberg, M., Werb, Z. and Noble-Haeusslein, L. J. (2003) Blood-spinal cord barrier after spinal cord injury: relation to revascularization and wound healing. *J Neurosci Res*, **74**, 227–239
- Widenfalk, J., Lipson, A., Jubran, M., Hofstetter, C., Ebendal, T., Cao, Y. and Olson, L. (2003) Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience*, **120**, 951–960
- Zeilhofer, h. U. (2007) Prostanoids in nociception and pain. *Biochem Pharmacol*, **73**, 165–174
- Zhao, P., Waxman, S. G. and Hains, B. C. (2007a) Extracellular signal-regulated kinase-regulated microglia-neuron signaling by prostaglandin E2 contributes to pain after spinal cord injury. *J Neurosci*, **27**, 2357–2368
- Zhao, P., Waxman, S. G. and Hains, B. C. (2007b) Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J Neurosci*, **27**, 8893–8902

RAR/RXR and PPAR/ RXR signalling in neurological and psychiatric diseases

2

S.G.A. van Neerven^{1,2}, E. Kampmann¹ and J. Mey^{1,2}

¹Institute of Biology II, RWTH, Aachen University,
52056 Aachen, Germany

²EURON European Graduate School of Neuroscience,
6229 Maastricht, The Netherlands

Progress in Neurobiology, 2008; 85(4): 433–51

Abstract

Retinoids are important signals in brain development. They regulate gene transcription by binding to retinoic acid receptors (RAR) and, as was discovered recently, a peroxisome proliferator-activated receptor (PPAR). Traditional ligands of PPAR are best known for their functions in lipid metabolism and inflammation. RAR and PPAR are ligand activated transcription factors, which share members of the retinoid X receptor (RXR) family as heterodimeric partners. Both signal transduction pathways have recently been implicated in the progression of neurodegenerative and psychiatric diseases. Since inflammatory processes contribute to various neurodegenerative diseases, the anti-inflammatory activity of retinoids and PPAR γ agonists recommends them as potential therapeutic targets. In addition, genetic linkage studies, transgenic mouse models and experiments with vitamin A deprivation provide evidence that retinoic acid signaling is directly involved in physiology and pathology of motoneurons, of the basal ganglia and of cognitive functions. The activation of PPAR/RXR and RAR/RXR transcription factors has therefore been proposed as a therapeutic strategy in disorders of the central nervous system.

Keywords: Retinoic acid, PPAR, Neurodegeneration, Inflammation, Alzheimer, Parkinson, ALS, MS, Schizophrenia, Depression

1. Introduction

With increasing life expectancy in modern societies, neurodegenerative diseases are gaining major socioeconomic relevance. At present the financial burden of brain disorders constitutes 35% of the total costs of all diseases in Europe (Andlin-Sobocki *et al.*, 2005). For neurological diseases, such as multiple sclerosis or Parkinson's and Huntington's disease as well as for the psychiatric disorders depression, schizophrenia and dementia, the costs in terms of quality of life are enormous. However, for none of these diseases is an etiological therapy yet available. Therefore, the importance of basic research for the development of therapeutic strategies has met growing awareness. A class of signalling molecules, whose relevance for neurodegenerative diseases has recently become apparent, is the transcriptional regulators of the nuclear receptor superfamily. This group includes retinoic acid receptors (RAR) and peroxisome proliferator-activated receptors (PPAR), both of which form heterodimers with retinoid X receptors (RXR). The structural similarity of the two pathways is further reflected by a functional link as it was recently discovered that retinoic acid can elicit different, even opposing effects by activating either RAR/RXR or PPAR β δ /RXR heterodimers (Schug *et al.*, 2007). It has been suggested that these ligand-activated transcription factors are potential pharmacological targets for the treatment of spinal cord injury (Mey, 2006). Since retinoid signaling affects neuronal proliferation, differentiation and physiology in the adult brain, its potential therapeutic benefits are not limited to traumatic lesions but extend to other pathological conditions. Retinoids and PPAR ligands participate in the modulation of inflammatory processes, and immune reactions are implicated in several neurodegenerative diseases. The immunomodulatory function of PPAR/RXR and RAR/RXR activity may therefore provide another pharmacological rationale. In this review we will discuss the results and implications of studies that focused on multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), motor disorders involving the basal ganglia, on Alzheimer's disease (AD), depression and schizophrenia.

1.1. RAR/RXR signal transduction

The RAR/RXR transcription factor complex is activated by ligand binding, its natural ligand being all-*trans* retinoic acid (all-*trans* RA). Retinoids are obtained from the diet in form of vitamin A (retinol and retinal), retinyl esters or β -carotene. Following cellular uptake of all-*trans* retinol from the plasma, the intracellular synthesis of retinoic acid occurs in two steps: retinol is oxidized first to retinal and subsequently to retinoic acid (Fig. 1). The second NADH-dependent, thermodynamically irreversible step is catalyzed by retinal-dehydrogenases (Blomhoff and Blomhoff, 2006). Three known RA-synthesizing aldehyde dehydrogenases are designated as RALDH-1, -2, and -3 (also: ALDH1A1, A2, A3). Retinoic acid acts in a paracrine or autocrine fashion. The fact that RA activates nuclear transcription factors was discovered in 1987 (Giguere *et al.*, 1987; Petkovich *et al.*, 1987).

Retinoid receptors belong to the same superfamily as PPAR, vitamin D receptor, thyroid hormone receptor (TR) and steroid receptors. They can be grouped into two families, the RAR and the RXR, each consisting of three isoforms encoded by separate genes: RAR α , RAR β , RAR γ (also: NR1B1–3) and RXR α , RXR β , RXR γ (NR2B1–3). All-*trans* RA and 9-*cis* RA bind to the RAR family, whereas only 9-*cis* RA is a high affinity ligand for the RXR (Bastien and Rochette-Egly, 2004). The active transcription factor complex consists of an RAR/RXR heterodimer, ligand and co-activators. It interacts with retinoic acid response elements (RARE) in the promoters of target genes (Glass and Rosenfeld, 2000). About 500 genes have been suggested to be regulated by RAR/RXR signalling (Blomhoff and Blomhoff, 2006); however, a much lower number was experimentally shown to be activated via the classical RARE driven pathway. Proven target genes include enzymes, transcription factors, cytokines and cytokine receptors (Mey, 2001, 2006; Lane and Bailey, 2005). In addition, many cases of gene suppression and non-genomic modes of action of RA and its receptors have been described. Rapid actions of RA include the regulation of gap junctions (Zhang and McMahon, 2000), spinule formation in the retina (Dirks *et al.*, 2004), and effects on dendritic spines in the hippocampus (Chen and Napoli, 2008). A direct influence on phosphorylation of the transcription factor CREB and the mitogen-activated kinase ERK1/2 has been observed (Cañón *et al.*, 2004; Chen and Napoli, 2008). Another path of action is the repression of AP-1 (Jun, Fos) activity, which involves RAR/RXR heterodimers, but not the RARE (Li *et al.*, 1996). In an oxygen/glucose-deprivation/reperfusion model to simulate ischemia, RA protected hippocampal neurons from cell death. In this case, RA also increased phosphorylation of ERK1/2 but reduced phosphorylation of the kinases JNK and p38 (Shinozaki *et al.*, 2007).

During the last decade it became apparent that the retinoid signal transduction system is activated after CNS lesions and during peripheral nerve regeneration. For instance, spinal cord contusion injury in the rat caused a local increase in protein expression and enzyme activity of the RA-synthesizing RALDH-2 (Mey *et al.*, 2005; Kern *et al.*, 2007). In macrophages at the lesion site RAR α , RXR α and RXR β were observed to translocate from the cytosol to the cell nuclei, where they might control gene transcription (Schrage *et al.*, 2006). Following sciatic nerve injury transcriptional activity of RA was also detected, and the expression of several mediators of the RA signaling pathway increased strongly in the regenerating nerve segment (Zhelyaznik *et al.*, 2003; Zhelyaznik and Mey, 2006). Cell culture studies and experiments with RAR β -deficient mice indicated that RAR β is required for RA-induced axonal regeneration of sensory neurons (Corcoran *et al.*, 2000; So *et al.*, 2006). Since RALDH-2 (Corcoran and Maden, 1999) and RAR β (Cosgaya and Aranda, 2001) are induced by NGF, RA appears to act downstream of this neurotrophin, while additional neurotrophin-independent RA/RAR β activity contributes to axon outgrowth (So *et al.*, 2006). In a mouse model of diabetic neuropathy, RA treatment raised serum levels of NGF, improved myelination in peripheral nerves and caused behavioral improvements (Arrieta *et al.*, 2005).

Fig. 1. Retinoic acid signal transduction. The upper cell illustrates synthesis and release of RA. Blood-derived retinyl esters and retinol are bound to retinol binding protein (RBP) and taken up by RA-synthesizing cells. In two redox reactions retinol (ROH) is oxidized via retinaldehyde (RAL) to RA, which can leave the cell through the plasma membrane. In target cells RA binds to cytosolic binding proteins and enters the nucleus where it regulates gene expression by activating RAR/RXR heterodimers. Retinoic acid can be transcriptionally active in the absence of CRABP II. It is inactivated by cytochrome 450 oxidases of the Cyp26 family. See also list of abbreviations.

1.2. PPAR/RXR signal transduction

Peroxisome proliferator-activated receptors (PPAR) belong to the same superfamily as the RAR. The first PPAR was discovered, cloned and sequenced in 1990, and it was named after its property to be activated by drugs that cause proliferation of peroxisomes in hepatocytes (Issemann and Green, 1990). Until now, three different isoforms of PPAR, encoded by separate genes, have been identified: PPAR α (NR1C1), PPAR β/δ (NUC1, NR1C2) and PPAR γ (NR1C3). The different isoforms have similar structural features; however, each isoform exhibits its own specific tissue expression pattern and distinct physiological functions depending on ligand activation (Kliewer *et al.*, 1994; Kota *et al.*, 2005). PPAR

also heterodimerize with RXR, then bind as a complex to their response elements (PPRE) (Gervois *et al.*, 1999). Different promoter-specific co-activators participate to regulate the activity of PPAR γ and other nuclear receptors (Glass and Rosenfeld, 2000). Natural ligands of PPAR are fatty acids, prostaglandins and oxidized fatty acid derivatives. They are also activated by synthetic ligands like the lipid-lowering fibrates, the anti-diabetic glitazones (Moraes *et al.*, 2006) and by some non-steroid anti-inflammatory drugs (NSAIDs) like ibuprofen or indomethacin (Lehmann *et al.*, 1997). Recently, RA was shown to activate not only RAR/RXR but also PPAR/RXR heterodimers (Schug *et al.*, 2007). Different lipid binding proteins selectively cooperate with different nuclear receptors, for instance, CRABP-II with RAR α and the fatty acid binding protein FABP-5 with PPAR β/δ . When a high CRABP-II concentration was available in the cell, RA activated RAR α (Kd ca. 0.1–0.2 nM), but in the presence of a high FABP-5/CRABP-II ratio RA bound PPAR β/δ with high affinity (Kd 10–50 nM), which induced expression of endogenous PPRE target genes in a human keratinocyte cell line (Schug *et al.*, 2007).

The PPARs, which have been implicated in lipid metabolism, cellular proliferation and inflammatory responses, are widely expressed, including in monocytes, dendritic cells, endothelial cells, megakaryocytes and lymphocytes, where they may be related to immune functions (Kota *et al.*, 2005; Moraes *et al.*, 2006). PPAR γ can also influence gene expression independently of PPRE. The activity of a number of transcription factors, e.g., NF- κ B, AP-1 and STAT1, is inhibited by PPAR γ via direct interaction or by competition for limiting supplies of co-activators (Kielian and Drew, 2003). PPAR α and PPAR β/δ but not PPAR γ were found in cervical, thoracic and lumbar segments of the spinal cord, in the thalamus and cerebral cortex (Braissant and Wahli, 1998; Moreno *et al.*, 2004). Immunohistochemical staining showed that PPAR β/δ is the main isoform present in neuronal cell bodies of the spinal cord gray matter. Both receptors, PPAR α and β/γ , were concentrated in cell nuclei. In the white matter PPAR α appeared particularly strong in PPAR β/δ negative astrocytes, whereas oligodendrocytes expressed only PPAR β/δ (Benani *et al.*, 2003). PPAR β/δ is a factor in neuronal differentiation, and functions of this receptor in various aspects of neural physiology have been suggested (Benani *et al.*, 2003; Cimini *et al.*, 2005). While conflicting results about the expression of PPAR γ in the CNS have been published (Braissant and Wahli, 1998; Benani *et al.*, 2003; Moreno *et al.*, 2004), this receptor is expressed in microglial primary cultures (Bernardo *et al.*, 2000) and may be upregulated after injury. Endogenous PPAR ligands may mitigate the inflammatory response after spinal cord injury. To examine the effects of endogenous PPAR α ligands, experimental spinal cord injury was induced in wild-type and in PPAR α deficient (-/-) mice. The injury resulted in severe trauma characterized by edema, loss of myelin, neutrophil infiltration, apoptosis and increased production of TNF α . Compared to wildtype animals all these parameters were augmented in PPAR α -/- mice. The absence of PPAR α also interfered with recovery of limb function (Genovese *et al.*, 2005).

1.3. Polyunsaturated fatty acids as PPAR and RXR ligands

Polyunsaturated fatty acids (PUFA) play a crucial role in brain function as well as in general growth and development. Among them, the ω -3 fatty acids, and specifically a high ratio of ω -3 to ω -6 fatty acids, have been shown to be neuroprotective, to reduce inflammation and to correlate with behavioral recovery after spinal cord injury (King *et al.*, 2006), ischemia and age-related deficits (Lauritzen *et al.*, 2000; Dyall *et al.*, 2007). They are implicated in several neurological and psychiatric diseases that will be discussed below.

Omega-3 fatty acids cannot be synthesized by the body but must be obtained from the food. The three major forms are α -linolenic acid (α -LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the latter being the most important form in the brain, where it is highly concentrated in myelin. Since dietary uptake of fatty acids is of concern for the entire population and since it affects several medical conditions, the number of publications about this subject is enormous. On the other hand, fatty acid derivatives do not yield patentable thus commercially profitable products, and therefore many studies do not match size or clinical standards required for drug approval by the FDA. Nevertheless, nutritional and epidemiological research demonstrated various biological effects on the brain. While PUFA constitute a structural component of myelin and maintain the fluidity of cell membranes (Bourre, 2005; Hashimoto *et al.*, 2006), they also act as specific signaling molecules. In the context of this review this is of interest in so far as ω -3 fatty acids, most notably DHA, have been found to be direct ligands of PPAR and of RXR (Chawla *et al.*, 2001). Although the 9-*cis* isomer of RA was previously thought to be the natural ligand of RXR (Solomin *et al.*, 1998), its endogenous occurrence has been difficult to confirm (Kane *et al.*, 2005). The discovery that DHA can act as a direct RXR ligand therefore strengthened the concept of RXR activation independent of its heterodimeric partner (RAR, PPAR, TR, etc.). While initially the required concentrations of DHA seemed to be super-physiologically high (Mata de Urquiza *et al.*, 2000), values of stronger binding affinities were recently obtained. In addition, other naturally occurring PUFAs, e.g., arachidonic acid, were also found to activate RXR at physiological concentrations and with similar efficiency as DHA (Lengqvist *et al.*, 2004). This opens the possibility that many of the effects of fatty acids on neurological diseases are based on direct transcriptional regulation.

It should be mentioned that other fatty acid signaling pathways have been described, which are not within the range of the present review: Omega-6 arachidonic acid, synthesized from α -LA in the liver, is a very important substrate for synthesis of eicosanoids, which are absolutely necessary to regulate blood flow as well as platelet, gastric and renal functions. Some eicosanoids such as prostaglandin E2 or leukotriene B4 are potent mediators early in the course of inflammation. In contrast, ω -3 fatty acids exert mainly anti-inflammatory effects, primarily because they compete with arachidonic acid

as a substrate for cyclooxygenases and 5-lipoxygenase (Simopoulos, 2002), they suppress NF- κ B activity (Moon *et al.*, 2007) and act as antioxidants (Sarsilmaz *et al.*, 2003). By binding and activating a family of background K⁺ channels PUFA reduce neuronal excitability and glutamate cytotoxicity (Lauritzen *et al.*, 2000).

Tab. 1. RAR/RXR, PPAR/RXR signaling in multiple sclerosis

ligand/ receptor	major results	experimental approach	references
RA	anti-inflammatory, Th2-phenotype	<i>in vitro</i>	Correale <i>et al.</i> , 1995; Racke <i>et al.</i> , 1995; Lovett-Racke <i>et al.</i> , 2002; rev.: van Neerven and Mey, 2007
RA	suppresses neurological symptoms	oral administration in EAE rat model	Massacesi <i>et al.</i> , 1987, 1991
PPAR α	anti-inflammatory, Th2 phenotype	<i>in vitro</i>	Lovett-Racke <i>et al.</i> , 2004; Xu <i>et al.</i> , 2007a
PPAR β/δ	improvement: histology, behavior	EAE mice	Polak <i>et al.</i> , 2005
PPAR γ	involvement in EAE, Th1 immune response	heterozygous mice, EAE	Natarajan <i>et al.</i> , 2003; Raikwar <i>et al.</i> , 2005, 2006; Xu <i>et al.</i> , 2007b
PPAR γ	T-cell adhesion and transmigration	PPAR γ overexpression in endothelial cells	Klotz <i>et al.</i> , 2007
PPAR γ	anti-inflammatory, reduced demyelination	EAE mouse model	Natarajan and Bright, 2002; Feinstein <i>et al.</i> , 2002; Diab <i>et al.</i> , 2004
PPAR γ	clinical improvement	case study	Pershadsingh <i>et al.</i> , 2004
ω -3 PUFA	anti-inflammatory	nutritional studies	rev.: Simopoulos, 2002
ω -3 PUFA	possibly beneficial for MS	nutritional studies	rev.: Steward and Bowling, 2005
ω -3 PUFA	anti-inflammatory, improvement	EAE rodent models	Harbige, 2003

Tab. 2. RAR/RXR, PPAR/RXR signaling in amyotrophic lateral sclerosis

ligand/ receptor	major results	experimental approach	references
RA/RAR α	involved in motoneuron disease	retinoid deficient rats	Corcoran <i>et al.</i> , 2002
RBP, CRABP-II, RALDH-2 RAR α , RAR β	marginally upregulated in ALS animal model reduced with motoneuron degeneration	gene expression screen and immunohistochemistry of SOD-1 mutant rats	Jokic <i>et al.</i> , 2007
RAR α , RXR γ , CRABP-I, RBP	involved in ALS	postmortem analysis of ALS patients	Malaspina <i>et al.</i> , 2001; Dangond <i>et al.</i> , 2004; Jiang <i>et al.</i> , 2005
PPAR γ	anti-inflammatory, motoneuron survival, clinical benefit	SOD-1 mutant mice	Kiaei <i>et al.</i> , 2005; Schutz <i>et al.</i> , 2005
ω -3 PUFA	negative correlation with ALS	clinical study	Veldink <i>et al.</i> , 2007

2. Multiple sclerosis and amyotrophic lateral sclerosis

Multiple sclerosis (MS), a chronic disease of the CNS, is characterized by inflammation, demyelination and degeneration of sensory- and motoneurons. About 2.5 million people have been diagnosed with MS worldwide (Feinstein, 2003). The disease has its onset during early adult life and is recognized by patches of demyelination in cerebrum, cerebellum, brainstem and spinal cord. In MS, periods of autoimmune attacks are followed by remission. The relapsing-remitting phase often lasts several years. About 30% of patients enter into a secondary, chronic progressive state (Steinman *et al.*, 2000). Animal experiments demonstrating that autoreactive T-cells are able to induce demyelination of the CNS supported the theory that MS is an immune-mediated disorder with an underlying T-cell response to antigens located in the myelin of the CNS (Pette *et al.*, 1990; Steinman *et al.*, 2000) (Tables 1 and 2).

2.1. Effect of retinoids on experimental allergic encephalomyelitis

Experimental allergic (or: autoimmune) encephalomyelitis (EAE) is the most important animal model to study MS. In EAE, inflammation with variable degrees of demyelination and axonal damage is induced by injecting mice with myelin antigens such as oligoden-

drocyte-myelin glycoprotein or myelin basic protein (MBP) (Hemmer *et al.*, 2006). Immunization results in a Th1-mediated immune response, monocyte recruitment, microglia activation (Ando *et al.*, 1989) and causes pathology comparable to MS, although its progression in time is different (Zamvil and Steinman, 1990; Hemmer *et al.*, 2006). Excessive Th1 response is typical for several autoimmune diseases. With macrophage activation, respiratory burst and activation of killer cells, the Th1 response leads to severe tissue damage, whereas cytokines produced by Th2 cells seem to inhibit inflammatory processes in the CNS (Aloisi *et al.*, 2000). Therefore, switching the immune response towards a Th2 phenotype could be a successful strategy to limit autoreactive diseases of the CNS (Kennedy *et al.*, 1992; Racke *et al.*, 1994). Cell culture experiments indicate that retinoids may be useful in this regard (Fig. 2): Proliferation of murine MBP-specific T-cells was inhibited by RA, accompanied with an increase in IL-4 mRNA levels and decreased transcript concentrations of IL-2, TNF α and IFN γ (Racke *et al.*, 1995). A similar anti-proliferative effect was reported with human autoreactive T-cells (Massaccesi *et al.*, 1987; Lovett-Racke and Racke, 2002). In MBP-specific T-cell lines derived from relapsing-remitting MS patients, RA promoted T-cell differentiation towards the Th2 phenotype, indicated by production of IL-4 in the presence of RA, and production of IFN γ in the absence of RA (Correale *et al.*, 1995; Racke *et al.*, 1995; Dawson *et al.*, 2008). Retinoids were also therapeutically beneficial *in vivo*. Oral administration of 13-*cis* RA suppressed neurological symptoms in EAE rats and reduced lymphocyte responsiveness to T-cell mitogens (Massaccesi *et al.*, 1987, 1991). Although the 13-*cis* isomer of RA does not directly bind to retinoic acid receptors, as a drug (Isotretinoin, Accutane) it is effective in the treatment of dermatological diseases. The pharmacological activity of 13-*cis* RA is believed to result either from non-genomic effects or a gradual isomerization to all-*trans* RA (Nelson *et al.*, 2006). The fact that a non-toxic dose of 13-*cis* RA decreased T-cell mediated immunity strong enough to suppress an ongoing immune response in the EAE model (Massaccesi *et al.*, 1991) appears to be highly relevant with respect to possible therapeutic applications. Side effects of Accutane on the nervous system are another issue and will be discussed below (Section 5.1).

Retinoic acid has beneficial effects in other diseases with an inflammatory based pathology including asthma, arthritis and atherosclerosis. Signals that were found to be down-regulated by retinoids include transcriptional activity of NF- κ B, expression of cytokines IL-1 α , IL-1 β , IL-6, TNF α , IL-8, IL-12, production of reactive oxygen species and release of lysosomal enzymes. Anti-inflammatory effects of retinoids and PPAR ligands which have been identified with microglia and astrocyte primary cultures are discussed in detail in a recent review (van Neerven and Mey, 2007). While new data suggest that the inflammatory synthesis of prostaglandin E2 is modulated by retinoic acid in microglia (enhanced: Kim *et al.*, 2008) and astrocytes (reduced: Kampmann *et al.*, 2008), it remains to be seen how these results contribute to the design of translational experiments.

2.2. Effect of peroxisome proliferator-activated receptor ligands on EAE

When cells of the immune system are challenged with LPS or other immunogenic stimuli, activation of PPAR α and PPAR γ reduces inflammatory responses (van Neerven and Mey, 2007).

Not surprisingly, PPAR activation has been tested to counter symptoms of EAE. The specific PPAR α agonists gemfibrozil and fenofibrate were able to increase the production of the Th2-related cytokine IL-4, inhibited release of IL-12p40, IL-12p70, IL-23, IL-27p28 and reduced NO production by microglia. They suppressed proliferation of transgenic T-cells that were specific for MBP (Ac1-11), which had been used for immunization (Lovett-Racke *et al.*, 2004; Xu *et al.*, 2007). Similarly, gemfibrozil shifted the cytokine secretion of human T-cell lines by inhibiting secretion of INF γ and promoting that of IL-4. Most importantly, oral administration of gemfibrozil and fenofibrate reduced clinical signs of EAE (Lovett-Racke *et al.*, 2004; Fig. 3).

The PPAR β/δ agonist GW0742 also improved clinical and histological symptoms in EAE. While GW0742 did not suppress INF γ production *in vivo* or *in vitro*, it reduced astroglial and microglial activation and IL-1 β levels in EAE brains. Several myelin genes were elevated (Polak *et al.*, 2005).

In the EAE model PPAR γ appears to be even more important. A study with PPAR γ -heterozygous mice demonstrated that these animals developed exacerbated EAE with prolonged clinical symptoms, increased expansion of CD4(+) and CD8(+) T-cells and expression of CD40 and MHC class II molecules, which are crucial for antigen presentation and activation of adaptive immune responses. T-cell proliferation and Th1 response were also stronger in these animals (Natarajan *et al.*, 2003). In accordance with these results, PPAR γ antagonists increased the severity and duration of EAE in both C57BL/6 wild-type and PPAR γ (+/-) mice, again associated with increased T-cell proliferation, Th1 differentiation and INF γ production (Raikwar *et al.*, 2005, 2006). Consequently, the activation of PPAR γ is beneficial: Treatment of SJL/J mice with the PPAR γ agonists 15d-PGJ2 or ciglitazone inhibited EAE in association with a decrease in neural antigen-specific Th1 cells and in IL-12 production. These observations were corroborated in cell cultures. PPAR γ agonists inhibited IL-12-induced activation of the JAK-STAT signalling pathway and Th1 differentiation of activated T-cells (Natarajan and Bright, 2002). Similar inhibition of several cytokines of the IL-12 family was obtained with LPS-stimulated primary microglia and astrocyte cultures (Xu and Drew, 2007). At histological examination the PPAR γ effect was associated with diminished lymphocyte infiltration and reduced demyelination (Feinstein *et al.*, 2002). Lentiviral overexpression of the nuclear receptor in endothelial cells blocked adhesion and transmigration of T-cells (Klotz *et al.*, 2007). Pioglitazone was effective even when provided after onset of the disease. On the molecular level, decreased chemokine and cytokine expression, and increased expression of I κ B were found in the brain and in EAE-derived T-cells, suggesting that the protective effect of PPAR γ activation was mediated by an increased inhibition of NF- κ B (Feinstein *et al.*, 2002).

Fig. 2. Retinoic acid affects leukocyte differentiation. Upon activation by the innate immune system and antigen presentation, CD4+ T-lymphocytes differentiate either to IFNγ producing Th1 effector cells or to IL-4 producing Th2 effector cells. The cytotoxic Th1 response is mainly directed against intracellular pathogens, whereas the humoral Th2 response is mainly against extracellular pathogens. Both responses differ in their patterns of cytokine and cellular activities. RA- and PPAR-agonists promote Th2 differentiation at the expense of the Th1 pathway. Under the influence of TGFβ and IL-6 CD4+ and CD8+ cells can be induced to generate IL-17 (Th17 differentiation). RA inhibits this process (Mucida *et al.*, 2007). The interaction between CD4 and MHC II is essential for successful antigen presentation. Activation of CD40 by its ligand triggers the antigen presenting cell to supply co-stimulatory signals, which are necessary for proper T-cell activation. These processes are also inhibited by PPARγ agonists. Positive and negative effects are indicated by arrows and blunt ending lines, respectively; arrows with broken lines show paths of differentiation or cytokine release. Refer to the list of abbreviations.

A case report, where the safety and therapeutic potential of oral pioglitazone was tested in a patient with secondary MS, demonstrated improved motor function and coordination, better orientation, short-term memory and attention. Treatment with pioglitazone continued for 3 years without adverse events, and the patient remained clinically stable. However, magnetic resonance imaging displayed no perceptible change in overall brain atrophy or the extent of demyelination. As these preliminary data suggest that PPARγ agonists offer a therapeutic benefit to MS patients (Pershadsingh *et al.*, 2004) more clinical studies are in progress now (e.g., Takeda Pharmaceuticals, Univ. of Illinois). We note that drugs used as PPAR agonists can be beneficial in EAE independent of PPAR/RXR activation (Dasgupta *et al.*, 2007).

Fig. 3. Oral administration of PPAR agonists has a therapeutic benefit in EAE.

(a) Mice were fed 500 mg of gemfibrozil (GEM) daily by gavage for 21 days, beginning the day before immunization with 200 mg MBP Ac1-11/complete Freund's adjuvant. Control mice were immunized only (control) or were fed PBS by gavage (PBS). Severity of the symptoms (clinical score) is indicated on the ordinate; disease incidence is shown in parenthesis. (b) Mice were given a diet supplemented with fenofibrate (FEN, 0.25%, w/w) beginning 3 days before immunization. Half of the mice in the fenofibrate group died within the first 10 days, and the FEN-supplemented diet was stopped. (c) Animals received a diet supplemented with GEM (0.25%, w/w) beginning 3 days before immunization. (d) Mice were immunized with MBP, and at the first signs of disease, the animals were fed 500 mg GEM, FEN or PBS by gavage for 21 days. Positive effects of the PPAR γ agonists were highly significant in all cases. Source: Lovett-Racke *et al.* (2004).

2.3. PPAR γ and RAR α signaling in motoneuron disease

Amyotrophic lateral sclerosis is characterized by the loss of motoneurons in the cerebral cortex, brain stem and spinal cord leading to muscular weakness and atrophy. About 90% of all ALS cases are sporadic, whereas 10% include familial forms (Jackson and Bryan, 1998). Mutations in the Cu/Zn superoxide dismutase gene (SOD)-1 have been found in 20% of the familial ALS patients and have been implicated in the pathophysiology of the disease, albeit in a small percentage of all cases. This is important nonetheless, because several lines of transgenic mice have been engineered that carry mutant forms of the SOD-1 gene,

and these animals serve as experimental models of ALS. Similar to human ALS patients, they display abnormal accumulation of neurofilaments in motoneurons, which is partly responsible for the cellular degeneration (Lee *et al.*, 1994; Straube-West *et al.*, 1996).

Using such SOD-1 mutant mice, PPAR agonists were assessed as a possible treatment of ALS. The PPAR γ agonist pioglitazone, fed with the diet, improved motoneuron survival, muscle strength and motor performance. The animals exhibited a delayed disease onset and longer survival (Kiaei *et al.*, 2005; Schutz *et al.*, 2005). Gliosis was also reduced, as were microglial activation and molecular parameters of inflammation in the spinal cord, including NF- κ B, iNOS and 3-nitrotyrosine immunoreactivity, which correlates with produced NO. Quantification of motor neurons of the spinal cord revealed complete neuroprotection by pioglitazone, whereas nontreated SOD1-G93A mice lost 30% of motoneurons.

Retinoids are also implicated in the disease, because dietary retinoid deficiency in adult rats induced motoneuron degeneration, intracellular accumulation of neurofilaments and increased astrogliosis. These features were interpreted as a syndrome of motoneuron disease similar to ALS. Receptor analysis demonstrated that the effects were mediated by a lack of RAR α signaling. This receptor was also pathologically reduced in motoneurons of patients with spontaneous ALS, shown with in situ hybridization in the post mortem tissue from ten cases. Moreover, the lack of RAR α was accompanied by RALDH-2 deficiency (Corcoran *et al.*, 2002b). These results suggest that PPAR γ agonists and retinoids have a therapeutic potential for human ALS (Schutz *et al.*, 2005). Several publications report analyses of post mortem human spinal cord tissue from ALS patients by gene micro-arrays. Changed patterns of transcription affected a large number of genes that are involved in many cell functions, including the RA signaling pathway. Specifically, the genes of cellular retinoic acid binding protein I (CRABP-I), RAR α and RXR γ were found to be expressed at lower levels, and the gene for retinol binding protein (RBP) was upregulated (Malaspina *et al.*, 2001; Dangond *et al.*, 2004; Jiang *et al.*, 2005). This is somewhat corroborated by a recent gene expression analysis of SOD-1 mutant rats (Jokic *et al.*, 2007). With progression of the disease a marginal increase in the expression of RPB-1, CRABP-II and RALDH-2 occurred. Receptors RAR α and RAR β were detected immunohistochemically in motoneurons. Although quantitative changes in protein expression were not significant, this specific staining disappeared with the degeneration of these cells. Although variable findings regarding the expression of retinoid receptors in the spinal cord have been published (Krezel *et al.*, 1999; Corcoran *et al.*, 2002a; Schrage *et al.*, 2006; Jokic *et al.*, 2007), RAR α appears to be important for motoneuron survival. It is especially high in γ -motoneurons, which survive longer in SOD-1 mutant mice and rats. Also consistently, reactive astrocytes in the spinal cord show strong RXR β immunoreactivity (Schrage *et al.*, 2006; Jokic *et al.*, 2007). It is not known at this point whether RA signaling is causally related to the progress of the disease, part of compensatory physiological mechanisms or only a pathological sign without significant therapeutic relevance.

2.4. Supplementation of polyunsaturated fatty acids in MS and ALS

As noted in the introduction, PUFA can regulate gene transcription by activating PPAR or RXR. Epidemiological observations suggested that dietary PUFA supplementation might be beneficial in demyelinating diseases. Consequently there have been a number of prospective randomized clinical trials to test ω -3 fatty acids (either with α -LA or with fish oil containing EPA and DHA) among patients with relapsing-remitting MS. In most cases a positive trend favoring the treatment group was reported, but possible benefits of the ω -3 PUFA never reached significance. No effects of ω -6 γ -LA on disease progression or severity were found (reviewed in: Stewart and Bowling, 2005). Although animal experiments showed more conclusive results with positive influences of ω -3 PUFA on survival and progression in EAE the mechanisms of action are not clear (Harbige, 2003). Their anti-inflammatory effects appear mostly to be due to indirect influences on prostaglandin metabolism (Simopoulos, 2002).

A recent clinical study involving 132 ALS patients and 220 healthy controls revealed a negative correlation between PUFA and vitamin E in the food and the occurrence of ALS. Other dietary components such as glutamate, cholesterol or vitamin C were not significantly associated with the risk of developing ALS; vitamin A was not investigated in this study (Veldink *et al.*, 2007). Again, it remains to be seen whether any effects of dietary fatty acids can be attributed to regulatory functions on the level of gene transcription.

Tab. 3. RAR/RXR, PPAR/RXR signaling in subcortical neurodegenerative diseases

ligand/ receptor	major results	experimental approach	references
RAR, RXR, binding proteins	involved extrapyramidal motor functions	rodents, biochemistry, histology	Zetterström <i>et al.</i> , 1999; Liao and Liu, 2005; Gbate <i>et al.</i> , 2007
RA, RALDH-1, other enzymes	involved extrapyramidal motor functions	in situ hybridization, transgenic mice	Abu-Abed <i>et al.</i> , 2002; Galter <i>et al.</i> , 2003; McCaffery and Dräger, 2004
RA	involved in dopamine signaling	<i>in vitro</i> , molecular biology	Samad <i>et al.</i> , 1997; Valdenaire <i>et al.</i> , 1998; Wang and Liu, 2005
RAR β , RXR β , RXR γ	involved in extrapyramidal motor functions, dopamine signaling	knockout mice	Krezel <i>et al.</i> , 1998; Saga <i>et al.</i> , 1999
RA	involved in extrapyramidal motor functions	retinoid deficient rats	Carta <i>et al.</i> , 2006
RALDH-1	involved in striatal functions	clinical observations	Fisher, 1989; Laplane <i>et al.</i> , 1992
RA, RXR γ	reduced in HD model	gene expression analysis in HD mouse model	Luthi-Carter <i>et al.</i> , 2000
PPAR α , PPAR β/δ , PPAR γ	rescued dopaminergic neurons of the substantia nigra	MPTP treated monkeys and rats	Breidert <i>et al.</i> , 2002; Dehmer <i>et al.</i> , 2004; Kreisler <i>et al.</i> , 2007; Iwashita <i>et al.</i> , 2007
PUFA	clinical benefit in HD	clinical studies	Vaddadi <i>et al.</i> , 2002; Puri <i>et al.</i> , 2002

3. Subcortical neurodegenerative diseases

Parkinson's disease (PD) and Huntington's disease (HD) are two well-known neurodegenerative diseases that affect subcortical structures of the brain. Both diseases share disturbances in the nigrostriatal projections and extrapyramidal system, which are accompanied by severe movement disorders. Parkinson's disease has onset at a mean age of 55 and more than 0.5% of individuals older than 70 years are affected. The disease becomes clinically apparent after the degeneration of approximately 70% of the dopaminergic neurons of the substantia nigra (SN). With a variety of contributing genetic and external factors, idiopathic PD is the common clinical appearance of different pathological processes. The degenerating neurons typically contain Lewy bodies, cytoplasmic inclusions mainly

consisting of α -synuclein and ubiquitin. As neuronal degeneration progresses, symptoms are resting tremor, rigidity, bradykinesia and postural instability. Although several treatments to stabilize and improve functions of the basal ganglia were developed, most patients are bedridden after approximately 15 years (Rowland, 2000) (Table 3).

3.1. Physiological functions of retinoic acid in the nigrostriatal system

Several lines of experimental evidence suggest that the RA system is involved in development and function of the nigrostriatal pathway.

(1) A first indicator is the presence of RA-synthesizing enzymes, binding proteins and retinoid receptors. RAR α and RAR β are expressed in the striatum of neonatal rats and mice, RAR β also in adult animals (Ghate *et al.*, 2007). All three RXRs are expressed continuously during ontogenesis (Zetterström *et al.*, 1999; Liao and Liu, 2005). Using a reporter cell line, McCaffery and Dräger detected endogenous RA in striatal tissue of adult mice (McCaffery and Dräger, 1994). They identified dopaminergic neurons of the substantia nigra as the source of RA synthesis. These cells express enzymes required for the conversion of vitamin A to RA, namely alcohol dehydrogenase ADH-3 (Galter *et al.*, 2003) and retinaldehyde dehydrogenase RALDH-1. Interestingly, the RA-synthesizing enzyme RALDH-1 appears to be transported in axons to the forebrain, implying that projection neurons supply the striatum with RA. Additional aldehyde dehydrogenases were found in the striatum during embryonic development (McCaffery and Dräger, 1994). The striatal expression of retinoid binding proteins CRBP-I, CRABP-I, CRABP-II (Zetterström *et al.*, 1999) and of the RA-degrading enzyme CYP26B1 (Abu-Abed *et al.*, 2002) further support a physiological function of RA in this system.

(2) Functional studies revealed that RA influences the dopamine signal transduction system. The D2 receptor gene has a functional RA response element in its promoter (Samad *et al.*, 1997). RA treatment of neuronal cultures from embryonic striatum raised the number of D2 receptor binding sites (Valdenaire *et al.*, 1998). Using RNase expression assays Wang and coworkers showed that RA also regulated expression of the D1 receptor gene and of other genes that are part of the dopamine signal transduction cascade in the lateral ganglionic eminence. These included the heterotrimeric G-protein Golf, adenylyl cyclase type V and dopamine- and cAMP-regulated phosphoprotein (Wang and Liu, 2005). It was assumed that RA might influence D1 receptor expression via activation of the transcription factors Meis2 and Brn4. So far, most of the effects of RA on genes necessary for dopaminergic signaling were described in development, and little is known about these influences in the adult brain.

(3) A causal link between RA signaling and extrapyramidal motor symptoms was more convincingly demonstrated by the interference with RA signaling *in vivo*. Since RA seems to be produced by neurons of the substantia nigra, it is likely that in addition to the dopamine deficiency, caused by the disappearance of substantia nigra neurons,

a reduction in RA signaling contributes to the neurological appearance of PD. This hypothesis is clinically supported by experience with Antabuse, a drug which is used in the treatment of alcoholism. Antabuse, which is a potent inhibitor of the RA-synthesizing enzyme RALDH-1 (McCaffery and Dräger, 1994), can induce PD symptoms accompanied by striatal lesions (Fisher, 1989; Laplane *et al.*, 1992). It should be noted, however, that Antabuse can directly influence the action of enzymes through copper chelation; for instance, it inhibits dopamine- β -hydroxylase thereby blocking the reaction from dopamine to norepinephrine. RAR β /RXR β , RAR β /RXR γ and RXR β /RXR γ double knockout mice exhibited a strong reduction of striatal D2- and D1 receptor expression and show reduced locomotor activity and impaired motor performance in the rotarod test (Krezel *et al.*, 1998), resembling the Parkinson-like phenotype of D2 receptor knockout mice (Baik *et al.*, 1995). Carta and coworkers also demonstrated motor impairments in vitamin A deprived rats, though in this case, the behavioral effect seemed to be caused by reduced acetylcholine levels in the striatum and not by a reduced expression of dopamine receptors (Carta *et al.*, 2006). In accordance with this interpretation, lower expression of choline acetyltransferase was found in striatal cholinergic interneurons of RXR γ -deficient mice (Saga *et al.*, 1999). Since acetylcholine is a physiological antagonist of dopamine in the striatum (Rowland, 2000), these results seem to be contradictory. Perhaps RA serves generally as a trophic and differentiation factor in the striatum, thus maintaining the homeostasis of both neurotransmitter systems.

The down-regulation of D2 receptors observed in patients with advanced PD is exacerbated by long-term dopaminergic treatment (Rowland, 2000; Thobois *et al.*, 2004). Therefore, by strengthening the postsynaptic dopamine system, RA could also delay the development of drug insensitivity. On the other hand, the pharmacological potential of RA may be limited by effects on the cholinergic system.

3.2. Immunotoxicity, oxidative stress and cell viability in the basal ganglia

Parkinson's disease is accompanied by inflammatory processes (Hirsch *et al.*, 2003), and the involvement of neuroinflammation in Huntington's disease (HD) is also likely (Crocker *et al.*, 2006). As noted above, RAR/RXR and PPAR/RXR signals can reduce immunotoxicity in the CNS (van Neerven and Mey, 2007). Activation of these signaling pathways might therefore be helpful in the treatment of PD and HD. By stabilizing the redox balance PPAR can mitigate pathological processes caused by respiratory stress or mitochondrial dysfunction, which are discussed as primary factors for both diseases (Rego and Oliveira, 2003; Lin and Beal, 2006). While there are no clinical studies published yet, experiments with animal models of PD and HD indicate that PPAR agonists influence the course of both diseases beneficially. One established animal model for PD consists of systemic injections of the neurotoxin MPTP in monkeys or rodents, which causes a degeneration of dopaminergic neurons. In this type of experiment, treatment with the PPAR α agonist

fenofibrate (Kreisler *et al.*, 2007), PPAR β/δ agonists L-165041 and GW501516 (Iwashita *et al.*, 2007) and the PPAR γ agonists pioglitazone (Breidert *et al.*, 2002) and rosiglitazone (Dehmer *et al.*, 2004) resulted in significant rescue effects of dopaminergic neurons in the substantia nigra. The actions of the PPAR β/δ agonists involved inhibition of apoptosis via reduced activation of caspase-3 (Iwashita *et al.*, 2007). The PPAR γ agonists exerted their effects by I κ B α activation and NF- κ B inhibition leading to reduced microglia activation. This nearly abolished NO-mediated cell death (Dehmer *et al.*, 2004).

Effects of PPAR ligands in HD were investigated in fewer studies. This is an autosomal dominant hereditary trinucleotide repeat disorder. The triplet repeat CAG codes for part of the protein huntingtin, whose physiological function is still unknown. The extent of the trinucleotide extension correlates directly with the severity of symptoms and inversely with the age at onset, on average 35–40 years. One prominent pathological feature is the massive loss of striatal medium-sized GABA-ergic spiny projection neurons, leading to a marked atrophy of the neostriatum. Generalized brain atrophy is due to additional pathological processes affecting the cortex, thalamus, cerebellum and further structures (Rowland, 2000). In a gene expression screen of mice that were transgenic for the 50 end of the human huntingtin gene, Luthi-Carter *et al.* found that the mRNA concentrations of RXR γ and a number of RA-responsive genes including the D2 receptor were significantly reduced (Luthi-Carter *et al.*, 2000). While this link between RA signaling and HD is rather weak, unsaturated fatty acids which activate PPARs had beneficial effects on the clinical course of HD (Vaddadi *et al.*, 2002). Puri and coworkers demonstrated that 6 month ethyl-EPA treatment of HD patients improved orofacial dyskinesia and significantly reduced progressive cerebral atrophy measured by MRI (Puri *et al.*, 2002). A more recent study could reproduce a positive effect of ethyl-EPA only for a subset of HD patients with a later age at onset and lower CAG repeat value. After 12 months of therapy these patients showed better motor performance than patients of the control group. Unfortunately, the treated patients still suffered from significant deterioration of behavioral symptoms (Puri *et al.*, 2005). Since ethyl-EPA elicits several effects in addition to activating PPARs (Jump, 2002) it is uncertain whether these observations involve the PPAR system. With respect to a therapeutic influence on extrapyramidal symptoms the anti-inflammatory effects or RAR/RXR signaling have not been studied so far.

4. Alzheimer's disease and aging

Alzheimer's disease (AD) is a progressive degenerative disease of the death commences in brain regions that control memory and cognitive function but progresses to other areas. Alzheimer's disease is pathologically characterized by the extracellular accumulation of β amyloid (A β) plaques and intracellular neurofibrillary tangles of hyperphospho-

rylated tau protein. The idea that PPAR/RXR and RAR/RXR signaling are candidates for therapeutic strategies against AD was suggested by three lines of evidence. (1) Given that inflammatory reactions contribute substantially to the neurodegenerative processes (Kielian and Drew, 2003; Sastre *et al.*, 2006b), their anti-inflammatory properties recommend PPAR/RXR activating drugs and retinoids for therapeutic use (Delerive *et al.*, 2001; Diab *et al.*, 2004; Delayre-Orthez *et al.*, 2005). (2) RA is implicated in neural plasticity and memory processes which deteriorate in the disease (Chiang *et al.*, 1998; Misner *et al.*, 2001; Alfos *et al.*, 2001; Cocco *et al.*, 2002; Etchamendy *et al.*, 2003; Shinozaki *et al.*, 2007; mingaud *et al.*, 2008). (3) Genes for RA signaling are found within the boundaries of many of the chromosomal regions genetically linked to AD (Goodman and Pardee, 2002). A weak association exists also between late-onset AD and PPAR γ polymorphisms (Koivisto *et al.*, 2006; Hamilton *et al.*, 2007; Scacchi *et al.*, 2007) (Table 4).

4.1. Anti-inflammatory treatment with PPAR agonists

While the causal relationship between the formation of amyloid plaques, deposition of neurofibrillary tangles and the following neuronal degeneration is still not completely understood, several hypotheses compete in the field (Mudher and Lovestone, 2002; Sastre *et al.*, 2006b). Regardless of the etiology of the disease, an inflammatory process, including microglia and astrocyte activity, cytokine production and prostaglandin synthesis, is believed to contribute to the AD pathology (Kielian and Drew, 2003; Hoozemans *et al.*, 2006). As mentioned above, RAR/RXR as well as PPAR/RXR signaling suppresses several aspects of inflammatory activity in microglia and astrocytes. A specific target of NSAIDs with relevance for AD is the PPAR γ , which can inhibit expression of iNOS, COX-2 and of the cytokines IL-1 β , TNF α , IL-6, IL-2 and IFN γ (Li *et al.*, 2000; Sastre *et al.*, 2003, 2006a; Heneka *et al.*, 2005; Townsend and Pratico, 2005). In addition to direct transcriptional regulation by PPAR/RXR, effects via inhibition of transcription factors STAT1, NF- κ B and AP-1 were also observed (Ricote *et al.*, 1998; van Neerven and Mey, 2007).

Oxidative stress arising with increasing age and during inflammation seems to be etiologically associated with AD. In aged mice NF- κ B becomes constitutively active in many tissues due to the oxidative stress, which eventually leads to the production of pro-inflammatory cytokines. Administration of PPAR α activators was found to restore the cellular redox balance, to eliminate the constitutive activation of NF- κ B and the spontaneous production of IL-6 and IL-12 (Spencer *et al.*, 1997; Poynter and Daynes, 1998). During the aging process inflammatory reactions are accompanied by reduced PPAR γ levels. Specific PPAR γ ligands were therefore explored as therapeutic tools in this context. For instance, 2,4-thiazolidinedione (2,4-TZD) and pioglitazone reduced age-related oxidative stress, the translocation of NF- κ B p65 subunit to the cell nuclei and NF- κ B-regulated transcription of iNOS, COX-2, IL-1 β , IL-6, and adhesion molecules VCAM-1 and P-selectin (Sung *et al.*, 2006). Oral administration of pioglitazone or the NSAID ibuprofen,

also a ligand of PPAR γ , resulted in a reduction of the number of activated microglia and reactive astrocytes in hippocampus and cerebral cortex of APPV717I mice, an animal model for inflammation in AD (Heneka *et al.*, 2005). One effect of PPAR γ agonists consists in its ability to counteract the induction of β -secretase by inflammatory cytokines. This is one of the enzymes involved in A β deposition because it is responsible for proteolytic cleavage of amyloid precursor protein (APP). In transfected neuroblastoma cells NSAIDs, via PPAR γ activation, inhibited activity of β -secretase and release of A β (Sastre *et al.*, 2003). It was further shown that this effect is caused by direct transcriptional inhibition of the β -secretase gene, BACE1 (Sastre *et al.*, 2006a). In accordance with this mechanism, brain extracts from AD patients contained more β -secretase and less PPAR γ protein than controls, and there was less PPRE binding activity in their tissue (Sastre *et al.*, 2006a). A genetic association between PPAR γ and late-onset AD was recently revealed (Koivisto *et al.*, 2006; Hamilton *et al.*, 2007; Scacchi *et al.*, 2007). PPAR γ overexpression dramatically increased ubiquitinylation and degradation of APP in proteasomes, though effects on A β clearance were independent of secretase activity in these experiments (Camacho *et al.*, 2004; D'Abramo *et al.*, 2005).

Fig. 4. Treatment of AD patients with the PPAR γ agonist rosiglitazone. Numbers of patients who completed the study were: 106 (placebo), 109 (2 mg RSG), 114 (4 mg RSG) and 113 (8 mg RSG). Although the effect of the drug on cognitive performance was not significant, the analysis of ApoE e4-negative vs. ApoE e4 positive cohorts showed a significant interaction between genotype and treatment (AD Assessment Scale-Cognitive score, negative number indicates improvement). Shown are the individual ADAS-Cog scores in patients with ApoE e4/e4 and ApoE e4/x genotypes. Symbol size is proportional to number of subjects with same change in score. Source: Risner *et al.* (2006).

Tab. 4. RAR/RXR, PPAR/RXR signaling in Alzheimer's disease

ligand/ receptor	major results	experimental approach	references
RA, RAR α	genetic linkage between genes associated with AD and RA signaling	genetic evidence, human AD patients	Goodman, 1998, 2006; Goodman, Pardee, 2002
RARs, RXRs, PPARs	expressed in hippocampus	knockout mice, histology; transgenic reporter mice	Krezel <i>et al.</i> , 1999; Moreno <i>et al.</i> , 2004; Sakai <i>et al.</i> , 2004
RA	reduces A β accumulation	<i>in vitro</i>	Ono <i>et al.</i> , 2004; Holback <i>et al.</i> , 2005
RA	involved in A β deposition	retinoid deficient rats	Corcoran <i>et al.</i> , 2004; Husson <i>et al.</i> , 2006
RA, RAR β , RXR γ	involved in learning/memory, aging; necessary for LTP, LTD in hippocampus	knockout mice, retinoid deficient rodents, behavior, physiology	Enderlin <i>et al.</i> , 1997; Chiang <i>et al.</i> , 1998; Misner <i>et al.</i> , 2001; Cocco <i>et al.</i> , 2002; Etchamendy <i>et al.</i> , 2002, 2003; rev.: Mey, McCaffery, 2004; Lane, Bailey, 2005
RAR β /ethanol	blocking RAR β reduced alcohol memory deficits	chronic alcohol consumption in mice	Alfos <i>et al.</i> , 2001
13- <i>cis</i> RA	reduced hippocampal neurogenesis and memory performance	chronic administration in mice (i.p.)	Crandall <i>et al.</i> , 2004; Sakai <i>et al.</i> , 2004
PPAR α	anti-inflammatory	mouse models for AD	Spencer <i>et al.</i> , 1997; Poynter, Daynes, 1998
PPAR α	neuroprotective with A β exposure	<i>in vitro</i>	Santos <i>et al.</i> , 2005
PPAR γ	genetic linkage with AD	genetic evidence, human AD patients	Koivisto <i>et al.</i> , 2006; Hamilton <i>et al.</i> , 2007; Scacchi <i>et al.</i> , 2007
PPAR γ	anti-inflammatory	<i>in vitro</i>	Ricote <i>et al.</i> , 1998; Li <i>et al.</i> , 2000; Sastre <i>et al.</i> , 2006b
PPAR γ	anti-inflammatory	mouse models for AD	Heneka <i>et al.</i> , 2005; Sung, 2006
PPAR γ	inhibits A β release	<i>in vitro</i>	Sastre <i>et al.</i> , 2003, 2006a
PPAR γ	degradation of APP	over-expression <i>in vivo</i>	Camacho <i>et al.</i> , 2004; D'Abramo <i>et al.</i> , 2005
PPAR γ	behavioral benefit	mouse model for AD	Pedersen <i>et al.</i> , 2006
PPAR γ	limited clinical benefit	clinical studies	Watson <i>et al.</i> , 2005; Risner <i>et al.</i> , 2006
ω -3 PUFA	reduces plaque formation	rodent models for AD	Gamoh <i>et al.</i> , 2001; Calon <i>et al.</i> , 2004; Lim <i>et al.</i> , 2005
ω -3 PUFA	low in AD brains	postmortem analyses	Prasad <i>et al.</i> , 1998; Heude <i>et al.</i> , 2003; Hashimoto <i>et al.</i> , 2006
ω -3 PUFA	cognitive benefit	epidemiological studies	Kalmijn <i>et al.</i> , 1997; Morris <i>et al.</i> , 2006

4.2. PPAR activation as a therapeutic tool for Alzheimer's disease

Pedersen and coworkers used the PPAR γ agonist rosiglitazone to treat learning and memory deficits in another animal model for AD, the transgenic Tg2576 mouse (Pedersen *et al.*, 2006). These mice overexpress a mutated form of human APP, which leads to increased levels of A β that eventually forms β -amyloid plaques and causes memory deficits (Hsiao *et al.*, 1996). This is similar to the pathology in AD, although neurofibrillary tangles and neuronal degeneration are not commonly observed in the animal model. In the rosiglitazone study, transgenic animals that received the PPAR γ agonist in their diet showed spatial memory performance similar to wild-type mice, in contrast to non-treated Tg2576 mice, whose abilities deteriorated significantly. Rosiglitazone also attenuated the reduction of insulin-degrading enzyme in the hippocampus, an enzyme that also degrades monomeric A β . The authors speculated that beneficial effects of rosiglitazone were due to lowering of the glucocorticoid level because glucocorticoids reduce the activity of this enzyme, insulin increases it and PPAR γ supports peripheral insulin sensitivity (Pedersen *et al.*, 2006). This mode of action is particularly relevant because the drug does not cross the blood brain barrier. PPAR agonists which do penetrate into the brain tissue, however, may directly protect nerve cells from A β -induced degeneration. That was recently shown in neuronal primary cultures from embryonic rat hippocampus (Santos *et al.*, 2005), and may also be a property of RA (Shinozaki *et al.*, 2007). Since FDA-approved PPAR γ agonists are available, clinical studies have already been started to test these drugs in age-related dementia. A preliminary experiment, where rosiglitazone was given to patients with mild AD/cognitive impairment, demonstrated better delayed recall and selective attention after 6 months of treatment (Watson *et al.*, 2005). Apart from anti-inflammatory effects a major function of PPARs is the regulation of lipid homeostasis and insulin sensitivity (Kota *et al.*, 2005). This provides another link to AD, because lipoprotein metabolism, specifically apolipoprotein E (ApoE) is associated with the disease. One or two copies of the e4 allele of the ApoE gene are present in approximately half of the AD patients, while in contrast the e2 allele confers decreased risk and later onset (Risner *et al.*, 2006). A connection between ApoE and PPAR γ treatment became apparent in the first large clinical investigation involving 67 clinical centers and 449 patients. This study reported a statistical association between the effect of rosiglitazone and the ApoE genotype of the patients (Risner *et al.*, 2006). At the highest concentration ApoE e4-negative patients benefited from the treatment. While Landreth and colleagues conclude that these results support the use of PPAR γ agonists in the treatment of AD (Sundararajan *et al.*, 2006) in our opinion the results give less reason for optimism (Fig. 4). No significant benefit of rosiglitazone was found across the entire population of patients, and the authors report that the interaction between rosiglitazone treatment and allelic status was primarily the result of unexplained improvement at week 24 in ApoE e4-positive patients receiving placebo (Risner *et al.*, 2006). Nonetheless, possible long-term benefits of PPAR-related treatment of AD will certainly be investigated in the future.

A similar conclusion can be drawn with respect to fatty acid ligands of PPAR and RXR, specifically DHA. Epidemiological studies suggested that a diet rich in ω -3 PUFA is associated with decreased risk of cognitive decline (Kalmijn *et al.*, 1997; Morris *et al.*, 2006), and cell membranes of AD patients contained lower amounts of DHA (Prasad *et al.*, 1998; Heude *et al.*, 2003; Hashimoto *et al.*, 2006). Experiments with DHA administration in mice and rats corroborate this interpretation (Gamoh *et al.*, 2001). For instance, Lim and coworkers found in aged Tg2576 mice that high intake of DHA reduced amyloid plaque formation. Supported by gene expression data, they conclude that DHA influenced APP processing (Lim *et al.*, 2005). This may have been caused by transcriptional effects of RXR or indirectly by decreasing oxidative stress and inflammatory reactions (Calon *et al.*, 2004). Although in astrocytes LXR/RXR activity induces ApoE expression (Liang *et al.*, 2005), this was not significantly altered in the DHA experiments (Lim *et al.*, 2005), and it is not certain that dietary effects of ω -3 PUFA in transgenic animals involved RXR signaling.

4.3. Retinoic acid signaling is involved in learning and memory

Although most publications on retinoic acid are related to embryonic development, dermatology or cancer research, increasing attention is being paid to its physiological functions in the adult brain (Mey and McCaffery, 2004; Lane and Bailey, 2005). Investigations of retinoid receptor knockout mice and vitamin A deprived rats showed that these animals were compromised in memory tasks (Chiang *et al.*, 1998; Misner *et al.*, 2001; mingaud *et al.*, 2008). Transgenic mice lacking either RAR β or RXR γ or both genes were deficient in the formation of spatial memory as tested with the Morris water maze. Adult rats, which received a vitamin A free diet for 12 weeks, developed a severe deficit in spatial learning and memory, and this cognitive impairment was fully restored when vitamin A was replaced in the food (Cocco *et al.*, 2002). Chronic ethanol consumption, which produces cognitive deficits, also interferes with retinoid signaling, though the mechanisms are far from understood. Ethanol can induce RAR β and RXR β/γ expression *in vivo*, and blocking RAR β activity was shown to reverse alcohol-induced working memory deficits (Alfos *et al.*, 2001). Astrocytes *in vitro* produced more RA when treated with ethanol (McCaffery *et al.*, 2004); indeed astroglial-derived RA may even be an important signal for neurogenesis in the dentate gyrus of the hippocampus (Környei *et al.*, 2007). The brain structure most closely associated with spatial learning and memory consolidation is the hippocampus, which in mice shows a high expression of RAR α , RAR γ , RXRs and PPARs (Krezel *et al.*, 1999; Moreno *et al.*, 2004). Accordingly, transcriptional activity of RA was observed in the hippocampus of transgenic reporter mice (Luo *et al.*, 2004; Sakai *et al.*, 2004; Környei *et al.*, 2007; Fig. 5). Potential physiological correlates of associative learning are long-term potentiation (LTP) and longterm depression (LTD). These were studied in brain slices of retinoid receptor knockout mice. RAR β and RAR β /RXR γ mutant mice lacked LTP almost completely, while LTP was normal in RXR γ -deficient animals. LTD was severely reduced in all three knockout mice including the RXR γ mutants (Chiang *et al.*, 1998).

Fig. 5. Retinoic acid activity in the mouse brain. Serial slices through the rostral brain of a 7-week-old RARE-lacZ transgenic mouse show endogenous transcriptional activity of RA (blue staining); aha: amygdala/hippocampal area, am: amygdala, ccx: cingulate cortex, DG: dentate gyrus, hyp: hypothalamus. Source: Luo *et al.* (2004). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In aging mice, the declining memory performance in radial maze tasks as well as diminished amplitude of LTP correlated with a decrease in the expression of RAR β and RXR β/γ in the brain. RA administration led to a reversal and the application of an RAR antagonist exacerbated the age-related deficits (Enderlin *et al.*, 1997; Etchamendy *et al.*, 2001). In line with these results, life-long dietary supplementation with vitamin A prevented age-related deterioration in the performance of short-term working memory and long-term declaratory memory tasks in mice. Aged animals which had received the high vitamin A diet showed no decline in RAR β and GAP43 expression in the hippocampus, and learning induced expression of Fos was specifically enhanced in hippocampus CA1–CA3, the prefrontal cortex and the mediodorsal striatum (Mingaud *et al.*, 2008). Since ageing is also associated with a decrease in the brain content of ω -3 PUFA, dietary supplementation with EPA and DHA was tested in old rats. The treatment reversed an age-related decline in the expression of GluR2 and NR2B glutamate receptor subunits in prefrontal cortex, hippocampus and striatum (Dyall *et al.*, 2007).

The fact that memory impairment due to vitamin A deficiency is reversible points to a specific physiological, opposed to a general developmental function of RA (Misner *et al.*, 2001; Cocco *et al.*, 2002). That may include long-term structural changes, however.

Cocco and colleagues found that vitamin A deprivation led to reduced sizes of pyramidal neurons in the hippocampus. In an eight-arm radial maze discrimination task it took 39 weeks of vitamin A deprivation to produce significant memory impairment in young mice, and this effect was not reversible with systemic injections of RA (150 mg/kg; Etchamendy *et al.*, 2003).

Although little is known about the physiological mechanisms that underlie effects of RA on cognitive functions, several pertinent processes are regulated or at least influenced by RA. These are neurotransmitter- and neurotrophin-signaling pathways, including genes for acetylcholine esterase and choline acetyl transferase (Malik *et al.*, 2000; Lane and Bailey, 2005), and the regulation of neuronal differentiation and even of neurogenesis in the dentate gyrus and subventricular zone of adult mammals (Crandall *et al.*, 2004; Wang *et al.*, 2005). That RA may play a role in the plasticity of neural networks has most recently been demonstrated by Chen and Napoli (2008). They report that all-*trans* RA increased dendritic spine formation and synapse staining (PSD-95, synaptophysin) in cultured hippocampal neurons within 30 min, indicating that RA directly affects the formation of synapses. These rapid changes were RAR α -dependent (as checked with siRNA and shRNA) and involved Ras activation, transient phosphorylation of kinase Erk1/2 and an effect on local dendritic translation of the glutamate receptor GluR1 (Chen and Napoli, 2008).

4.4. Connections between retinoid signaling and Alzheimer's disease

In a pioneering study Goodman investigated genetic linkages between RA signaling and neurological and psychiatric disorders (Goodman, 1998). Based on the observation that gene loci thought to be involved in AD were clustered around genes for CYP26 enzymes, RAR α , RXR β , RXR γ , CRABP-II, and retinol binding proteins, she hypothesized that a decreased availability of RA and subsequent dysregulation of retinoid genes and their targets contribute to late-onset AD (Goodman, 2006). Biochemical data support this idea as they indicate lower retinoid supply, transport and signaling as factors associated with the disease. For instance, ApoE is a transport protein for retinyl esters in chylomicrons (Ishibashi *et al.*, 1996), and the ApoE e2 allele has a better ability to carry retinoids and DHA (the putative RXR ligand). Compared to ApoE e4 the e2 allele then is associated with decreased risk of AD in humans and memory impairment in rats (Goodman and Pardee, 2002). The insulin-degrading enzyme, which also degrades A β plaques, has an RARE in its promoter, and its expression is induced by RA (Melino *et al.*, 1996). Since either increase of α -secretase or decrease of β -secretase can reduce the relative accumulation of A β , it was also an interesting finding that RA and BDNF shift APP processing towards the α -secretase pathway *in vitro* (Holback *et al.*, 2005). The promotor of the α -secretase gene (ADAM10) contains an RARE, and RA induces ADAM10 promotor-mediated gene expression *in vitro* (Prinzen *et al.*, 2005). Also in cell cultures, retinol destabilizes preformed A β fibrils (Ono *et al.*, 2004). Corcoran *et al.* (2004) discovered that after 1 year of vitamin A deprivation rats

developed A β deposits in the forebrain, this being associated with a loss of choline acetyl transferase positive neurons. As corroborating evidence they found lower expression of RAR α and of the RA-synthesizing enzyme RALDH-2 in cortex samples of AD patients (Corcoran *et al.*, 2004). While a contrasting report showed lower amyloid precursor proteins and β -secretase in retinoid deficient rats (Husson *et al.*, 2006), in general, RA activity seems to be beneficial with respect to memory functions. Acute administration of 150 mg/kg RA raised retinoid receptor levels in the hippocampus of aged mice, and this treatment alleviated deficits in memory performance of these animals (Etchamendy *et al.*, 2001; mingaud *et al.*, 2008). While we are still ignorant of the precise contribution of RAR/RXR signaling in AD, raising the levels of RA through dietary or pharmacological intervention appears to be a promising strategy. For this approach synthetic RAR/RXR ligands (with diminished toxicity compared to RA), the activation of RA-synthesizing RALDH enzymes or inhibition of RA-degrading CYP26 enzymes have been suggested (Goodman and Pardee, 2002).

5. Psychiatric disorders

The observation that retinoids participate in brain physiology and cognitive functions suggests they may also be involved in disorders of mood. In this area, interest was at first not caused by scientific research but came from clinical experience (Table 5).

5.1. Does RA treatment cause depression?

Since in 1982 isotretinoin (13-*cis* RA, Accutane) was approved by the American Food and Drug Administration for the treatment of cystic acne, this medication has been given to more than eight million patients worldwide (Bremner, 2003). Reports of suicides among the treated population raised public awareness and scientific interest about the possible relationship between isotretinoin administration and increased risk of depression and suicide. The first safety warnings about possible psychiatric side effects were issued in 1985, and since then extensive litigation resulted in the US. However, while RA remains the best available treatment against acne and several other dermatological disorders, the issue whether it causes depressive symptoms remains controversial. While a large number of case studies have been published that either support or reject the purported psychiatric effects of isotretinoin (Wysowski *et al.*, 2001; Bremner, 2003; Magin *et al.*, 2005), possible mechanisms have only recently been investigated.

Clinical depression is described as a state of intense sadness, melancholia or despair that has advanced to the point of being disruptive to an individual's social functioning or activities of daily life. One current idea about the etiology of depression proposes that this disease is the result of reduced neurogenesis in the hippocampus (Vogel, 2000; Santarelli *et al.*, 2003). This hypothesis was based on the findings that hippocampal vol-

ume is reduced in depressive patients as well as on observations that steroid hormones, which signal stress-induced depression, lower hippocampal neurogenesis. Moreover, anti-depressants have been shown to reverse these effects by promoting the formation of new nerve cells in the hippocampus (Cameron *et al.*, 1998; McEwen, 1999; Malberg *et al.*, 2000). Since RA is an important regulator of cell proliferation and neuronal differentiation, McCaffery and co-workers investigated whether 13-*cis* RA can affect neurogenesis in the hippocampus, which may thereby explain psychiatric side effects, in case these are substantiated. In contrast to short-term effects of retinoids or the replenishment of a vitamin A deficient diet, where RA was beneficial for cognitive functions, the chronic administration of a clinical dose (1 mg/kg/ day) of 13-*cis* RA suppressed hippocampal neurogenesis in mice (Sakai *et al.*, 2004) and interfered with spatial memory performance in a radial maze task (Crandall *et al.*, 2004). Here isotretinoin is believed to be isomerized to all-*trans* RA and/or 9-*cis* RA because the 13-*cis* isomer does not directly bind to RAR/RXR. As mentioned above, RA is endogenously synthesized in the hippocampus where it affects granule cells of the dentate gyrus (Luo *et al.*, 2004; Sakai *et al.*, 2004; Fig. 5). Since direct effects of RA on the physiology of neural functions including aminergic neurotransmitter systems and synaptic plasticity are also discussed (see Sections 3.1 and 4.3; O'Reilly *et al.*, 2007; Chen and Napoli, 2008), other lines of interaction between RA and the disorders of mood are not unlikely.

First, however, it must be established empirically that such a connection exists. Several clinical studies suggested a possible link between chronic use of oral isotretinoin and depression (Hazen *et al.*, 1983; Hull and Demkiw-Bartel, 2000; Bremner *et al.*, 2005; O'Reilly *et al.*, 2006). Bremner and others used functional brain imaging to test whether 13-*cis* RA would result in decreased metabolic activity in the orbitofrontal cortex, a feature seen in depressive patients (Bremner *et al.*, 1997, 2003). The data demonstrated that 4 months of isotretinoin, but not antibiotic treatment, decreased brain metabolism in the orbitofrontal cortex. However, treatment was not associated with any changes in depressive symptoms (Bremner *et al.*, 2005).

Tab. 5. RAR/RXR, PPAR/RXR signaling in psychiatric disorders

ligand/ receptor	major results	experimental approach	references
13- <i>cis</i> RA	possible influence on depression (conflicting results)	case reports	Hazen <i>et al.</i> , 1983; Hull and Demkiw- Bartel, 2000; Ferahas <i>et al.</i> , 2004; Bremner <i>et al.</i> , 2003; rev.: Wysowski <i>et al.</i> , 2001; Bremner, 2003; Magin <i>et al.</i> , 2005
13- <i>cis</i> RA	effect on cortical brain metabolism	brain imaging study	Bremner <i>et al.</i> , 2003, 2005
13- <i>cis</i> RA	reduced hippocampal neurogenesis and memory performance	chronic administration in mice, histology	Crandall <i>et al.</i> , 2004; Sakai <i>et al.</i> , 2004
13- <i>cis</i> RA	effects on depressive behaviour but not on anxiety	behavioral tests with mice	O'Reilly <i>et al.</i> , 2006
13- <i>cis</i> RA	no effect on depressive behaviour	behavioral tests with rats	Ferguson <i>et al.</i> , 2005
13- <i>cis</i> RA	no effect on depression	clinical studies	Jick <i>et al.</i> , 2000; Chia <i>et al.</i> , 2005; critique: Wysowski and Beitz, 2001; Kellett and Gawkrödger, 2005
RA, RAR α	involved in Schizophrenia	effects of nutrition, genetic evidence	Goodman, 1998; LaMantia, 1999; St.Clair <i>et al.</i> , 2005, Feng <i>et al.</i> , 2005; Rioux and Arnold, 2005, Goodman, 2006; Palha and Goodman, 2006; Wan <i>et al.</i> , 2006
PPAR α , RXR β	no association with Schizophrenia	genetic polymorphisms	Ishiguro <i>et al.</i> , 2002
RXR	involved in neurogenesis, autism	indirect evidence	rev.: McCaffery and Deutsch, 2005
ω -3 PUFA	lower risk of depression (conflicting results)	epidemiology	LLorente <i>et al.</i> , 2003; Peet and Stokes, 2005; Saduyu <i>et al.</i> , 2005; Lee <i>et al.</i> , 2006; Parker <i>et al.</i> , 2006; rev.: Schachter <i>et al.</i> , 2005

One animal study revealed that mice treated with isotretinoin displayed behavioural changes (O'Reilly *et al.*, 2006). In the forced swim test and the tail suspension test animals spent more time being immobile, which can be interpreted as depression-related behavior. No significant effects on anxiety were reported as assessed with behavior in the open field.

Although this result argues for an association between depression and isotretinoin, another behavioral study with rats (Ferguson *et al.*, 2005), several case reports (Ferahbas *et al.*, 2004; Chia *et al.*, 2005) and a large clinical study provide evidence to the contrary (Jick *et al.*, 2000). In a cohort study over a 3- to 4-months period, patients with moderate

to severe acne and treated with isotretinoin or antibiotics were monitored to determine whether they experienced increases in depressive symptoms during treatment. Data from this analysis show that there was no difference in severity or incidence of depressive symptoms when comparing isotretinoin- with antibiotic-treated patients (Chia *et al.*, 2005). A prospective study, which was completed by 16 patients, found even improvements in the Beck depression inventory and hopelessness scale during treatment with 13-*cis* RA. The positive effect of RA was significant for the cognitive-affective scale after the first 8 weeks of treatment (Kellett and Gawkrödger, 2005).

In a large population-based cohort study, data of 7535 isotretinoin and 14,376 oral antibiotic users with acne were analyzed. Relative risk estimates for newly diagnosed depression or psychosis, attempted suicide between treatment groups (isotretinoin vs. antibiotic treatment) or within treatment groups (before vs. after treatment) also gave no evidence that RA treatment is associated with an increased risk of depression, suicide or other psychiatric disorders (Jick *et al.*, 2000). It should be mentioned that this industry-sponsored study was criticized on methodological grounds. For instance, psychiatric diagnoses were recorded only when patients were hospitalized or evaluated by a psychiatrist for depression, and therefore changes in depressive mood symptoms below the clinical level could not be assessed (Wysowski and Beitz, 2001; Bremner, 2003). In their review of the clinical studies conducted so far Magin and colleagues conclude that the incidence of depression and suicide during isotretinoin therapy may be no greater than the background incidence and that no causal relationship had been demonstrated (Magin *et al.*, 2005; similar: Marqueling and Zane, 2007).

5.2. Relevance of RA signaling for schizophrenia as a developmental disease

Schizophrenia is a psychiatric diagnosis that describes a condition characterized by cognitive and affective deficits (e.g., disorganized thought, apathy), combined with positive symptoms, especially delusions or hallucinations. The underlying mental disorder results in significant social or occupational dysfunction. The diagnosis is based on the self-reported experience of the patient in combination with secondary clinical signs (American Psychiatric Association, 1994). Increasing evidence supports the hypothesis that schizophrenia is a neurodevelopmental disease leading to a disconnection between different brain regions. Since drugs that interfere with dopaminergic neurotransmission in the mesolimbic system are effective against psychotic symptoms, this neurotransmitter is implicated in the disorder. Goodman (1998) was the first to suggest that retinoids may be involved in the development of schizophrenia. She introduced three lines of evidence (1) developmental symptoms of vitamin A deprivation (2) genetic linkage studies supporting a relation between schizophrenia and RA signaling and (3) effects of RA on the dopaminergic system. (1) Evidence for this hypothesis was revealed by an investi-

gation of Ashkenazi Jews with and without symptoms related to schizophrenia (e.g., thought disorder or mental deficits) and their relatives. In this population, symptoms similar to those caused by embryonic RA intoxication, overlap frequently with a familial incidence of schizophrenia. These were enlarged ventricles, agenesis of the corpus callosum, microcephaly, and a variety of major and minor congenital malformations, the most prominent being craniofacial and digital anomalies (Palha and Goodman, 2006). Two studies demonstrated that prenatal nutritional deprivation increased the risk of schizophrenia later in life (Susser and Lin, 1992; St Clair *et al.*, 2005). In line with the idea of a developmental connection between retinoids and schizophrenia, animal studies showed that abnormalities in retinoid signaling (excess or deprivation of retinoic acid) caused changes in neural crest cell differentiation and migration during development (LaMantia, 1999). This may provide an explanation for the observed association between brain and craniofacial malformations and schizophrenia noted above. (2) The second line of evidence came from studies demonstrating that chromosomal regions previously implicated in schizophrenia contained genes involved in retinoid signaling (listed in: Goodman, 1998). A genetic linkage has been shown between schizophrenia and the G-protein Golf, a downstream signal of the D1 receptor, which is regulated by RA in some tissues (Schwab *et al.*, 2000; Wang and Liu, 2005). An analysis of the six RAR/RXR genes in schizophrenia patients for variants that affect protein structure found only a low frequency of structural variants not seen in controls. The conclusion of this study was that the RAR/RXR genes, while not a major cause in the etiology of schizophrenia in general, may play a role in some patients (Feng *et al.*, 2005). The link is somewhat corroborated by indications of altered RA signaling in the disease. Schizophrenic patients expressed higher levels of RAR α in the dentate gyrus when compared to control subjects. Overall expression of retinoid receptors also seemed to be elevated; however differences were not statistically significant (Rioux and Arnold, 2005). In the cerebral fluid of schizophrenic patients protein levels of retinoid transporters (ApoE and transthyretin tetramer) were significantly reduced when compared to healthy controls (Wan *et al.*, 2006). Recently, a genetic link was also found between a PPAR γ allele and cognitive decline (Yaffe *et al.*, 2008). (3) Schizophrenic patients have been treated with antipsychotic drugs (neuroleptics) since the 1950s, the first of these being a phenothiazine-derived compound. In 1975 it was discovered that phenothiazines bind to a dopamine receptor, now named D2R. Antipsychotic drugs tend to antagonize D2R of the mesolimbic system of the brain and have been shown to be effective in most schizophrenic patients. The third line of evidence supporting Goodman's hypothesis includes studies that implicate retinoids in the modulation of this pathway by influencing dopaminergic neurotransmission. This was mentioned when we discussed Parkinson's disease. Brain areas that receive dopaminergic innervation, e.g., corpus striatum, nucleus accumbens and olfactory tubercle, express retinoid receptors (Zetterström *et al.*, 1999), and expression of the D1 and D2 receptor was shown to be regulated by retinoic acid (Samad *et al.*, 1997) or reduced in retinoid

receptor deficient mice (Krezel *et al.*, 1998). Retinoid X receptors form heterodimers with other members of the superfamily of ligand-activated transcription factors. One of these, Nur77, has been implicated in the adaptation and homeostatic regulation of dopaminergic systems (Levesque and Rouillard, 2007). While these are all very indirect pieces of evidence, one study investigated mutations in the genes of RXR β , PPAR α and nuclear-related receptor 1 in schizophrenic patients. Polymorphisms were identified in RXR β and PPAR α genes, yet there was no association found between schizophrenia and the RXR β or PPAR α polymorphisms (Ishiguro *et al.*, 2002).

A case can be made for erroneous nuclear receptor activation in the development of autism (McCaffery and Deutsch, 2005). Similar to schizophrenia there are some indirect lines of evidence that link the diagnosis to RAR/RXR signal transduction. Macrocephaly has been described as an endophenotype of autism, where it results from excessive neurogenesis and gliogenesis during the first years after birth (Herbert *et al.*, 2003). Nuclear receptors, especially RAR/ RXR but also TR/RXR and steroid receptors are potent regulators of neuronal progenitor cell division and differentiation. Teratogens, including RA itself, that affect these pathways have been linked to autism. McCaffery and Deutsch suggested therefore that reduced transcriptional inhibition of RXR contributes to the development of autism (McCaffery and Deutsch, 2005).

5.3. Involvement of ω -3 fatty acids in psychiatric disorders

The last decades of the 20th Century saw a dramatic rise in the incidence of clinical depression. This observation could merely reflect changes in diagnosis, help seeking, etc., or it could indicate a real increase in the occurrence of the disease. In support of the latter interpretation it was suggested that strong increases in depression and neurological disorders were partially caused by higher consumption of vegetable oils rich in ω -6 fatty acids in North America and Europe (Smith, 1991; Hibbeln and Salem, 1995). Accordingly, a link between seafood intake and lower rate of depression, specifically postpartum depression was observed (Parker *et al.*, 2006), though not always (Llorente *et al.*, 2003), and several studies reported that a high ratio of ω -3 to ω -6 PUFA alleviated symptoms in psychiatric patients (Sagduyu *et al.*, 2005; Peet and Stokes, 2005; Lee *et al.*, 2006). However, 3 years ago, Schachter and colleagues conducted a thorough meta-analysis that seemed to have covered all controlled clinical trials concerning ω -3 fatty acids and psychiatric diseases (Schachter *et al.*, 2005). Out of 257 relevant reports 79 unique studies met their methodological criteria and could be evaluated. Depression and schizophrenia were the most frequently investigated disorders with 22 and 28 reports, respectively. The meta-analysis produced either no significant effects or no conclusive evidence regarding the use of EPA or DHA for depression, bipolar disorder, attention deficit/ hyperactivity disorder, obsessive-compulsive disorder and other diagnoses. A more recent review on ω -3 PUFA as treatment of mood disorders revealed significant beneficial effects in four out of

six double-blind placebo-controlled trials. The authors argue for studies clarifying the efficacy of EPA or DHA supplementation for unipolar and bipolar depressive disorders (Parker *et al.*, 2006). In contrast to the inconclusive results for most psychiatric conditions, the meta-analysis mentioned above found that three of four clinical trials investigating the treatment of schizophrenia reported significant effects in favor of EPA (Schachter *et al.*, 2005). While positive results with ω -3 PUFA on clinical symptoms may have been due to interference with inflammatory processes and other indirect paths of action (Sarsilmaz *et al.*, 2003), the evidence can also be interpreted to corroborate the concept that RXR signaling is directly involved in mood disorders.

6. Summary

In the context of increasing research on RAR/RXR and PPAR/RXR in the brain, a number of recent studies have explored the relevance of these nuclear receptors for neurological and psychiatric diseases. While in most cases the current evidence is far from conclusive, promising results point toward new therapeutic strategies. Both transcription factor families, RARs and PPARs possess anti-inflammatory properties that may be useful when pathological processes involve microglia, macrophages, astrocytes or activation of the peripheral immune system. This is of particular importance not only for multiple sclerosis but also for Alzheimer's, Parkinson's and other neurodegenerative diseases. Retinoids and PPAR γ agonists inhibit the formation of amyloid plaques or protect neurons from A β -induced degeneration. In this regard, selective agonists for specific receptors will most likely be employed in the future.

Indirect evidence and studies with transgenic animals indicate that retinoids are involved in the physiology of the extrapyramidal motor system and of the hippocampus. While direct neurophysiological functions of RA are likely, the precise mechanisms have yet to be discovered. Since several of the respective ligands have already been approved for other medical conditions, e.g., retinoids for dermatological diseases and cancer, PPAR agonists for diabetes, clinical studies for neurodegenerative diseases are in progress or soon to be expected. A lively but so far inconclusive debate has developed about the possible role of RA and nuclear receptors in the etiology of psychiatric disorders, especially clinical depression and schizophrenia.

Acknowledgements

Sabien van Neerven is supported by a Marie-Curie fellowship of the EURON Graduate School of Neuroscience. We are grateful for helpful comments from three anonymous reviewers.

References

- Abu-Abed, S., MacLean, G., Fraulob, V., Chambon, P., Petkovich, M., Dollé, P., 2002. Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. *Mech. Dev.* **110**, 173–177
- Alfos, S., Boucheron, C., Pallet, V., Higuieret, D., Enderlin, V., Be´racoche´ a, D., Jaffard, R., Higuieret, P., 2001. A retinoic acid receptor antagonist suppresses brain retinoic acid receptor overexpression and reverses a working memory deficit induced by chronic ethanol consumption in mice. *Alcohol Clin. Exp. Res.* **25**, 1506–1514
- Aloisi, F., Serafini, B., Adorini, L., 2000. Glia-T cell dialogue. *J. Neuroimmunol.* **107**, 111–117
- American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition (DSM-IV). <http://www.behavenet.com/capsules/disorders/dsm4TRclassification.htm>. Andlin-Sobocki, P., Jonnsson, N., Wittchen, H.-U., Olesen, J., 2005. Cost of disorders of the brain in Europe. *Eur. J. Neurol.* **12** (Suppl. 1), 1–27
- Ando, D.G., Clayton, J., Kono, D., Urban, J.L., Sercarz, E.E., 1989. Encephalitogenic T-cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell. Immunol.* **124**, 132–143
- Arrieta, O., Garcí’a-Navarrete, R., Zúñiga, S., Ordoñez, G., Ortiz, A., Palencia, G., Morales-Espinosa, D., Hernández-Pedro, N., Sotelo, J., 2005. Retinoic acid increases tissue and plasma contents of nerve growth factor and prevents neuropathy in diabetic mice. *Eur. J. Clin. Invest.* **35**, 201–297
- Baik, J.H., Picetti, R., Saiardi, A., Thiriet, G., Dierich, A., Depaulis, A., Le Meur, M., Borrelli, E., 1995. Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. *Nature* **377**, 424–428
- Bastien, J., Rochette-Egly, C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1–16
- Benani, A., Kre´marik-Bouillard, P., Bianchi, A., Netter, P., Minn, A., Dauc, a, M., 2003. Evidence for the presence of both peroxisome proliferator-activated receptors alpha and beta in the rat spinal cord. *J. Chem. Neuroanat.* **25**, 29–38
- Bernardo, A., Levi, G., Minghetti, L., 2000. Role of the peroxisome proliferator-activated receptor-gamma (PPAR-g) and its natural ligand 15-deoxy 12,14-prostaglandin J2 in the regulation of microglial functions. *Eur. J. Neurosci.* **12**, 2223
- Blomhoff, R., Blomhoff, H.K., 2006. Overview of retinoid metabolism and function. *J. Neurobiol.* **66**, 606–630
- Bourne, J.M., 2005. Dietary omega-3 fatty acids and psychiatry: mood, behaviour, stress, depression, dementia and aging. *J. Nutr. Health Aging* **9**, 31–38
- Braissant, O., Wahli, W., 1998. Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. *Endocrinology* **139**, 2748–2754
- Breider, T., Callebort, J., Heneka, M.T., Landreth, G., Launay, J.M., Hirsch, E.C., 2002. Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. *J. Neurochem.* **82**, 615–624
- Bremner, J.D., 2003. Does isotretinoin cause depression and suicide? *Psychopharmacol. Bull.* **37**, 64–78

- Bremner, J.D., Fani, N., Ashraf, A., Votaw, J.R., Brummer, M.E., Cummins, T., Vaccarino, V., Goodman, M.M., Reed, L., Siddiq, S., Nemeroff, C.B., 2005. Functional brain imaging alteration in acne patients treated with isotretinoin. *Am. J. Psychiatry* **162**, 983–991
- Bremner, J.D., Innis, R.B., Salomon, R.M., Staib, L.H., Ng, C.K., Miller, h.L., Bronen, R.A., Krystal, J.H., Duncan, J., Rich, D., Price, L.H., Malison, R., Dey, h., Soufer, R., Charney, D.S., 1997. Positron emission tomography measurement of cerebral metabolic correlates of tryptophan depletion-induced depressive relapse. *Arch. Gen. Psychiatry* **54**, 364–374
- Bremner, J.D., Vythilingam, M., Ng, C.K., Vermetten, E., Nazeer, A., Oren, D.A., Berman, R.M., Charney, D.S., 2003. Regional brain metabolic correlates of alpha methylparatyrosine-induced depressive symptoms: implications for the neural circuitry of depression. *JAMA* **289**, 3125–3134
- Calon, F., Lim, G.P., Yang, F., Morihara, T., Teter, B., Ubeda, O., Rostaing, P., Triller, A., Salem Jr., N., Ashe, K.H., Frautschy, S.A., Cole, G.M., 2004. Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model. *Neuron* **43**, 633–645
- Camacho, I.E., Serneels, L., Spittaels, K., Merchiers, P., Dominguez, D., De Strooper, B., 2004. Peroxisome-proliferator-activated receptor gamma induces a clearance mechanism for the amyloid-beta peptide. *J. Neurosci.* **24**, 10908–10917
- Cameron, h.A., Tanapat, P., Gould, E., 1998. Adrenal steroids and N-methyl Daspartate receptor activation regulate neurogenesis in the dentate gyrus of adult rats through a common pathway. *Neuroscience* **82**, 349–354
- Cañón, E., Cosgaya, J.M., Scsucova, S., Aranda, A., 2004. Rapid effects of retinoic acid on CREB and ERK phosphorylation in neuronal cells. *Mol. Biol. Cell* **15**, 5583–5592
- Carta, M., Stancampiano, R., Tronci, E., Collu, M., Usiello, A., Morelli, M., Fadda, F., 2006. Vitamin A deficiency induces motor impairments and striatal cholinergic dysfunction in rats. *Neuroscience* **139**, 1163–1172
- Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J., 2001. Nuclear receptors and lipid physiology: opening the X-files. *Science* **294**, 1866–1870
- Chen, N., Napoli, J.L., 2008. All-*trans*-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARa. *FASEB J.* **22**, 236–245
- Chia, C.Y., Lane, W., Chibnall, J., Allen, A., Siegfried, E., 2005. Isotretinoin therapy and mood changes in adolescents with moderate to severe acne: a cohort study. *Arch. Dermatol.* **141**, 557–560
- Chiang, M.-Y., Misner, D., Kempermann, G., Schikorski, T., Gigue`re, V., Sucov, h.M., Gage, F.H., Stevens, C.F., Evans, R.M., 1998. An essential role for retinoid receptors RARb and RXRg in long-term potentiation and depression. *Neuron* **21**, 1353–1361
- Cimini, A., Benedetti, E., Cristiano, L., Sebastiani, P., D'Amico, M.A., D'Angelo, B., Di Loreto, S., 2005. Expression of peroxisome proliferator-activated receptors (PPARs) and retinoic acid receptors (RXRs) in rat cortical neurons. *Neuroscience* **130**, 325–337
- Cocco, S., Diaz, G., Stancampiano, R., Diana, A., Carta, M., Curreli, R., Sarais, L., Fadda, F., 2002. Vitamin A deficiency produces spatial learning and memory impairment in rats. *Neuroscience* **115**, 475–482
- Corcoran, J., Maden, M., 1999. Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nat. Neurosci.* **2**, 307–308

- Corcoran, J., Shroot, B., Pizzey, J., Maden, M., 2000. The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *J. Cell Sci.* **113**, 2567–2574
- Corcoran, J., So, P.-L., Barber, R.D., Vincent, K.J., Mazarakis, N.D., Mitrophanous, K.A., Kingsman, S.M., Maden, M., 2002a. Retinoic acid receptor beta2 and neurite outgrowth in the adult mouse spinal cord *in vitro*. *J. Cell Sci.* **115**, 3779–3786
- Corcoran, J., So, P.-L., Maden, M., 2002b. Absence of retinoids can induce motoneuron disease in the adult rat and a reinoid defect is present in motoneuron disease patients. *J. Cell Sci.* **115**, 4735–4741
- Corcoran, J., So, P.-L., Maden, M., 2004. Disruption of the retinoid signalling pathway causes a deposition of amyloid b in the adult brain. *Eur. J. Neurosci.* **20**, 896–902
- Correale, J., Gilmore, W., McMillan, M., Li, S., McCarthy, K., Le, T., Weiner, L.P., 1995. Patterns of cytokine secretion by autoreactive proteolipid protein-specific T cell clones during the course of multiple sclerosis. *J. Immunol.* **154**, 2959–2968
- Cosgaya, J.M., Aranda, A., 2001. Nerve growth factor activates the RARb2 promoter by a Ras-dependent mechanism. *J. Neurochem.* **76**, 661–670
- Crandall, J., Sakai, Y., Zhang, J., Koul, O., mineur, Y., Crusio, W.E., McCaffery, P., 2004. 13-*cis*-Retinoic acid suppresses hippocampal cell division and hippocampaldependent learning in mice. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 5111–5116
- Crocker, S.F., Costain, W.J., Robertson, h.A., 2006. DNA microarray analysis of striatal gene expression in symptomatic transgenic Huntington's mice (R6/2) reveals neuroinflammation and insulin associations. *Brain Res.* **1088**, 176–186
- D'Abramo, C., Massone, S., Zingg, J.-M., Pizzuti, A., Marambaud, P., Dalla Piccola, B., Azzi, A., Marinari, U.M., Pronzato, M.A., Ricciarelli, R., 2005. Role of peroxisome proliferator-activated receptor g in amyloid precursor protein processing and amyloid b-mediated cell death. *Biochem. J.* **391**, 693–698
- Dangond, F., Hwang, D., Camelo, S., Pasinelli, P., Frosch, M.P., Stephanopoulos, G., Stephanopoulos, G., Brown Jr., R.H., Gullans, S.R., 2004. Molecular signature of late-stage human ALS revealed by expression profiling of postmortem spinal cord gray matter. *Physiol. Genomics* **16**, 229–239
- Dasgupta, S., Roy, A., Jana, M., Hartley, D.M., Pahan, K., 2007. Gemfibrozil ameliorates relapsing-remitting experimental autoimmune encephalomyelitis independent of peroxisome proliferator-activated receptor-alpha. *Mol. Pharmacol.* **72**, 934–946
- Dawson, h.D., Collins, G., Pyle, R., Key, M., Taub, D.D., 2008. Retinoic acid receptoralpha mediates human T-cell activation and Th2 cytokine production. *BMC BMC Immunol.* 2008 Apr **16**;9:16
- Dehmer, T., Heneka, M.T., Sastre, M., Dichgans, J., Schulz, J.B., 2004. Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I kappa B alpha induction and block of NF kappa B and iNOS activation. *J. Neurochem.* **88** 494–501
- Delayre-Orthez, C., Becker, J., Guenon, I., Lagente, V., Auwerx, J., Frossard, N., Pons, F., 2005. PPARa down-regulates airway inflammation induced by lipopolysaccharide in the mouse. *Respiratory Res.* **6**, doi:10.1186/1465-9921-6-91
- Delerive, P., Fruchart, J.C., Staels, B., 2001. Peroxisome proliferator-activated receptors in inflammation control. *J. Endocrinol.* **169**, 453–459

- Diab, A., Hussai, R.Z., Lovett-Racke, A.E., Chavis, J.A., Drew, P.D., Racke, M.K., 2004. Ligands for the peroxisome proliferator-activated receptor- γ and retinoid X receptor exert additive anti-inflammatory effects on experimental autoimmune encephalomyelitis. *J. Immunol.* **148**, 116–126
- Dirks, P., Tieding, S., Schneider, I., Mey, J., Weiler, R., 2004. Characterization of retinoic acid neuromodulation in the carp retina. *J. Neurosci. Res.* **78**, 177–185
- Dyall, S.C., Michael, G.J., Whelpton, R., Scott, A.G., Michael-Titus, A.T., 2007. Dietary enrichment with omega-3 polyunsaturated fatty acids reverses age-related decreases in the GluR2 and NR2B glutamate receptor subunits in rat forebrain. *Neurobiol. Aging* **28**, 424–439
- Enderlin, V., Pallet, V., Alfios, S., Dargelos, E., Jaffard, R., Garcin, h., Higuieret, P., 1997. Age-related decreases in mRNA for brain nuclear receptors and target genes are reversed by retinoic acid treatment. *Neurosci. Lett.* **229**, 125–129
- Etchamendy, N., Enderlin, V., Marighetto, A., Pallet, V., Higuieret, P., Jaffard, R., 2003. Vitamin A deficiency and relational memory deficit in adult mice: relationships with changes in brain retinoid signalling. *Behav. Brain Res.* **145**, 37–49
- Etchamendy, N., Enderlin, V., Marighetto, A., Vouimba, R.-M., Pallet, V., Jaffard, R., Higuieret, P., 2001. Alleviation of a selective age-related relational memory deficit in mice by pharmacologically induced normalization of brain retinoid signaling. *J. Neurosci.* **21**, 6423–6429
- Feinstein, D.L., 2003. Therapeutic potential of peroxisome proliferator-activated receptor agonists for neurological disease. *Diab. Technol. Ther.* **5**, 67–73
- Feinstein, D.L., Galea, E., Gavriluk, V., Brosnan, C.F., Whitacre, C.C., Dumitrescu- Ozimek, L., Landreth, G.E., Perashadsingh, h.A., Weinberg, G., Heneka, M.T., 2002. Peroxisome proliferator-activated receptor- γ agonists prevent experimental autoimmune encephalomyelitis. *Ann. Neurol.* **51**, 694–702
- Feng, J., Chen, J., Yan, J., Jones, I.R., Craddock, N., Cook Jr., E.H., Goldman, D., Heston, patients with schizophrenia and other psychiatric diseases. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **133**, 50–53
- Ferahbas, A., Turan, M.T., Esel, E., Utas, S., Kutlugun, C., Kilic, C.G., 2004. A pilot study evaluating anxiety and depressive scores in acne patients treated with isotretinoin. *J. Dermatolog. Treat.* **15**, 153–157
- Ferguson, S.A., Cisneros, F.J., Gough, B., Hanig, J.P., Berry, K.J., 2005. Chronic oral treatment with 13-*cis*-retinoic acid (isotretinoin) or all-*trans*-retinoic acid does not alter depression-like behaviors in rats. *Toxicol. Sci.* **87**, 451–459
- Fisher, C.M., 1989. 'Catatonia' due to disulfiram toxicity. *Arch. Neurol.* **46**, 798–804. Galter, D., Carmine, A., Buervenich, S., Dueter, G., Olson, L., 2003. Distribution of class I, III and IV alcohol dehydrogenase mRNAs in the adult rat, mouse and human brain. *Eur. J. Biochem.* **270**, 1316–1326
- Gamoh, S., Hashimoto, M., Hossain, S., Masumura, S., 2001. Chronic administration of docosahexaenoic acid improves the performance of radial arm maze task in aged rats. *Clin. Exp. Pharmacol. Physiol.* **28**, 266–270
- Genovese, T., Mazzon, E., Di Paola, R., Cannavo, G., Muia, C., Bramanti, P., Cuzzocrea, S., 2005. Role of endogenous ligands for the peroxisome proliferators activated receptors α in the secondary damage in experimental spinal cord trauma. *Exp. Neurol.* **194**, 267–278.

- Gervois, P., Chopin-Delannoy, S., Fadel, A., Dubois, G., Kosykh, V., Fruchart, J.C., Najib, J., Laudet, V., Staels, B., 1999. Fibrates increase human REV-ERB α expression in liver via a novel peroxisome proliferator-activated receptor response element. *Mol. Endocrinol.* **13**, 400–409
- Ghate, A., Befort, K., Becker, J.A., Filliol, D., Bole-Feysot, C., Demebele, D., Jost, B., Koch, M., Kieffer, B.L., 2007. Identification of novel striatal genes by expression profiling in adult mouse brain. *Neuroscience* **146**, 1182–1192
- Giguere, V., Ong, E.S., Segui, P., Evans, R.M., 1987. Identification of a receptor for the morphogen retinoic acid. *Nature* **330**, 624–629
- Glass, C.K., Rosenfeld, M.G., 2000. The coregulator exchange in transcriptional functions of nuclear receptors. *Genes Dev.* **14**, 121–141
- Goodman, A.B., 1998. Three independent lines of evidence suggest retinoids as causal to schizophrenia. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7240–7244
- Goodman, A.B., 2006. Retinoid receptors, transporters, and metabolizers as therapeutic targets in late onset Alzheimer Disease. *J. Cell. Physiol.* **209**, 598–603
- Goodman, A.B., Pardee, A.B., 2002. Evidence for defective retinoid transport and function in late onset Alzheimer's disease. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2901–2905
- Hamilton, G., Proitsi, P., Jehu, L., Morgan, A., Williams, J., O'Donovan, M.C., Owen, M.J., Powell, J.F., Lovestone, S., 2007. Candidate gene association study of insulin signaling genes and Alzheimer's disease: evidence for SOS2, PCK1, and PPARGgamma as susceptibility loci. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **144**, 508–516
- Harbige, L.S., 2003. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. *Lipids* **38**, 323–341
- Hashimoto, M., Hossain, S., Shimada, T., Shido, O., 2006. Docosahexaenoic acid-induced protective effect against impaired learning in amyloid beta-infused rats is associated with increased synaptosomal membrane fluidity. *Clin. Exp. Pharmacol. Physiol.* **33**, 934–939
- Hazen, P.G., Carney, J.F., Walker, A.E., Stewart, J.J., 1983. Depression—a side effect of 13-*cis*-retinoic acid therapy. *J. Am. Acad. Dermatol.* **9**, 278–279
- Hemmer, B., Nessler, S., Zhou, D., Kieseier, B., Hartung, h.P., 2006. Immunopathogenesis and immunotherapy of multiple sclerosis. *Nat. Clin. Pract. Neurol.* **2**, 201–211
- Heneka, M.T., Sastre, M., Dumitrescu-Ozimek, L., Hanke, A., Dewachter, I., Kuiperi, C., O'Banion, K., Klockgether, T., Van Leuven, F., Landreth, G.E., 2005. Acute treatment with the PPARGgamma agonist pioglitazone and ibuprofen reduces glial inflammation and Abeta1–42 levels in APPV717I transgenic mice. *Brain* **128**, 1442–1453
- Herbert, M.R., Ziegler, D.A., Deutsch, C.K., O'Brien, L.M., Lange, N., Bakardjiev, A., Hodgson, J., Adrien, K.T., Steele, S., Makris, N., Kennedy, D., Harris, G.J., Caviness Jr., V.S., 2003. Dissociations of cerebral cortex, subcortical and cerebral white matter volumes in autistic boys. *Brain* **126**, 1182–1192
- Heude, B., Ducimetiere, P., Berr, C., 2003. Cognitive decline and fatty acid composition of erythrocyte membranes—the EVA Study. *Am. J. Clin. Nutr.* **77**, 803–808
- Hibbeln, J.R., Salem Jr., N., 1995. Dietary polyunsaturated fats and depression: when cholesterol alone doesn't satisfy. *Am. J. Clin. Nutr.* **62**, 1–9

- Hirsch, E.C., Breidert, T., Rousset, E., Hunot, S., Hartmann, A., Michel, P.P., 2003. The role of glial reaction and inflammation in Parkinson's disease. *Ann. N. Y. Acad. Sci.* **991**, 214–228
- Holback, S., Adlerz, L., Iverfeldt, K., 2005. Increased processing of APLP2 and APP with concomitant formation of APP intracellular domains in BDNF and retinoic acid differentiated human neuroblastoma cells. *J. Neurochem.* **95**, 1059–1068
- Hoozemans, J.J.M., Veerhuis, R., Rozemuller, J.M., Eikelenboom, P., 2006. Neuroinflammation and regeneration in the early stages of Alzheimer's disease pathology. *Int. J. Dev. Neurosci.* **24**, 157–165
- Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., Cole, G., 1996. Correlative memory deficits, Ab elevation and amyloid plaques in transgenic mice. *Science* **274**, 99–102
- Hull, P.R., Demkiw-Bartel, C., 2000. Isotretinoin use in acne: prospective evaluation of adverse events. *J. Cutan. Med. Surg.* **4**, 66–70
- Husson, M., Enderlin, V., Delacourte, A., Ghenimi, N., Alfos, S., Pallet, V., Huguieret, P., 2006. Retinoic acid normalizes nuclear receptor mediated hypo-expression of proteins involved in beta-amyloid deposits in the cerebral cortex of vitamin A deprived rats. *Neurobiol. Dis.* **23**, 1–10
- Ishibashi, S., Perrey, S., Chen, Z., Osuga, J., Shimada, M., Ohashi, K., Harada, K., Yazaki, Y., Yamada, N., 1996. Role of the low density lipoprotein (LDL) receptor pathway in the metabolism of chylomicron remnants. A quantitative study in knockout mice lacking the LDL receptor, apolipoprotein E, or both. *J. Biol. Chem.* **271**, 22422–22427
- Ishiguro, h., Okubo, Y., Ohtsuki, T., Yamakawa-Kobayashi, K., Arinami, I., 2002. Mutation analysis of the retinoid X receptor beta, nuclear-related receptor 1, and peroxisome proliferator-activated receptor alpha genes in schizophrenia and alcohol dependence: possible haplotype association of nuclear-related receptor 1 gene to alcohol dependence. *Am. J. Med. Genet.* **114**, 15–23
- Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645–650
- Iwashita, A., Muramatsu, Y., Yamazaki, T., Muramoto, M., Kita, Y., Yamazaki, S., Mihara, K., Moriguchi, A., Matsuo, N., 2007. Neuroprotective efficacy of the peroxisome proliferator-activated receptor δ -selective agonists *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.* **320**, 1087–1096
- Jackson, C.E., Bryan, W.W., 1998. Amyotrophic lateral sclerosis. *Semin. Neurol.* **18**, 27–39
- Jiang, Y.M., Yamamoto, M., Kobayashi, Y., Yoshihara, T., Liang, Y., Terao, S., Takeuchi, h., Ishigaki, S., Katsuno, M., Adachi, h., Niwa, J., Tanaka, F., Doyu, M., Yoshida, M., Hashizume, Y., Sobue, G., 2005. Gene expression profile of spinal motor neurons in sporadic amyotrophic lateral sclerosis. *Ann. Neurol.* **57**, 236–251
- Jick, S.S., Kremers, h.M., Vasilakis-Scaramozza, C., 2000. Isotretinoin use and risk of depression, psychotic symptoms, suicide, and attempted suicide. *Arch. Dermatol.* **136**, 1231–1236
- Jokic, N., Ling, Y.Y., Ward, R.E., Michael-Titus, A.T., Priestley, J.V., Malaspina, A., 2007. Retinoid receptors in chronic degeneration of the spinal cord: observations in a rat model of amyotrophic lateral sclerosis. *J. Neurochem.* **103**, 1821–1833
- Jump, D.B., 2002. The biochemistry of n – 3 polyunsaturated fatty acids. *J. Biol. Chem.* **277**, 8755–8758
- Kalmijn, S., Launer, L.J., Ott, A., Witteman, J.C., Hofman, A., Breteler, M.M., 1997
- Dietary fat intake and the risk of incident dementia in the Rotterdam Study. *Ann. Neurol.* **42**, 776–782

- Kampmann, E., Johann, S., van Neerven, S., Beyer, C., Mey, J., 2008. Anti-inflammatory effect of retinoic acid on prostaglandin synthesis in cultured cortical astrocytes. *J Neurochem.* **106**, 320–32
- Kane, M.A., Chen, N., Sparks, S., Napoli, J.L., 2005. Quantification of endogenous retinoic acid in limited biological samples by LC/MS/MS. *Biochem. J.* **388**, 363–369
- Kellett, S.C., Gawkrödger, D.J., 2005. A prospective study of the responsiveness of depression and suicidal ideation in acne patients to different phases of isotretinoin therapy. *Eur. J. Dermatol.* **15**, 484–488
- Kennedy, M.K., Torrance, D.S., Picha, K.S., Mohler, K.M., 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol.* **149**, 2496–2505
- Kern, J., Schrage, K., Koopmans, G.C., Joosten, E.A., McCaffery, P., Mey, J., 2007. Characterization of retinaldehyde dehydrogenase-2 induction in NG2-positive glia after spinal cord contusion injury. *Int. J. Dev. Neurosci.* **25**, 7–16
- Kiaei, M., Kipiani, K., Chen, J., Calingasan, N.Y., Beal, M.F., 2005. Peroxisome proliferator-activated receptor-gamma agonist extends survival in transgenic mouse model of amyotrophic lateral sclerosis. *Exp. Neurol.* **191**, 331–336
- Kielian, T., Drew, P.D., 2003. Effects of peroxisome proliferator-activated receptor-gamma agonists on central nervous system inflammation. *J. Neurosci. Res.* **71**, 315–325
- Kim, B., Lee, J.-H., Yang, M.-S., Jou, I., Joe, E., 2008. Retinoic acid enhances PGE2 production through increased expression of cyclooxygenase-2 and microsomal PGE synthase-1 in rat brain microglia. *J. Neurosci. Res.* **86**, 1353–1360
- King, V.R., Huang, W.L., Dyall, S.C., Curran, O.E., Priestley, J.V., Michael-Titus, A.T., 2006. Omega-3 fatty acids improve recovery, whereas omega-6 fatty acids worsen outcome, after spinal cord injury in the adult rat. *J. Neurosci.* **26**, 4672–4680
- Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K., Evans, R.M., 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7355–7359
- Klotz, L., Diehl, L., Dani, I., Neumann, h., von Oppen, N., Dolf, A., Endl, E., Klockgether, T., Engelhardt, B., Knolle, P., 2007. Brain endothelial PPARgamma controls inflammation-induced CD4+ T cell adhesion and transmigration *in vitro*. *J. Neuroimmunol.* **190**, 34–43
- Koivisto, A.M., Helisalmi, S., Pihlajamäki, J., Hiltunen, M., Koivisto, K., Moilanen, L., Kuusisto, J., Helkala, E.L., Hanninen, T., Kervinen, K., Kesaniemi, Y.A., Laakso, M., Soininen, h., 2006. Association analysis of peroxisome proliferator-activated receptor gamma polymorphisms and late onset Alzheimer's disease in the Finnish population. *Dement. Geriatr. Cogn. Disord.* **22**, 449–453
- Kota, B.P., Huang, T.H., Roufogalis, B.D., 2005. An overview on biological mechanisms of PPARs. *Pharmacol. Res.* **51**, 85–94
- Környei, Z., Gócza, E., Rühl, R., Orsolits, B., Vörös, E., Szabó, B., Vágovits, B., Madarász, E., 2007. Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis. *FASEB J.* **21**, 2496–2509
- Kreisler, A., Gele, P., Wiart, J.F., Lhermitte, M., Destee, A., Bordet, R., 2007. Lipidlowering drugs in the MPTP mouse model of Parkinson's disease: Fenofibrate has a neuroprotective effect, whereas bezafibrate and HMG-CoA reductase inhibitors do not. *Brain Res.* **1135**, 77–84

- Krezel, W., Ghyselinck, N.B., Samad, T.A., Dupe', V., Kastner, P., Borelli, E., Chambon, P., 1998. Impaired locomotion and dopamine signaling in retinoid receptor mutant mice. *Science* **279**, 864–867
- Krezel, W., Kastner, P., Chambon, P., 1999. Differential expression of retinoid receptors in the adult mouse central nervous system. *Neuroscience* **89**, 1291–1300
- LaMantia, A.-S., 1999. Forebrain induction, retinoic acid, and vulnerability to schizophrenia: Insights from molecular and genetic analysis in developing mice. *Biol. Psychiat.* **46**, 19–30
- Lane, M.A., Bailey, S.J., 2005. Role of retinoid signalling in the adult brain. *Progr. Neurobiol.* **75**, 275–293
- Laplante, D., Attal, N., Sauron, B., de Billy, A., Dubois, B., 1992. Lesions of basal ganglia due to disulfiram neurotoxicity. *J. Neurol. Neurosurg. Psychiatry* **55**, 925–929. Lauritzen, I., Blondeau, N., Heurteaux, C., Widmann, C., Romey, G., Lazdunski, M., 2000. Polyunsaturated fatty acids are potent neuroprotectors. *EMBO J.* **19**, 1784–1793
- Lee, M.K., Marszalek, J.R., Cleveland, D.W., 1994. A mutant neurofilament subunit causes massive, selective motor neuron death: implications for the pathogenesis of human motor neuron disease. *Neuron* **13**, 975–988
- Lee, S., Gura, K.M., Kim, S., Arsenault, D.A., Bistran, B.R., Puder, M., 2006. Current clinical applications of omega-6 and omega-3 fatty acids. *Nutr. Clin. Pract.* **21**, 323–341
- Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **272**, 3406–3410
- Lengqvist, J., Mata, d.U., Bergman, A.C., Willson, T.M., Sjoval, J., Perlmann, T., Griffiths, W.J., 2004. Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Mol. Cell. Proteomics* **3**, 692–703
- Levesque, D., Rouillard, C., 2007. Nur77 and retinoid X receptors: crucial factors in dopamine-related neuroadaptation. *Trends Neurosci.* **30**, 22–30
- Li, A.C., Brown, K.K., Silvestre, M.J., Willson, T.M., Palinski, W., Glass, C.K., 2000. Peroxisome proliferator-activated receptor g ligands inhibit development of atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest.* **106**, 523–531
- Li, J.-J., Dont, Z., Dawson, M.I., Colburn, N., 1996. Inhibition of tumor promoter-induced transformation by retinoids that transrepress AP-1 without transactivating retinoic acid response element. *Cancer Res.* **56**, 483–489
- Liang, Y., Lin, S., Beyer, T., Zhang, Y., Wu, X., Bales, K.R., DeMattos, R.B., May, P.C., Li, S.D., Jiang, X.-C., Eacho, P.I., Cao, G., Paul, S.M., 2005. A liver X receptor and retinoid X receptor heterodimer mediates apolipoprotein E expression, secretion and cholesterol homeostasis in astrocytes. *J. Neurochem.* **88**, 623–630
- Liao, W.L., Liu, F.C., 2005. RARbeta isoform-specific regulation of DARPP-32 gene expression: an ectopic expression study in the developing rat telencephalon. *Eur. J. Neurosci.* **21**, 3262–3268
- Lim, G.P., Calon, F., Moriham, T., Yang, F., Teter, B., Ubeda, O., Salem Jr., N., Frautschy, S.A., Cole, G.M., 2005. A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. *J. Neurosci.* **25**, 3032–3040
- Lin, M.T., Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443**, 787–795

- Llorente, A.M., Jensen, C.L., Voigt, R.G., Fraley, J.K., Berretta, M.C., Heird, W.C., 2003. Effect of maternal docosahexaenoic acid supplementation on postpartum depression and information processing. *Am. J. Obstet. Gynecol.* **188**, 1348–1353
- Lovett-Racke, A.E., Hussain, R.Z., Northrop, S., Choy, J., Rocchini, A., Matthes, L., Chavis, J.A., Diab, A., Drew, P.D., Racke, M.K., 2004. Peroxisome proliferator-activated receptor alpha agonists as therapy for autoimmune disease. *J. Immunol.* **172**, 5790–5798
- Lovett-Racke, A.E., Racke, M.K., 2002. Retinoic acid promotes the development of Th2-like human myelin basic protein-reactive T cells. *Cell. Immunol.* **215**, 54–60
- Luo, T., Wagner, E., Gruen, F., Draeger, U.C., 2004. Retinoic acid signaling in the brain marks formation of optic projections, maturation of the dorsal telencephalon, and function of limbic sites. *J. Comp. Neurol.* **470**, 297–316
- Luthi-Carter, R., Strand, A., Peters, N.L., Solano, S.M., Hollingsworth, Z.R., Menon, A.S., Frey, A.S., Spektor, B.S., Penney, E.B., Schilling, G., Ross, C.A., Borchelt, D.R., Tapscott, S.J., Young, A.B., Cha, J.H., Olson, J.M., 2000. Decreased expression of striatal signaling genes in a mouse model of Huntington's disease. *Hum. Mol. Gen.* **9**, 1259–1271
- Magin, P., Pond, D., Smith, W., 2005. Isotretinoin, depression and suicide: a review of the evidence. *Br. J. Gen. Pract.* **55**, 134–138
- Malaspina, A., Kaushik, N., de Belleruche, J., 2001. Differential expression of 14 gene in amyotrophic lateral sclerosis spinal cord detected using gridded cDNA arrays. *J. Neurochem.* **77**, 132–145
- Malberg, J.E., Eisch, A.J., Nestler, E.J., Duman, R.S., 2000. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J. Neurosci.* **20**, 9104–9110
- Malik, M.A., Blusztajn, J.K., Greenwood, C.E., 2000. Nutrients as trophic factors in neurons and the central nervous system: role of retinoic acid. *J. Nutr. Biochem.* **11**, 2–13
- Marqueling, A.L., Zane, L.T., 2007. Depression and suicidal behavior in acne patients treated with Isotretinoin: a systematic review. *Semin. Cutan. Med. Surg.* **26**, 210–220
- Massacesi, L., Abbamondi, A.L., Giorgi, C., Sarlo, F., Lolli, F., Amaducci, L., 1987. Suppression of experimental allergic encephalomyelitis by retinoic acid. *J. Neurol. Sci.* **80**, 55–64
- Massacesi, L., Castigli, E., Vergelli, M., Olivetto, J., Abbamondi, A.L., Sarlo, F., Amaducci, L., 1991. Immunosuppressive activity of 13-*cis*-retinoic acid and prevention of experimental autoimmune encephalomyelitis in rats. *J. Clin. Invest.* **88**, 1331–1337
- Mata de Urquiza, A., Liu, S., Sjöberg, M., Zetterström, R.H., Griffiths, W., Sjövall, J., Perlmann, T., 2000. Docosahexaenoic acid, a ligand for the retinoic X receptor in mouse brain. *Science* **290**, 2140–2144
- McCaffery, P., Deutsch, C.K., 2005. Macrocephaly and the control of brain growth in autistic disorders. *Prog. Neurobiol.* **77**, 38–56
- McCaffery, P., Draeger, U.C., 1994. High levels of retinoic acid-generating dehydrogenases in the meso-telencephalic dopamine system. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7772–7776
- McCaffery, P., Koul, O., Smith, D., Napoli, J.L., Chen, N., Ullman, D., 2004. Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum. *Dev. Brain Res.* **153**, 233–241
- McEwen, B.S., 1999. Stress and hippocampal plasticity. *Ann. Rev. Neurosci.* **22**, 105–122

- Melino, G., Draoui, M., Bernardini, S., Bellincampi, L., Reichert, U., Cohen, P., 1996. Regulation by retinoic acid of insulin-degrading enzyme and of a related endoprotease in human neuroblastoma cell lines. *Cell Growth Diff.* **7**, 787–796
- Mey, J., 2001. Retinoic acid as a regulator of cytokine signaling after nerve injury. *Z. Naturforsch. C* **56c**, 163–176
- Mey, J., 2006. A new target for CNS injury? The role of retinoic acid signaling after nerve lesions. *J. Neurobiol.* **66**, 757–779
- Mey, J., McCaffery, P., 2004. Retinoic acid signaling in the nervous system of adult vertebrates. *Neuroscientist* **10**, 409–421
- Mey, J., Morassutti, D., Brook, G., Liu, R.-H., Zhang, Y.-P., Koopmans, G., McCaffery, P., 2005. Retinoic acid synthesis by a population of NG-2 positive cells in the injured spinal cord. *Eur. J. Neurosci.* **21**, 1555–1568
- Mingaud, F., Mormede, C., Etchamendy, N., Mons, N., Niedergang, B., Wietrzycz, M., Pallet, V., Jaffard, R., Krezel, W., Huguieret, P., Marighetto, A., 2008. Retinoid hyposignaling contributes to aging-related decline in hippocampal function in short term/working memory organization and long-term declarative memory encoding in mice. *J. Neurosci.* **28**, 279–291
- Misner, D.L., Jacobs, S., Shimizu, Y., de Urquiza, A.M., Solomin, L., Perlmann, T., De Luca, L.M., Stevens, C.F., Evans, R.M., 2001. Vitamin A deprivation results in reversible loss of hippocampal long-term synaptic plasticity. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 11714–11719
- Moon, D.O., Kim, K.C., Jin, C.Y., Han, M.H., Park, C., Lee, K.J., Park, Y.M., Choi, Y.H., Kim, G.Y., 2007. Inhibitory effects of eicosapentaenoic acid on lipopolysaccharide-induced activation in BV2 microglia. *Int. Immunopharmacol.* **7**, 222–229
- Moraes, L.A., Piqueras, L., Bishop-Bailey, D., 2006. Peroxisome proliferator-activated receptors and inflammation. *Pharmacol. Ther.* **110**, 371–385
- Moreno, S., Farioli-Vecchioli, S., Ceru, M.P., 2004. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience* **123**, 131–145
- Morris, M.C., Evans, D.A., Tangney, C.C., Bienias, J.L., Wilson, R.S., 2006. Associations of vegetable and fruit consumption with age-related cognitive change. *Neurology* **67**, 1370–1376
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., Cheroutre, h., 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260
- Mudher, A., Lovestone, S., 2002. Alzheimer's disease—do tauists and baptists finally shake hands? *Trends Neurosci.* **25**, 22–25
- Natarajan, C., Bright, J.J., 2002. Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. *Genes Immun.* **3**, 59–70
- Natarajan, C., Muthian, G., Barak, Y., Evans, R.M., Bright, J.J., 2003. Peroxisome proliferator-activated receptor-gamma-deficient heterozygous mice develop an exacerbated neural antigen-induced Th1 response and experimental allergic encephalomyelitis. *J. Immunol.* **171**, 5743–5750
- Nelson, A.M., Gilliland, K.L., Cong, Z., Thiboutot, D.M., 2006. 13-*cis* Retinoic acid induces apoptosis and cell cycle arrest in human SEB-1 sebocytes. *J. Invest. Dermatol.* **126**, 2178–2189

- O'Reilly, K.C., Shumake, J., Gonzalez-Lima, F., Lane, M.A., Bailey, S.J., 2006. Chronic administration of 13-*cis*-retinoic acid increases depression-related behavior in mice. *Neuropsychopharmacology* **31**, 1919–1927
- O'Reilly, K.C., Trent, S., Bailey, S.J., Lane, M.A., 2007. 13-*cis*-Retinoic acid alters intracellular serotonin, increases 5-HT1A receptor, and serotonin reuptake transporter levels *in vitro*. *Exp. Biol. Med.* **232**, 1195–1203
- Ono, K., Yoshiike, Y., Takashima, A., Hasegawa, K., Naiki, h., Yamada, M., 2004. Vitamin A exhibits potent anti-amyloidogenic and fibril-destabilizing effects *in vitro*. *Exp. Neurol.* **189**, 380–392
- Palha, J.A., Goodman, A.B., 2006. Thyroid hormones and retinoids: a possible link between genes and environment in schizophrenia. *Brain Res. Rev.* **51**, 61–71
- Parker, G., Gibson, N.A., Brotchie, h., Heruc, G., Rees, A.M., Hadzi-Pavlovic, D., 2006. Omega-3 fatty acids and mood disorders. *Am. J. Psychiatry* **163**, 969–978
- Pedersen, W.A., McMillan, P.J., Kulstad, J.J., Leverenz, J.B., Craft, S., Haynatzki, G.R., 2006. Rosiglitazone attenuates learning and memory deficits in Tg2576 Alzheimer mice. *Exp. Neurol.* **199**, 237–265
- Peet, M., Stokes, C., 2005. Omega-3 fatty acids in the treatment of psychiatric disorders. *Drugs* **65**, 1051–1059
- Pershad Singh, h.A., Heneka, M.T., Saini, R., Amin, N.M., Broeske, D.J., Feinstein, D.L., 2004. Effect of pioglitazone treatment in a patient with secondary multiple sclerosis. *J. Neuroinflammation* **1**, In: <http://www.jneuroinflammation.com/content/1/1/3>
- Petkovich, M., Brand, N.J., Krust, A., Chambon, P., 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330**, 444–450
- Pette, M., Fujita, K., Kitze, B., Whitaker, J.N., Albert, E., Kappos, L., Wekerle, h., 1990. Myelin basic protein-specific T lymphocyte lines from MS patients and healthy individuals. *Neurology* **40**, 1770–1776
- Polak, P.E., Kalinin, S., Dello, R.C., Gavriluk, V., Sharp, A., Peters, J.M., Richardson, J., Willson, T.M., Weinberg, G., Feinstein, D.L., 2005. Protective effects of a peroxisome proliferator-activated receptor-beta/delta agonist in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **168**, 65–75
- Poynter, M.E., Daynes, R.A., 1998. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappaB signaling, and reduces inflammatory cytokine production in aging. *J. Biol. Chem.* **273**, 32833–32841
- Prasad, M.R., Lovell, M.A., Yatin, M., Dhillon, h., Markesbery, W.R., 1998. Regional membrane phospholipid alterations in Alzheimer's disease. *Neurochem. Res.* **23**, 81–88
- Prinzen, C., Muller, U., Endres, K., Fahrenholz, F., Postina, R., 2005. Genomic structure and functional characterization of the human ADAM10 promoter. *FASEB J.* **19**, 1522–1524
- Puri, B.K., Bydder, G.M., Counsell, S.J., Corridan, B.J., Richardson, A.J., Hajnal, J.V., Appel, C., Mckee, h.M., Vaddadi, K.S., Horrobin, D.F., 2002. MRI and neuropsychological improvement in Huntington disease following ethyl-EPA treatment. *Neuroreport* **13**, 123–126
- Puri, B.K., Leavitt, B.R., Hayden, M.R., Ross, C.A., Rosenblatt, A., Greenamyre, J.T., Hersch, S., Vaddadi, K.S., Sword, A., Horrobin, D.F., Manku, M., Murck, h., 2005. Ethyl-EPA in Huntington disease: a double-blind, randomized, placebo-controlled trial. *Neurology* **65**, 286–292

- Racke, M.K., Bonomo, A., Scott, D.E., Cannella, B., Levine, A., Raine, C.S., Shevach, E.M., Rocken, M., 1994. Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med.* **180**, 1961–1966
- Racke, M.K., Burnett, D., Pak, S.H., Albert, P.S., Cannella, B., Raine, C.S., McFarlin, D.E., Scott, D.E., 1995. Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. *J. Immunol.* **154**, 450–458
- Raikwar, h.P., Muthian, G., Rajasingh, J., Johnson, C., Bright, J.J., 2005. PPARgamma antagonists exacerbate neural antigen-specific Th1 response and experimental allergic encephalomyelitis. *J. Neuroimmunol.* **167**, 99–107
- Raikwar, h.P., Muthian, G., Rajasingh, J., Johnson, C.N., Bright, J.J., 2006. PPARgamma antagonists reverse the inhibition of neural antigenspecific Th1 response and experimental allergic encephalomyelitis by Ciglitazone and 15-deoxy-Delta12,14-prostaglandin J2. *J. Neuroimmunol.* **178**, 76–86
- Rego, A.C., Oliveira, C.R., 2003. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. *Neurochem. Res.* **28**, 1563–1574
- Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J., Glass, C.K., 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* **391**, 79–82
- Rioux, L., Arnold, S.E., 2005. The expression of retinoic acid receptor alpha is increased in the granule cells of the dentate gyrus in schizophrenia. *Psychiatry Res.* **133**, 13–21
- Risner, M.E., Saunders, A.M., Altman, J.F., Ormandy, G.C., Craft, S., Foley, I.M., Zvartau-Hind, M.E., Hosford, D.A., Roses, A.D., 2006. Efficacy of rosiglitazone in a genetically defined population with mild-to-moderate Alzheimer's disease. *Pharmacogenomics J.* **6**, 246–254
- Rowland, L.P., 2000. *Merritt's Neurology*, vol. **10**. Lippincott, Williams & Wilkins, Philadelphia
- Saga, Y., Kobayashi, M., Ohta, h., Murai, N., Nakai, N., Oshima, M., Taketo, M.M., 1999. Impaired extrapyramidal function caused by the targeted disruption of retinoid X receptor RXRgamma1 isoform. *Genes Cells* **4**, 219–228
- Sagduyu, K., Dokucu, M.E., Eddy, B.A., Craigen, G., Baldassano, C.F., Yildiz, A., 2005. Omega-3 fatty acids decreased irritability of patients with bipolar disorder in an add-on, open label study. *Nutr. J.* **4**, 6
- Sakai, Y., Crandall, J.E., Brodsky, J., McCaffery, P., 2004. 13-*cis* Retinoic acid (accutane) suppresses hippocampal cell survival in mice. *Ann. N. Y. Acad. Sci.* **1021**, 436–440
- Samad, T.A., Krezel, W., Chambon, P., Borelli, E., 1997. Regulation of dopaminergic pathways by retinoids: activation of the D2 receptor promoter by members of the retinoic acid receptor-retinoid X receptor family. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14349–14354
- Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., Weisstaub, N., Lee, J., Duman, R., Arancio, O., Belzung, C., Hen, R., 2003. Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**, 805–809
- Santos, M.J., Quintanilla, R.A., Toro, A., Grandy, R., Dinamarca, M.C., Godoy, J.A., Inestrosa, N.C., 2005. Peroxisomal proliferation protects from b-amyloid neurodegeneration. *J. Biol. Chem.* **280**, 41057–41068

- Sarsilmaz, M., Songur, A., Ozyurt, h., Kus, I., Ozen, O.A., Ozyurt, B., Sogut, S., Akyol, O., 2003. Potential role of dietary omega-3 essential fatty acids on some oxidant/ antioxidant parameters in rats' corpus striatum. *Prostaglandins Leukot. Essent. Fatty Acids* **69**, 253–259
- Sastre, M., Dewachter, I., Landreth, G.E., Wilson, T.M., Klockgether, T., Van Leuven, F., Heneka, M., 2003. Non-steroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. *J. Neurosci.* **23**, 9796–9804
- Sastre, M., Dewachter, I., Rossner, S., Bogdanovic, N., Rosen, E., Borghgraef, P., Evert, B.O., Dumitrescu-Ozimek, L., Thal, D.R., Landreth, G., Walter, J., Klockgether, T., Van Leuven, F., Heneka, M.T., 2006a. Nonsteroidal anti-inflammatory drugs repress b-secretase gene promoter activity by the activation of PPARγ. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 443–448
- Sastre, M., Klockgether, T., Heneka, M.T., 2006b. Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms. *Int. J. Dev. Neurosci.* **24**, 167–176
- Scacchi, R., Pinto, A., Gambina, G., Rosano, A., Corbo, R.M., 2007. The peroxisome proliferator-activated receptor gamma (PPAR-gamma2) Pro12Ala polymorphism is associated with higher risk for Alzheimer's disease in octogenarians. *Brain Res.* **1139**, 1–5
- Schachter, h.M., Kourad, K., Merali, Z., Lumb, A., Tran, K., Miguelez, M., 2005. Effects of omega-3 fatty acids on mental health. *Evid. Rep. Technol. Assess.* **116**, 1–11
- Schrage, K., Koopmans, G., Joosten, E.A., Mey, J., 2006. Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. *Eur. J. Neurosci.* **23**, 285–295
- Schug, T.T., Berry, D.C., Shaw, N.S., Travis, S.N., Noy, N., 2007. Opposing effects of retinoic acid on cell growth result from alternate activation of two different nuclear receptors. *Cell* **129**, 723–733
- Schutz, B., Reimann, J., Dumitrescu-Ozimek, L., Kappes-Horn, K., Landreth, G.E., Schurmann, B., Zimmer, A., Heneka, M.T., 2005. The oral antidiabetic pioglitazone protects from neurodegeneration and amyotrophic lateral sclerosis-like symptoms in superoxide dismutase-G93A transgenic mice. *J. Neurosci.* **25**, 7805–7812
- Schwab, S.G., Hallmayer, J., Albus, M., Lerer, B., Eckstein, G.N., Borrmann, M., Segman, R.H., Hanses, C., Freymann, J., Yakir, A., Tixler, M., Falkai, P., Rietschel, M., Maier, W., Wildenauer, D.B., 2000. A genome-wide autosomal screen for schizophrenia susceptibility loci in 71 families with affected siblings: support for loci on chromosome 10p and 6. *Mol. Psychiatry* **5**, 638–649
- Shinozaki, Y., Sato, Y., Koizumi, S., Ohno, Y., Nagao, T., Inoue, K., 2007. Retinoids acting through retinoid receptors protect hippocampal neurons from oxyglucose deprivation-mediated cell death by inhibition of JNK and p38 mitogenactivated protein kinase. *Neuroscience* **147**, 153–163
- Silver, J. and Miller, J. h. (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci*, **5**, 146–156
- Simopoulos, A.P., 2002. Omega-3 fatty acids in inflammation and autoimmune diseases. *J. Am. Coll. Nutr.* **21**, 495–505
- Smith, R.S., 1991. The macrophage theory of depression. *Med. Hypotheses* **35**, 298–306
- So, P.-L., Yip, P.K., Bunting, S., Wong, L.-F., Mazarakis, N.D., Hall, S., McMahon, S., Maden, M., Corcoran, J.P.T., 2006. Interactions between retinoic acid, nerve growth factor and sonic hedgehog signalling pathways in neurite outgrowth. *Dev. Biol.* **298**, 167–175

- Solomin, L., Johansson, C.B., Zetterstrom, R.H., Bissonnette, R.P., Heyman, R.A., Olson, L., Lendahl, U., Frisén, J., Perlmann, T., 1998. Retinoid-X receptor signalling in the developing spinal cord. *Nature* **395**, 398–402
- Spencer, N.F., Poynter, M.E., Im, S.Y., Daynes, R.A., 1997. Constitutive activation of NF-kappa B in an animal model of aging. *Int. Immunol.* **9**, 1581–1588
- St Clair, D., Xu, M., Wang, P., Yu, Y., Fang, Y., Zhang, F., Zheng, X., Gu, N., Feng, G., Sham, P., He, L., 2005. Rates of adult schizophrenia following prenatal exposure to the Chinese famine of 1959–1961. *JAMA* **294**, 557–562
- Steinman, L., Martin, R., Bernard, C., Conlon, P., Oksenberg, J.R., 2000. Multiple sclerosis: deeper understanding of its pathogenesis reveals new targets for therapy. *Ann. Rev. Neurosci.* **25**, 491–505
- Stewart, T.M., Bowling, A.C., 2005. Polyunsaturated fatty acid supplementation in MS. *Int. MS. J.* **12**, 88–93
- Straube-West, K., Loomis, P.A., Opal, P., Goldman, R.D., 1996. Alterations in neural intermediate filament organization: functional implications and the induction of pathological changes related to motor neuron disease. *J. Cell Sci.* **109**, 2319–2329
- Sundararajan, S., Jiang, Q., Heneka, M., Landreth, G., 2006. PPARγ as a therapeutic target in central nervous system diseases. *Neurochem. Int.* **49**, 136–144
- Sung, B., Park, S., Yu, B.P., Chung, H.Y., 2006. Amelioration of age-related inflammation and oxidative stress by PPARγ activator: suppression of NF-kappaB by 2,4-thiazolidinedione. *Exp. Gerontol.* **41**, 590–599
- Susser, E.S., Lin, S.P., 1992. Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944–1945. *Arch. Gen. Psychiatry* **49**, 983–988
- Thobois, S., Vingerhoets, F., Fraix, V., Xie-Brustolin, J., Mollion, H., Costes, N., Mertens, P., Benabid, A.L., Pollak, P., Broussolle, E., 2004. Role of dopaminergic treatment in dopamine receptor down-regulation in advanced Parkinson disease: a positron emission tomographic study. *Arch. Neurol.* **61**, 1705–1709
- Townsend, K.P., Pratico, D., 2005. Novel therapeutic opportunities for Alzheimer's disease: focus on non-steroidal anti-inflammatory drugs. *FASEB J.* **19**, 1592–1601.
- Vaddadi, K.S., Soosai, E., Chiu, E., Dingjan, P., 2002. A randomised, placebo controlled, double blind study of treatment of Huntington's disease with unsaturated fatty acids. *Neuroreport* **13**, 29–33
- Valdenaire, O., Maus-Moatti, M., Vincent, J.D., Mallet, J., Vernier, P., 1998. Retinoic acid regulates the developmental expression of dopamine D2 receptor in rat striatal primary cultures. *J. Neurochem.* **71**, 929–936
- van Neerven, S., Mey, J., 2007. RAR/RXR and PPAR/RXR signaling in spinal cord injury. *PPAR Res.*, doi:10.1155/2007/29275
- Veldink, J.H., Kalmijn, S., Groeneveld, G.-J., Wunderink, W., Koster, A., de Vries, J.H.M., van der Luyt, J., Wokke, J.H.J., van den Berg, L.H., 2007. Intake of polyunsaturated fatty acids and vitamin E reduces the risk of developing amyotrophic lateral sclerosis. *J. Neuro. Neurosurg. Psychiatry* **78**, 367–371
- Vogel, G., 2000. Neuroscience. New brain cells prompt new theory of depression. *Science* **290**, 258–259
- Wan, C., Yang, Y., Li, H., La, Y., Zhu, H., Jiang, L., Chen, Y., Feng, G., He, L., 2006. Dysregulation of retinoid transporters expression in body fluids of schizophrenia patients. *J. Proteome Res.* **5**, 3213–3216

- Wang, h.F., Liu, F.C., 2005. Regulation of multiple dopamine signal transduction molecules by retinoids in the developing striatum. *Neuroscience* **134**, 97–105
- Wang, T.-W., Zhang, h., Parent, J.M., 2005. Retinoic acid regulates postnatal neurogenesis in the murine sub-ventricular zone-olfactory bulb pathway. *Development* **132**, 2721–2732
- Watson, G.S., Cholerton, B.A., Baker, L.D., Plymate, S.R., Asthana, S., Fishel, M.A., Kulstad, J.J., Green, P.S., Cook, D.G., Kahn, S.E., Keeling, M.L., Craft, S., 2005. Preserved cognition in patients with early alzheimer disease and amnesic mild cognitive impairment during treatment with rosiglitazone. *Am. J. Geriatr. Psychiatry* **13**, 950–958
- Wysowski, D.K., Beitz, J., 2001. Methodological limitations of the study "Isotretinoin use and risk of depression, psychotic symptoms, suicide, and attempted suicide". *Arch. Dermatol.* **137**, 1102–1103
- Wysowski, D.K., Pitts, M., Beitz, J., 2001. An analysis of reports of depression and suicide in patients treated with isotretinoin. *J. Am. Acad. Dermatol.* **45**, 515–519
- Xu, J., Drew, P.D., 2007. Peroxisome proliferator-activated receptor-gamma agonists suppress the production of IL-12 family cytokines by activated glia. *J. Immunol.* **178**, 1904–1913
- Xu, J., Racke, M.K., Drew, P.D., 2007. Peroxisome proliferator-activated receptoralpha agonist fenofibrate regulates IL-12 family cytokine expression in the CNS: relevance to multiple sclerosis. *J. Neurochem.* **103**, 1801–1810
- Yaffe, K., Kanaya, A.M., Lindquist, K., Hsueh, W.C., Cummings, S.R., Beamer, B., Newman, A., Rosano, C., Li, R., Harris, T., 2008. PPAR-gamma Pro12Ala genotype and risk of cognitive decline in elders. *Neurobiol. Aging* **29**, 78–83
- Zamvil, S.S., Steinman, L., 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* **8**, 579–621
- Zetterström, R.H., Lindquist, E., Mata de Urquiza, A., Tomac, A., Eriksson, U., Perlmann, T., Olson, L., 1999. Role of retinoids in the CNS: differential expression of retinoid binding proteins and receptors and evidence for presence of retinoic acid. *Eur. J. Neurosci.* **11**, 407–416
- Zhang, D.-Q., McMahon, D.G., 2000. Direct gating by retinoic acid of retinal electrical synapses. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 14754–14759
- Zhelyaznik, N., Mey, J., 2006. Expression of retinoic acid receptors and retinoid X receptors after sciatic nerve injury. *Neuroscience* **141**, 1761–1774
- Zhelyaznik, N., Schrage, K., McCaffery, P., Mey, J., 2003. Activation of retinoic acid signaling after sciatic nerve injury: upregulation of cellular retinoid binding proteins. *Eur. J. Neurosci.* **18**, 1033–1040

RAR/RXR and PPAR/ RXR signalling in spinal cord injury

3

S.G.A. van Neerven^{1,2} and J. Mey^{1,2}

¹Institute of Biology II, RWTH Aachen University,
52056 Aachen, Germany

²EURON European Graduate School of Neuroscience,
6229 Maastricht, The Netherlands

PPAR Research, 2007; 2007: 29275

Abstract

The retinoid acid receptors (RAR) and peroxisome proliferator-activated receptors (PPAR) have been implicated in the regulation of inflammatory reactions. Both receptor families contain ligand activated transcription factors which form heterodimers with retinoid X receptors (RXR). We review data that imply RAR/RXR and PPAR/RXR pathways in physiological reactions after spinal cord injury. Experiments show how RAR signaling may improve axonal regeneration and modulate reactions of glia cells. While anti-inflammatory properties of PPAR are well documented in the periphery, their possible roles in the central nervous system have only recently become evident. Due to its anti-inflammatory function this transcription factor family promises to be a useful target after spinal cord or brain lesions.

1. Introduction

During the last decade much progress has been made disentangling the physiological responses after central nervous system (CNS) injury. Yet, there is still no effective treatment available to prevent the devastating effects that result from major CNS lesions. Although neurite sprouting and axonal growth is observed after spinal cord injury (SCI), successful regeneration beyond the lesion-induced scar does not occur. In contrast, axonal regeneration with functional recovery is possible in the peripheral nervous system (PNS). The different outcomes of CNS and PNS lesions are largely due to differences in the cellular and molecular signals that neurons encounter in these environments. Three important problems after CNS injury are (1) development of a growth inhibitory glial scar, (2) secondary neuronal and glial degeneration as a delayed consequence of the lesion, and (3) the failure of axonal regeneration in white matter tracts. Responding to these problems, most attention has been directed either to overcome the inhibitory barrier of the glial scar or to promote the growth of axon collaterals and thus compensate for permanently severed connections (Silver and Miller 2004; Liberto *et al.*, 2004; Makwana and Raivich 2005). A subsidiary approach is to modify inflammatory reactions in order to limit the secondary degeneration. Synthetic corticosteroids, e.g., methyl- prednisolone, are the only pharmacological tools currently in clinical use (Baptiste & Fehlings 2006). In this review, we will discuss two nuclear receptor families that have recently been discovered as possible therapeutic targets for the treatment of SCI and brain damage. Retinoic acid receptor (RAR) signaling may improve axonal regeneration, influence glial differentiation, and modulate inflammatory reactions (Mey 2006). Peroxisome proliferator- activated receptors (PPAR) have a major function in anti- inflammatory processes (Kielian & Drew 2003). Both receptor classes, RAR and PPAR, heterodimerize with retinoid X receptors (RXR) to form active transcription factors.

2. Endogenous RAR/RXR and PPAR/RXR activity in the injured nervous system

2.1. RAR/RXR signal transduction

The RAR/RXR transcription factor complex is activated by ligand binding, its natural ligand being all-*trans* retinoic acid (RA). Retinoids are obtained from the diet in the form of vitamin A (retinol and retinal), retinyl esters, or β -carotene. Following cellular uptake of all-*trans* retinol from the plasma, the intracellular synthesis of RA occurs in two steps. (1) Retinol is oxidized to retinal, predominantly involving the alcohol dehydrogenases ADH-1, -3, and -4. (2) The critical step is the subsequent oxidation of retinal to RA by retinaldehyde dehydrogenases (RALDH) (Blomhoff & Blomhoff, 2006). RA is released in a paracrine

or autocrine fashion. The fact that RA activates nuclear transcription factors was discovered in 1987 (Giguere *et al.*, 1987; Petkovich *et al.*, 1987). Retinoid receptors belong to the same superfamily as PPAR, thyroid hormone receptors (TR), and steroid receptors. They can be grouped into two families, the RARs and the RXRs, each consisting of three isoforms encoded by separate genes: RAR α , RAR β , RAR γ (also: NR1B1-3) and RXR α , RXR β , RXR γ (NR2B1-3). All-*trans* RA and 9-*cis* RA bind to the RAR family, whereas only 9-*cis* RA is a high-affinity ligand for the RXR (Blomhoff & Blomhoff, 2006; Bastien & Rochette-Egly, 2004). The active transcription factor complex consists of an RAR/RXR heterodimer, ligand, and co-activators. It interacts with retinoic acid response elements (RARE) in the promoters of target genes. About 500 genes have been suggested to be regulated by RAR/RXR signaling, however a much lower number was experimentally shown to be activated via the classical RARE driven pathway. Proven target genes include enzymes, transcription factors, cytokines, and cytokine receptors (Lane & Bailey, 2005; Mey, 2006). In addition, many cases of gene suppression and nongenomic modes of action of RA and its receptors have been described (Blomhoff & Blomhoff, 2006).

2.2. RAR/RXR signaling after spinal cord injury

The discovery that enzyme activity of a retinaldehyde dehydrogenase increased after spinal cord contusion injury was the first direct evidence that retinoids play a role in the physiological responses to SCI. Contusion injury caused a significant increase in RALDH2 enzyme activity, which peaked 8–14 days following the lesion (Mey *et al.*, 2005). While in the non-injured rat spinal cord RALDH2 is only present in the meninges, oligodendrocytes and in pericytes, around the lesion site, its immunoreactivity also appeared in a population of NG2-positive glia cells (Kern *et al.*, 2006) (Figure 1(a)). NG2 is a chondroitin sulfate proteoglycan, expressed in cells that have been described as oligodendrocyte precursor cells, synantocytes or polydendrocytes. These cells respond to injury with increased production of NG2 and a subpopulation of them, close to the lesion site, appears to be involved in the local production of RA. Alternatively or additionally, RA-synthesizing cells migrate from the adjacent arachnoidmembrane and from blood vessels toward the site of injury (Kern *et al.*, 2007). While only minor changes in the quantitative expression of retinoid receptors were observed after SCI, their cellular distribution changed remarkably (Figures 1(b), 1(c)). In the non-injured tissue, retinoid receptors were found in the cytosol of motoneurons and glia, but close to the injury site macrophages and surviving neurons displayed a nuclear localization of RAR α , RXR α , and RXR β (Schrage *et al.*, 2006). In the context of locally rising RA synthesis, the observation that retinoid receptors translocate into the cell nuclei indicates that neurons, glia, and macrophages are targets of RA signaling after SCI. This interpretation is consistent with data on spinal cord development, where RA has been shown to be a regulator of cell differentiation (Sockanathan *et al.*, 2003; Wuarin & Sidell, 1991; Wuarin *et al.*, 1990; Vermot *et al.*, 2005).

2.3. RAR/RXR signaling after peripheral nerve injury

RA signaling is implicated in the differentiation of neurons whose axons grow in the PNS, (Liu *et al.*, 2001; Holst *et al.*, 1997; Wyatt *et al.*, 1991) and again in neurite regeneration (Corcoran & Maden, 1999; Corcoran *et al.*, 2000). Several mediators of the RA signaling pathway have been shown to be induced after peripheral nerve injury in the rat. Experimental lesion of the sciatic nerve induced expression of the cellular retinol binding protein-I (CRBP-I) (Zhelyaznik *et al.*, 2003), which is implicated in retinoid metabolism (Belyaeva *et al.*, 2005). Similarly, transcript and protein concentrations of the cellular retinoic acid binding protein-II (CRABP-II) increased strongly (Zhelyaznik *et al.*, 2003). CRABP-II is probably involved in the intracellular transport of RA to the cell nucleus (Budhu & Noy, 2002). As after SCI, the RA-synthesizing enzyme RALDH2 was present in the injured nerve. After sciatic nerve injury in transgenic reporter mice, local activation of RARE was detectable in the regenerating nerve, indicating that RA-dependent gene expression is induced during peripheral nerve regeneration (Zhelyaznik *et al.*, 2003). The expression of retinoid receptors also changed significantly. Following sciatic nerve crush, mRNA of all RARs and of RXRa was increased, and at 4, 7, and 14 days after the injury protein levels of RAR α , RAR β , and RXRa were augmented (Zhelyaznik & Mey, 2006). To study Wallerian degeneration in the absence of axonal regeneration, the distal nerve segment can be cut off and sutured to the peroneus muscle. Under these conditions RAR α and RAR β were upregulated in the degenerating nerve. Cell culture experiments indicated that RAR β is required for RA-induced axonal regeneration (Corcoran *et al.*, 2000). Consequently, experiments with RAR β -deficient mice corroborated this interpretation because there were significantly less GAP43-positive, regenerating axons after sciatic nerve crush in the knockout animals (So *et al.*, 2006). Since RALDH2 (Corcoran & Maden, 1999) and RAR β (Cosgaya & Aranda, 2001) are induced by NGF, RA appears to act downstream of this neurotrophin. RA/RAR β signaling seemed to be necessary for the neurotrophic activity of NGF (Corcoran & Maden, 1999). In addition, neurotrophin-independent RA/RAR β activity contributes to axon outgrowth (So *et al.*, 2006). Double labeling with cell type specific markers in the sciatic nerve demonstrated expression of retinoid receptors mostly in macrophages and Schwann cells. In cultured primary Schwann cells, RA downregulated CNTF (Johann *et al.*, 2003) and raised expression of ErbB3, a neuregulin receptor that is necessary for normal myelination. Finally, RXRa labelling was shown to colocalize with some regenerating axons, indicative of a direct role for RXRa in nerve regeneration (Zhelyaznik & Mey, 2006).

2.4. PPAR/RXR signal transduction

Peroxisome proliferator-activated receptors belong to the same superfamily as the RAR. The first PPAR was discovered, cloned, and sequenced in 1990, and it was named after its property to be activated by drugs that cause proliferation of peroxisomes in hepato-

Fig. 1. Retinoic acid synthesis and nuclear translocation of RAR/RXR after spinal cord injury. (a) Double immunostaining of rat spinal cord sections shows RALDH-2 immunoreactivity (red) in a subpopulation of NG2-positive glia (green) 7 days after contusion injury. RALDH2/NG2 expressing cells were only detected in the vicinity of the lesion site, many NG2 cells do not contain the RA-synthesizing enzyme (yellow arrow heads). White arrows point to DAPI-stained cell nuclei of RALDH2/NG2 cells in superimposed photographs. (b) At 7 dpo, ED1-positive macrophages at the lesion site express RXRβ in their cell nuclei. (c) SCI-induced transient translocation of RARβ and RXRα from the cytosol into the nuclei of activated macrophages/microglia. All scale bars are 20 μm (sources: (Mey *et al.*, 2005; Schrage *et al.*, 2006).

cytes (Isseemann & Green, 1990). Until now, three different isoforms of PPAR, encoded by separate genes, have been identified: PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3). The different isoforms have similar structural features; however each isoform exhibits its own specific tissue expression pattern and distinct physiological functions depending on ligand activation (Kliwer *et al.*, 1994; Kota *et al.*, 2005). PPAR also heterodi-

merize with RXR, then bind as a complex to their response elements (PPRE). These consist of two repeats of the consensus sequence AGGTCA separated by one or two nucleotides (direct repeats DR-1 and DR-2) (Gervois *et al.*, 1999). Natural ligands of PPAR are fatty acids, prostaglandins, and oxidized fatty acid derivatives. They are also activated by synthetic ligands like the lipid-lowering fibrates and the anti-diabetic glitazones (Moraes *et al.*, 2006). PPAR, which have been implicated in lipid metabolism, cellular proliferation and inflammatory responses, are widely expressed, for example, in colon, spleen, retina, the cardiovascular system, liver, skeletal muscles, and in adipose tissue. Their expression by monocytes, dendritic cells, endothelial cells, megakaryocytes, and lymphocytes may be related to immune functions (Kota *et al.*, 2005; Moraes *et al.*, 2006). PPAR γ can also influence gene expression independently of PPRE. The activity of a number of transcription factors, for example, NF- κ B, AP-1, and STAT1, are inhibited by PPAR γ via direct interaction or by competition for limiting supplies of co-activators (Kielian & Drew, 2003).

2.5. PPAR/RXR signaling after spinal cord injury

PPAR are expressed in the developing and adult CNS. PPAR α and PPAR β/δ , but not PPAR γ were found in cervical, thoracic, and lumbar segments of the adult spinal cord, in the thalamus and cerebral cortex (Braissant & Wahli, 1998) (Figure 2). Immunohistochemical staining showed that PPAR β/δ is the main isoform present in neuronal cell bodies of the spinal cord gray matter. Both receptors, PPAR α and PPAR β/δ , were concentrated in cell nuclei. In the white matter PPAR α appeared particularly strong in PPAR β/δ -negative astrocytes, whereas oligodendrocytes expressed only PPAR β/δ (Benani *et al.*, 2003). PPAR β/δ is a factor in neuronal differentiation, and functions of this receptor in various aspects of neural physiology have been suggested (Benani *et al.*, 2003; Cimini *et al.*, 2005). While in two studies PPAR γ could not be detected in brain and spinal cord (Benani *et al.*, 2003; Braissant & Wahli, 1998), this receptor is expressed in microglial primary cultures (Bernardo *et al.*, 2000) and may be upregulated after injury. In a different report, all PPAR and RXR were found immunohistochemically throughout the adult rat CNS (Moreno *et al.*, 2004).

Endogenous PPAR ligands may play a role in modulating the inflammatory response after SCI, possibly preventing the expansion of the initial damage. Genovesi and coworkers (Genovesi *et al.*, 2005) examined the effects of endogenous PPAR α ligands in an experimental model of spinal cord trauma. SCI was induced in wild-type and in PPAR α -deficient (–/–) mice. The injury resulted in severe trauma characterized by edema, loss of myelin, neutrophil infiltration, apoptosis, and increased production of TNF α . Compared to wild-type animals, all these parameters were augmented in PPAR α –/– mice. The absence of PPAR α also interfered with recovery of limb function (Genovesi *et al.*, 2005). Studies using animal models of chronic pain indicate that PPAR α is involved in the neural processing of pain (Taylor *et al.*, 2002; LoVerme *et al.*, 2006).

3. Regulation of inflammatory processes by RAR/RXR signalling in the spinal cord

Although the inflammatory response limits the effects of a pathogenic insult, it is also responsible for most of the secondary damage incurred after spinal cord injury. The regulation of these events is therefore of primary therapeutic concern. Both pathways, RAR/RXR and PPAR/RXR signaling, are implicated.

3.1. Inflammatory reactions in the spinal cord

The physiological events following spinal cord injury can be differentiated into an acute phase (the first 24 hrs), followed by a subacute phase (24–72 hrs), and a late phase (3–90 days). Events after this time are considered chronic. CNS injury directly leads to release of inflammatory signals resulting in the production of vasoactive mediators and chemotactic factors. Primary damage consists mainly of severed axons, neuronal and oligodendrocyte cell death. Expansion of the initial damage is then caused by a disruption of the blood supply and an extended inflammatory reaction. Depending on the type of injury, the blood-brain barrier is disrupted. This event determines the extent to which blood derived cells participate in the inflammatory process (Raivich *et al.*, 1999; Popovich *et al.*, 2001). When the blood-brain barrier breaks down, the release of cytokines and chemokines leads to a massive recruitment of inflammatory cells from the periphery, including hematogenous neutrophils and perivascular/meningeal macrophages. These cells contribute to tissue damage by the production of proteolytic enzymes and reactive oxygen species. Microglia and macrophages phagocytose dead or injured neurons and glia, and are responsible for the clearance of cellular debris (Raivich *et al.*, 1999).

Microglia, perivascular macrophages, and activated astrocytes are the most important local sources of cytokines. Astrocytes are further involved in the formation of a glial scar, thereby insulating the healthy tissue from uncontrollable processes in the damaged area. Acute inflammation can develop into a chronic process when feedback mechanisms fail to inhibit amplification of the inflammatory response. Chronic inflammation leads to a continuous influx of neutrophils, macrophages, lymphocytes, and eosinophils from the circulation, causing more tissue destruction and scarring.

3.2. RAR/RXR signaling is involved in inflammation

Before the molecular mechanisms of the retinoid signalling pathway were understood, anti-inflammatory properties of RA had already been described. In 1983, a study showed that oral administration of retinoids affected dermal inflammatory cells and reduced elevated skin temperature (Orfanos & Bauer, 1983). Retinoic acid has beneficial effects in diseases with an inflammatory-based pathology including asthma, arthritis, and athero-

sclerosis (Fang *et al.*, 2004; Nozaki *et al.*, 2006). A number of cell culture studies support the hypothesis that RA reduces inflammatory activation of monocytes, macrophages, myeloma cells, and polymorphonuclear cells (neutrophil granulocytes). Signals that were found to be downregulated by retinoids include IL-1 α , IL-1 β , IL-6, TNF α , IL-8, prostaglandin E2, production of reactive oxygen species, and release of lysosomal enzymes. A table with these effects and references is presented in (5). In contrast to a majority of findings that indicate an anti-inflammatory role of RAR/RXR signaling, it was also reported that 9-*cis* RA induced secretion of MCP-1 and thereby stimulated monocyte migration (Zhu *et al.*, 1999). In combination with T-cell stimulating agents, all-*trans* RA, 9-*cis* RA, and an RAR agonist, TTNPB, indirectly increased proliferation of human T lymphocytes and IL-2 secretion (Ertesvag *et al.*, 2002).

One of the major transcription factors responsible for the regulation of inflammatory cytokines is NF- κ B. It was shown that RXR inhibits NF- κ B-dependent gene expression (IL-12) (Na *et al.*, 1999). In the experiments, interference of RXR with DNA binding of NF- κ B required the presence of the RXR ligand 9-*cis* RA. The negative RXR/NF- κ B interaction appears to be mutual because increasing levels of transfection with NF- κ B subunits also abrogated expression of a retinoid reporter construct (Na *et al.*, 1999).

3.3. The role of RAR/RXR in inflammatory reactions after CNS injury

Although retinoic acid has so far not been used to modify inflammatory reactions in the CNS *in vivo*, cell culture experiments demonstrate anti-inflammatory effects on microglia and astrocytes (Mey, 2006). Primary cultures of microglia and astrocytes are most frequently prepared from cerebral cortex of perinatal rats and mice. One can expose these cultures to LPS to simulate a bacterial infection, or treat them with β -amyloid peptide (A β) to mimic the immunogenic stimulus of Alzheimer's disease. In this kind of experiment, RA treatment suppressed the induction of TNF α and iNOS. Effects correlated with enhanced expression of RAR β , TGF β , and inhibition of NF- κ B nuclear translocation (Dheen *et al.*, 2005). Xu and Drew demonstrated that 9-*cis* RA suppressed LPS-induced production of NO as well as of pro-inflammatory cytokines TNF α , IL-1 β , and IL-12p40 in primary mouse microglia, while IL-6 secretion and MCP-1 production were not significantly affected (Figure 3) (Xu & Drew, 2006). In astrocytes from rat brain and in C6 astrogloma cells, 9-*cis* RA and all-*trans* RA inhibited IFN γ -induced inflammatory responses (Choi *et al.*, 2005).

Matrix metalloproteinases (MMP) are involved in the breakdown of the extracellular matrix and other proteins. These proteases are products of leukocytes and endothelial cells and are released in response to various cytokines and growth factors. Increased proteolytic activity of MMP can lead to disruption of the blood-brain barrier and escalate the inflammatory response (Noble *et al.*, 2002; Mun-Bryce & Rosenberg, 1998). Retinoic acid has negative effects on secretion and expression of these enzymes. It reduced mRNA, protein synthesis, and secretion of MMP-9 (gelatinase B) in lymphocytes from patients

Fig. 2. Localization of PPAR α and PPAR β/δ in astrocytes and oligodendrocytes in the spinal cord. (a) Western blotting shows PPAR α and PPAR β but not PPAR γ in spinal cord, telencephalon, and diencephalon. Identical expression patterns were detected with RT-PCR. (b) Detection of PPAR immunoreactive cells in the white matter of rat spinal cord. GFAP-positive/CNPase-negative astrocytes are immunoreactive for PPAR α (marked a, upper four panels) while GFAP-negative/CNPase-positive oligodendrocytes express PPAR β (marked ol, lower panels) (scale bars: 25 μ m, source: (Benani *et al.*, 2003). (c) Distribution of PPAR β/δ and PPAR γ immunoreactive cells in the spinal cord of the adult rat (cervical level, coronal sections, scale bar: 100 μ m, source: (Moreno *et al.*, 2004).

with chronic B-lymphocytic leukemia and in human monocytes (Nguyen *et al.*, 2006). Other metalloproteinases including MMP-1 and MMP-13 appeared to be influenced by RA as well (Ho *et al.*, 2005).

4. Regulation of inflammatory processes by PPAR/RXR signalling in the spinal cord

4.1. PPAR/RXR signaling counteracts inflammatory processes

For the PPAR family, anti-inflammatory effects are well documented. One mechanism involves direct interaction of PPAR with proinflammatory transcription factors, most importantly NF- κ B and AP-1, and the subsequent reduction of gene transcription. This has been observed *in vitro* in human vascular endothelial cells (Delerive *et al.*, 1999a), human aorta smooth muscle cells (Delerive *et al.*, 1999b), in C2C12 skeletal muscle cells (in a model for insulin resistance in type 2 diabetes) (Jove *et al.*, 2005), and in fibroblasts from rheumatoid

arthritis patients (Okamoto *et al.*, 2005). PPAR knockout studies supplement and confirm these results. One of the first reports indicating that PPAR α is involved in attenuating inflammation demonstrated that the eicosanoid LTB $_4$ binds and activates PPAR α . Subsequently, PPAR α -deficient mice were shown to have a prolonged inflammatory response when challenged with LTB $_4$ or arachidonic acid (Devchand *et al.*, 1996).

An interaction of PPAR α with NF- κ B is indicated by studies of inflammatory cytokine production in aging. In aged mice, NF- κ B becomes constitutively active in many tissues due to oxidative stress, which eventually leads to the production of cytokines. Administration of PPAR α activators was found to restore the cellular redox balance, to suppress the constitutive activation of NF- κ B and to eliminate the spontaneous production of IL-6 and IL-12 (Spencer *et al.*, 1997; Poynter *et al.*, 1998). Other examples where PPAR α -/- mice suffer from more severe inflammatory reactions are carrageenan-induced hypersensitivity (Cuzzocrea *et al.*, 2006), airway inflammation (Delayre *et al.*, 2005), and experimental colitis (Cuzzocrea *et al.*, 2004). On the molecular level, these investigations demonstrated Fas-ligand, IL-1 β , TNF α , keratinocyte-derived chemokine, MIP-2, MCP-1, ICAM-1, and enzyme activities of MMP-9, myeloperoxidase, and iNOS to be influenced by PPAR α signalling (Cuzzocrea *et al.*, 2004; Cuzzocrea *et al.*, 2006; Colville-Nash *et al.*, 1998).

PPAR γ -deficient mice die *in utero*, but heterozygotic PPAR γ +/- mice with 50% expression of the receptor survive and can be studied. In an investigation of PPAR γ functions after intestinal and gastric ischemia/reperfusion injury, PPAR γ +/- mice displayed more severe lesions of the gastric intestinal mucosa. Treatment with the PPAR γ ligand BRL-49653 limited the damage of intestinal injury and resulted in downregulation of cell adhesion molecules and proinflammatory cytokines in the intestine and stomach (Nakajima *et al.*, 2001; Wada *et al.*, 2004). As mentioned before, NF- κ B and inflammatory mediators are raised during the aging process. While this is accompanied by reduced PPAR γ levels, the PPAR γ ligand 2,4-TZD was shown to reduce age-related oxidative stress, the translocation of NF- κ B p65 subunit to the nucleus and NF- κ B-regulated transcription of iNOS, COX-2, IL-1 β , IL-6, and the cell-adhesion molecule VCAM-1 (Sung *et al.*, 2006). Another PPAR γ agonist, rosiglitazone, relieved renal injury in a nephrotoxicity model, also acting via inhibition of NF- κ B (Lee *et al.*, 2006). It seems therefore that both receptors PPAR α and PPAR γ play a general role in modulating the inflammatory response in a wide variety of tissues.

4.2. Effect of PPAR activation on macrophages, microglia, and astrocytes

Given that PPAR agonists influence peripheral macrophages (Li & Palinski, 2006), it was to be expected that they act on brain macrophages after disruption of the blood-brain barrier. PPAR α is already present in undifferentiated monocytes, while PPAR γ expression is induced during differentiation (Chinetti *et al.*, 1998; von Knethen & Brüne, 2001) and upregulated in the course of macrophage activation (Xu *et al.*, 2005; Petrova *et al.*, 1999; Ricote *et al.*, 1998; Jiang *et al.*, 1998; Combs *et al.*, 2000). A number of cell culture studies

Fig. 3. Anti-inflammatory effects of PPAR and RAR agonists in astrocytes. Astrocyte primary cultures from cortices of newborn mice were treated with 9-*cis* RA (RA) and/or the PPAR α agonist fenofibrate (F) for 1 hour and then stimulated with 2 µg/mL LPS for 24 hours. (a) Nitrite production as an indicator of NOS activity was reduced by 1 µM RA or 100 µM F, and completely suppressed with RA plus F. Pretreatment of RA and/or F also decreased the release of (b) the chemokine MCP-1 (RA: 2 µM, F: 200 µM), and cytokines (c) IL-1 β (RA: 1 µM, F: 100 µM), (d) IL-6 (RA: 2 µM, F: 50 µM), and (e) TNF α (RA: 1 µM, F: 100 µM) in response to LPS. Cytokine production was measured with ELISA. Experiments with microglia cultures revealed similar effects, except for MCP-1, whose production was stimulated by 9-*cis* RA in microglia. Error bars indicate SEM, asterisks indicate significant differences compared to the LPS condition (sources: (Xu & Drew, 2006; Xu *et al.*, 2005).

with macrophages and microglia demonstrate that agonists of PPAR α and PPAR γ elicit the same anti-inflammatory effects that we have discussed above. When cells were activated with bacterial antigens or A β , the PPAR ligands typically prevented or reduced the inflammatory response. This effect was observed on the following levels: (1) activity of transcription factors NF- κ B, AP-1, and STAT1, (2) secretion of proinflammatory cytokines and chemokines, (3) enzyme activities of COX-2, iNOS, and MMP-9, and (4) the formation of reactive oxygen radicals. A synopsis of these results is presented in Table 1.

Such effects were observed with natural ligands, for example, prostaglandins, with artificial PPAR activators, for example, antidiabetic thiazolidinones, and with non-steroid anti-inflammatory drugs (Heneka *et al.*, 2005). A very efficient endogenous agonist of PPAR γ is the prostaglandin 15d-PGJ2 (Colville-Nash *et al.*, 1998). However, this drug also interferes with the transcriptional activity of NF- κ B independently of PPAR γ activation because overexpression of PPAR γ or an antagonist of PPAR γ did not alter the 15d-PGJ2 effect on LPS/IFN γ -dependent inflammatory reaction. It was concluded that 15d-PGJ2 inhibits the inflammatory response by directly regulating the NF- κ B and PI3K/Akt pathways (Petrova *et al.*, 1999; Giri *et al.*, 2004).

With respect to microglia effects on nerve cells, PPAR activation with synthetic ligands can be neuroprotective. When cortical neurons were exposed to the cell-free supernatant from activated microglia, neurons survived better when microglia had also been incubated with PPAR γ agonists (Luna-Medina *et al.*, 2005). Some experiments *in vivo* also support this concept. Oral administration of pioglitazone or the nonsteroidal anti-inflammatory drug ibuprofen, which is also a ligand for PPAR γ , reduced the number of activated microglia and reactive astrocytes in the hippocampus and cerebral cortex. This was observed in 10-month-old APPV7171 mice, an inflammation model for Alzheimer's disease (Heneka *et al.*, 2005). Pioglitazone protected mice also from MPTP-induced dopaminergic neuronal cell loss and mitigated inflammatory response by microglia in an animal model of Parkinson's disease (Dehmer *et al.*, 2004).

As mentioned before, astrocytes participate in the secretion of cytokines and form a glial scar around the damaged tissue. Since the production of microglia primary cultures also involves isolation of astrocytes, it was a straightforward approach to test PPAR agonists on this type of glia as well (Figure 3) (Storer *et al.*, 2005; Xu *et al.*, 2006; Heneka *et al.*, 2000). Results, which were generally in line with the anti inflammatory effects on microglia, are also included in Table 5.

Fig. 4. Transfection of RAR β induces regeneration of corticospinal axons in vivo. (a) Longitudinal sections of the adult rat spinal cord were traced with BDA to determine fibre regeneration in the corticospinal tract; overview of the lesion site in an animal injected with the RAR β 2 expressing construct (EIAV-RAR β 2, Rostral: left). (b) Animals with RAR β 2-transfected cells displayed less fiber degeneration than control rats. (c) Labeled axons were detected up to the edge of the lesion, (d) at the distal edge of the lesion, and (e-g) at various distances caudal to the lesion. Growth cone-like endings were detected at the tips of some axons (white arrowheads in d). Axons appear to send collaterals (white arrowhead in g) from white matter to grey matter. (Scale bars in (a): 1 mm, in (b-g): 100 μ m.) (h-i) Quantification of BDA-labeled fibres in the corticospinal tract after spinal cord lesion. EIAV-RAR β 2-treated animals displayed increased fiber numbers rostral and caudal to the lesion compared to control animals ($P < .05$, two-way ANOVA) (source: (Yip *et al.*, 2006).

5. Application of retinoids and PPAR ligands after spinal cord and peripheral nerve injury

Retinoic acid is a well-characterized morphogenetic factor in embryonic development. Stem cells and several cell lines can be induced with RA to differentiate to a neuronal phenotype. A number of experiments with developing sympathetic neurons and with sensory neurons from dorsal root ganglia revealed that RA has neurotrophic effects or induces neurite growth. The question arises whether these properties can be exploited to support axonal regeneration (Mey, 2006; Clagett-Dame *et al.*, 2006).

5.1. Retinoic acid-induced axonal growth *in vitro* and *in vivo*

While the most convincing demonstration that RA can act as an axon-guiding chemo-attractant was recently published using the snail *Lymnaea stagnalis* (Dmetrichuk *et al.*, 2006), a number of earlier studies indicated that retinoids may fulfill similar functions in vertebrates. Neurites extending from chick neural tube cells responded to a gradient of RA (Maden *et al.*, 1998). Positive effects on axon outgrowth were also reported using explants from murine embryonic spinal cord (Quinn & Boni, 1991), embryonic cerebellum (Yamamoto *et al.*, 1996), and amphibian spinal cord (Hunter *et al.*, 1991). In a chick retinal explant assay, RA enhanced neurite outgrowth under the condition that the neurotrophin BDNF was subsequently added *in vitro* (Mey & Rombach, 1999). RA supported survival and morphological differentiation of cultured rat spinal cord neurons and increased neurite density. In this context, astrocytes were implicated as regulators of local RA concentration (Wuarin & Sidell, 1991; Wuarin *et al.*, 1990).

Corcoran, Maden, and coworkers suggested that the loss of regeneration in the adult mammalian CNS is related to developmental changes in retinoid signaling. They observed that the RAR β 2 receptor, which is present when neurite outgrowth occurs from embryonic spinal cord explants, was not detectable in the adult spinal cord (Corcoran *et al.*, 2002). A supporting result was that specifically RAR β mediated the induction of neurite outgrowth from subpopulations of sensory neurons (Corcoran *et al.*, 2000). In addition to the inhibitory glial environment (Silver & Miller, 2004), intrinsic changes in CNS neurons have been assumed to be responsible for the loss of regenerative ability during CNS development (Mey & Thanos, 1991). The declining expression of RAR β 2 may be one of those changes. To test this hypothesis, the RAR β 2 gene was transfected into spinal cord explants with a lentiviral vector. This treatment allowed the outgrowth of many neurites, implying the involvement of RAR β 2 in axonal regeneration (Corcoran *et al.*, 2002). Consequently, to demonstrate that a renewed expression of the appropriate receptor mechanism might induce axonal regeneration into the spinal cord *in vivo*, Wong and others (Wong *et al.*, 2006) transfected RAR β 2 in adult rats. They then analyzed regeneration of sensory axons into the spinal cord and performed behavioral experiments. Following

complete dorsal root transections by means of a crush injury, axons regenerated across the dorsal root entry zone into the CNS. Both myelinated and nonmyelinated fibers were found to have grown in the spinal cord where they projected into the gray matter, formed functional connections and improved sensorimotor recovery of the animals (Wong *et al.*, 2006). In another study, artificially induced RAR β 2 expression also supported regeneration of descending corticospinal tract fibers after midcervical spinal cord injury (Figure 4). Again, the induced regeneration of fibers was accompanied by improved sensory and locomotor behavior (Yip *et al.*, 2006). These data suggest not only that RAR β is involved in activating a physiological program of regeneration but also that retinoic acid signaling may be used as a therapeutic tool in spinal cord injury.

5.2. Manipulation of RAR/RXR signaling in the peripheral nervous system

As mentioned above, it has been known for some time that retinoids act as neurotrophic factors for sympathetic and sensory neurons of the peripheral nervous system (Holst *et al.*, 1997; Wyatt *et al.*, 1999; Rodriguez-Tebar & Rohrer, 1991; Corcoran & Maden, 1999; Corcoran *et al.*, 2000). However, very few studies have addressed the role of RA in peripheral nerve regeneration *in vivo*. Taha and coworkers tested whether local RA injections improved morphological and functional regeneration of tibial nerves undergoing anastomosis. They showed that animals injected locally with RA exhibited increased axonal density when compared to vehicle-treated animals (Taha *et al.*, 2004). Several cell culture experiments suggested that RA effects may involve an upregulation of neurotrophin receptors, and molecular studies indicate mutually synergistic influences of the RA- and neurotrophin-signaling pathways. Nerve growth factor induced the RA-synthesizing enzyme RALDH in dorsal root ganglia (Corcoran & Maden, 1999) and activated the RAR β promoter (Cosgaya & Aranda, 2001). RA, on the other hand, caused expression of various neurotrophin receptors (see Mey 2006, Clagett-Dame 2006 for review). That synergistic interactions between RA and neurotrophins take place *in vivo* was revealed in a study using a mouse model for diabetes (Arrieta *et al.*, 2005). Treatment of the animals with all-*trans* RA via subcutaneous injection in a dose of 20 mg/kg was able to prevent the NGF depletion normally seen in diabetic mice. Microscopic analysis subsequently showed that diabetes-associated loss of Schwann cells, of myelinated, and of nonmyelinated axons was strongly reduced by RA. This beneficial effect was also measurable in behavioral tests of sensory-motor functions (Arrieta *et al.*, 2005).

5.3. Retinoids and PPAR ligands in pain signaling

In a different line of research about spinal cord sensitization, some contrasting results were obtained recently. To investigate whether retinoids might be involved in the processing of nociceptive information, especially in situations of hyperalgesia due to inflammation, rats were given 10–15 mg/kg all-*trans* RA orally for 4 days. Electrophysiological record-

ings revealed that treated animals had decreased thresholds to mechanical and electrical stimulation of the paws and increased cutaneous receptive fields. This increased responsiveness caused by RA was similar to hyperalgesia induced by intraplantar administration of carrageenan (which leads to local inflammation) (Romero-Sandoval *et al.*, 2006). On the molecular level, RA induced changes of gene expression in the spinal cord, for instance, an increase of COX-2 and IL-1. Inhibition of these pathways with IL-1 receptor antagonist and the COX inhibitor dextetopfen reduced responses to mechanical or thermal stimulation when those had been sensitized with RA (Alique *et al.*, 2006). In these studies, it was concluded that all-*trans* RA induced changes in the spinal cord that are similar to inflammation. At this point, it is difficult to reconcile this conclusion with the anti-inflammatory properties when RA was combined with stimuli of bacterial infection or A β deposition.

In contrast to RA, administration of the PPAR α agonists GW7647, Wy14643, perfluorooctanoic acid, and PEA reduced nocifensive behaviors in various animal models of hyperalgesia, implicating a mitigating role of PPAR in the regulation of pain signaling (Taylor *et al.*, 2002; LoVerme *et al.*, 2006). GW7647 and PEA also prevented firing of spinal cord nociceptive neurons in rats after peripheral exposure to formalin (LoVerme *et al.*, 2006).

5.4. Use of PPAR ligands as anti-inflammatory treatment after spinal cord injury

The knowledge about anti-inflammatory properties of PPAR ligands obtained in cell cultures soon prompted animal experiments. In these, PPAR α ligands were indeed shown to exert neuroprotective effects after traumatic brain injury (Besson *et al.*, 2005). Recently, it was discovered that synthetic PPAR γ ligands rosiglitazone and pioglitazone are also neuroprotective by reducing the inflammation in the spinal cord. Treatment of rats directly after SCI resulted in a decreased lesion site, better survival of motor neurons, sparing of myelin, reduced astrogliosis, and less microglial activation. These features were accompanied by enhanced functional motor recovery and reduced hyperalgesia (Park *et al.*, 2007). Investigation of gene expression revealed that pioglitazone lowered levels of transcription factors IRF-1, NF- κ B, transmembrane proteins Egr-1, ICAM, and cytokine/chemokines IL-1 β , IL-6, MCP-1, but increased expression of neuroprotective genes and antioxidant enzymes. In a rat model for stroke (middle cerebral artery occlusion), PPAR γ ligands were able to improve neurological outcome, decreased infarct size, and reduced inflammation. Fewer microglia/macrophages appeared to be present, and transcript and protein levels of IL-1 β , COX-2, and iNOS were also lower (Sundararajan *et al.*, 2005).

Table 1. Anti-inflammatory effects of PPAR α and PPAR γ agonists on the molecular level.

Experimental system	Nuclear receptor	Regulated signals	Reference
Cytokine- and LPS-stimulated microglia	PPAR α , RXR	NO synthesis, IL-1 β , IL-6, IL-12p40, TNF α , MCP-1	Xu <i>et al.</i> , 2005
Activated macrophages	PPAR α , γ	iNOS, heme oxygenase (COX-2, HSP70 not affected)	Colville-Nash <i>et al.</i> , 1998
Activated macrophages	PPAR γ	iNOS, MMP-9, scavenger receptor A	Ricote <i>et al.</i> , 1998
Activated human monocytes	PPAR γ	IL-1 β , IL-6, TNF α	Jiang <i>et al.</i> , 1998
Activated macrophages	PPAR γ	Reactive oxygen species	von Knethen and Brüne., 2001
LPS-stimulated microglia	PPAR γ	IL-1 β , IL-6, TNF α , MCP-1, iNOS	Storer <i>et al.</i> , 2005
LPS-stimulated microglia	PPAR γ	IL-6, TNF α , iNOS, COX-2	Luna-medina <i>et al.</i> , 2005
A β -stimulated microglia	PPAR γ	IL-6, TNF α , COX-2	Combs <i>et al.</i> , 2000
LPS-stimulated astrocytes	PPAR α , RXR	IL-1 β , IL-6, TNF α , iNOS, MCP-1	Xu <i>et al.</i> , 2006
LPS-stimulated astrocytes	PPAR γ	IL-6, TNF α , iNOS, COX-2	Luna-medina <i>et al.</i> , 2005
LPS-stimulated astrocytes	PPAR γ	IL-1 β , IL-6, TNF α , MCP-1, iNOS	Storer <i>et al.</i> , 2005
Cerebellar injection of IFN γ , LPS	PPAR γ	iNOS, cell death	Heneka <i>et al.</i> , 2000
MPTP-treated mice	PPAR γ	iNOS, I κ B, cell death	Dehmer <i>et al.</i> , 2004
APPV717I transgenic mice	PPAR γ	iNOS, COX-2, β -amyloid	Heneka <i>et al.</i> , 2005
Rat spinal cord injury	PPAR γ	IRF-1, IL-1 β , IL-6, MCP-1, ICAM, Egr-1	Park <i>et al.</i> , 2007
Rat model for stroke	PPAR γ	IL-1 β , iNOS, COX-2	Sundararajan <i>et al.</i> , 2005

6. Conclusion

The studies discussed here demonstrate that RAR/RXR and PPAR/RXR signaling may be therapeutic targets after spinal cord injury. The main potential of PPAR ligands derives from their anti-inflammatory capacity, which has been corroborated in rodent models of inflammation involving various peripheral organs. After spinal cord injury, inflammatory reactions account for a large proportion of the secondary damage to neurons and oligodendrocytes. In this context, first experiments show that PPAR ligands are neuroprotective.

tive. Since PPAR activators are already in use to treat diabetes, clinical studies after stroke or other kinds of CNS damage are to be expected. While retinoids also have anti-inflammatory properties, they exert more direct effects on the nerve tissue. In several populations of neurons, axonal regeneration can be induced or supported by the activation of RAR/RXR signaling. Since systemic application of retinoic acid influences neural physiology within the CNS, therapeutic possibilities arise here as well. As in the case of cancer treatment, an important consideration is the expression of retinoid receptors. After spinal cord injury the induction of RAR β appears to be a requirement. In addition to the classical transcriptional effects, retinoids and PPAR ligands can act via receptor-independent mechanisms. This, however, is a largely uncharted territory where therapeutic benefits remain to be discovered.

Acknowledgements

S. van Neerven is supported by a Marie Curie Fellowship of the EURON Graduate School of Neuroscience. The authors thank Eric Kampmann for helpful discussions and proof-reading of the manuscript.

References

- Alique M., Lucio F. J., and Herrero J. F., 2006. Vitamin A active metabolite, all-*trans* retinoic acid, induces spinal cord sensitization. II. Effects after intrathecal administration," *British Journal of Pharmacology*, **149**, 65–72
- Arrieta O., García-Navarrete R., Zúñiga S., *et al.*, 2005. Retinoic acid increases tissue and plasma contents of nerve growth factor and prevents neuropathy in diabetic mice. *European Journal of Clinical Investigation*, **35**, 201–207
- Baptiste D. C. and Fehlings M. G., 2006. Pharmacological approaches to repair the injured spinal cord. *Journal of Neurotrauma* **23**, 318–334
- Bastien J. and Rochette-Egly C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene*, **328**, 1–16
- Belyaeva O. V., Korkina O. V., Stetsenko A. V., Kim T., Nelson P. S., and Kedishvili N. Y., 2005. Biochemical properties of purified human retinol dehydrogenase 12 (RDH12): catalytic efficiency toward retinoids and C9 aldehydes and effects of cellular retinol-binding protein type I (CRBPI) and cellular retinaldehyde-binding protein (CRALBP) on the oxidation and reduction of retinoids. *Biochemistry*, **44**, 7035–7047
- Benani A., Krémarik-Bouillaud P., Bianchi A., Netter P., minn A., and Dauc,a M., 2003. Evidence for the presence of both peroxisome proliferator-activated receptors alpha and beta in the rat spinal cord. *Journal of Chemical Neuroanatomy* **25**, 29–38
- Bernardo A., Levi G., and minghetti L., 2000 Role of the peroxisome proliferator-activated receptor- γ (PPAR- γ) and its natural ligand 15-deoxy- Δ 12,14-prostaglandin J2 in the regulation of microglial functions. *European Journal of Neuroscience*, **12**, 2215–2223
- Besson V. C., Chen X. R., Plotkine M., and Marchand-Verrecchia C., 2005. Fenofibrate, a peroxisome proliferator-activated receptor α agonist, exerts neuroprotective effects in traumatic brain injury. *Neuroscience Letters*, **388**, 7–12
- Blomhoff R. and Blomhoff h. K., 2006. Overview of retinoid metabolism and function. *Journal of Neurobiology*, **66**, 606–630
- Braissant O. and Wahli W., 1998. Differential expression of peroxisome proliferator-activated receptor- α , - β , and - γ during rat embryonic development. *Endocrinology*, **139**, 2748–2754
- Budhu A. S. and Noy N., 2002. Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. *Molecular and Cellular Biology*, **22**, 2632–2641
- Chinetti G., Griglio S., Antonucci M., *et al.*, 1998. Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. *Journal of Biological Chemistry*, **273**, 25573–25580
- Choi W.-H., Ji K.-A., Jeon S.-B., *et al.*, 2005. Anti-inflammatory roles of retinoic acid in rat brain astrocytes: suppression of interferon- γ -induced JAK/STAT phosphorylation. *Biochemical and Biophysical Research Communications*, **329**, 125–131
- Cimini A., Benedetti E., Cristiano L., *et al.*, 2005. Expression of peroxisome proliferator-activated receptors (PPARs) and retinoic acid receptors (RXRs) in rat cortical neurons. *Neuroscience*, **130**, 325–337, 2005

- Clagett-Dame M., McNeill E. M., and Muley P. D., 2006. Role of all-*trans* retinoic acid in neurite outgrowth and axonal elongation. *Journal of Neurobiology*, **66**, 739–756
- Colville-Nash P. R., Qureshi S. S., Willis D., and Willoughby D. A., 1998. Inhibition of inducible nitric oxide synthase by peroxisome proliferator-activated receptor agonists: correlation with induction of heme oxygenase 1. *Journal of Immunology*, **161**, 978–984
- Combs C.K., Johnson D.E., Karlo J. C., Cannady S. B., and Landreth G. E., 2000. Inflammatory mechanisms in Alzheimer's disease: inhibition of β - amyloid-stimulated proinflammatory responses and neurotoxicity by PPAR γ agonists. *Journal of Neuroscience*, **20**, 558–567
- Corcoran J. and Maden M., 1999. Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nature Neuroscience*, **2**, 307–308
- Corcoran J., Shroot B., Pizze J., and Maden M., 2000. The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *Journal of Cell Science*, **113**, 2567–2574
- Corcoran J., So P.-L., Barber R. D., *et al.*, 2002. Retinoic acid receptor β 2 and neurite outgrowth in the adult mouse spinal cord *in vitro*. *Journal of Cell Science*, **115**, 3779–3786
- Cosgaya J. M. and Aranda A., 2001. Nerve growth factor activates the RAR β 2 promoter by a Ras-dependent mechanism. *Journal of Neurochemistry*, **76**, 661–670
- Cuzzocrea S., Di Paola R., Mazzone E., *et al.*, 2004. Role of endogenous and exogenous ligands for the peroxisome proliferator-activated receptors α (PPAR- α) in the development of inflammatory bowel disease in mice. *Laboratory Investigation*, **84**, 1643–1654
- Cuzzocrea S., Mazzone E., Di Paola R., *et al.*, 2006. The role of the peroxisome proliferator-activated receptor- α (PPAR- α) in the regulation of acute inflammation. *Journal of Leukocyte Biology*, **79**, 999–1010
- Dehmer T., Heneka M. T., Sastre M., Dichgans J., and Schulz J. B., 2004. Protection by pioglitazone in the MPTP model of Parkinson's disease correlates with I κ B α induction and block of NF κ B and iNOS activation. *Journal of Neurochemistry*, **88**, 494–501
- Delayre-Orthez C., Becker J., Guenon I., *et al.*, 2005. PPAR α downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respiratory Research*, **6**, 91
- Delerive P., Martin-Nizard F., Chinetti G., *et al.*, 1999 Peroxisome proliferator-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. *Circulation Research*, **85**, 394–402
- Delerive P., De Bosscher K., Besnard S., *et al.*, 1999. Peroxisome proliferator-activated receptor α negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF- κ B and AP-1. *Journal of Biological Chemistry*, **274**, 32048–32054
- Dmetrichuk J. M., Carlone R. L., and Spencer G. E., 2006. Retinoic acid induces neurite outgrowth and growth cone turning in invertebrate neurons. *Developmental Biology*, **294**, 39–49,
- Devchand P. R., Keller h., Peters J.M., Vazquez M., Gonzalez F. J., and Wahli W., 1996. The PPAR α -leukotriene B4 pathway to inflammation control. *Nature*, **384**, 39–43
- Dheen S. T., Jun Y., Yan Z., Tay S. S. W., and Ling E. A., 2005. Retinoic acid inhibits expression of TNF- α and iNOS in activated rat microglia. *GLIA*, **50**, 21–31

- Ertesvag A, Engedal N, Naderi S, and Blomhoff h. K., 2002. Retinoic acid stimulates the cell cycle machinery in normal T cells: involvement of retinoic acid receptor-mediated IL-2 secretion. *Journal of Immunology*, **169**, 5555–5563
- Fang h., Jin h., and Wang h., 2004. Effect of all-*trans* retinoic acid on airway inflammation in asthmatic rats and its mechanism. *Journal of Huazhong University of Science and Technology—Medical Science*, **24**, 229–232
- Genovese T., Mazzon E., Di Paola R., *et al.*, 2005. Role of endogenous ligands for the peroxisome proliferators activated receptors alpha in the secondary damage in experimental spinal cord trauma. *Experimental Neurology*, **194**, 267–278
- Gervois P., Chopin-Delannoy S., Fadel A., *et al.*, 1999. Fibrates increase human REV-ERBa expression in liver via a novel peroxisome proliferator-activated receptor response element. *Molecular Endocrinology*, **13**, 400–409
- Giguere V., Ong E. S., Segui P., and Evans R. M., 1987. Identification of a receptor for the morphogen retinoic acid. *Nature*, **330**, 624–629
- Giri S., Rattan R., Singh A. K., and Singh I., 2004. The 15-deoxy- δ 12,14-prostaglandin J2 inhibits the inflammatory response in primary rat astrocytes via down-regulating multiple steps in phosphatidylinositol 3-kinase-akt-NF- κ B-p300 pathway independent of peroxisome proliferator-activated receptor γ . *Journal of Immunology*, **173**, 5196–5208, 2004
- Heneka M. T., Klockgether T., and Feinstein D. L., 2000. Peroxisome proliferator activated receptor- γ ligands reduce neuronal inducible nitric oxide synthase expression and cell death *in vivo*. *Journal of Neuroscience*, **20**, 6862–6867
- Heneka M. T., Sastre M., Dumitrescu-Ozimek L., *et al.*, 2005. Acute treatment with the PPAR γ agonist pioglitazone and ibuprofen reduces glial inflammation and A β 1–42 levels in APPV717I transgenic mice. *Brain*, **128**, 1442–1453
- Ho L.-J., Lin L.-C., Hung L.-F., *et al.*, 2005. Retinoic acid blocks pro-inflammatory cytokine-induced matrix metalloproteinase production by down-regulating JNK-AP-1 signaling in human chondrocytes. *Biochemical Pharmacology*, **70**, 200–208
- Holst A. V., Lefcort F., and Rohrer h., 1997. TrkA expression levels of sympathetic neurons correlate with NGF-dependent survival during development and after treatment with retinoic acid. *European Journal of Neuroscience*, **9**, 2169–2177
- Hunter K., Maden M., Summerbell D., Eriksson U., and Holder N., 1991. Retinoic acid stimulates neurite outgrowth in the amphibian spinal cord. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 3666–3670
- Issemann I. and Green S., 1990 Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, **347**, 645–650
- Jiang C., Ting A. T., and Seed B., 1998. PPAR- γ agonists inhibit production of monocyte inflammatory cytokines. *Nature*, **391**, 82–86
- Johann V., Jeliaznik N., Schrage K., and Mey J., 2003. Retinoic acid downregulates the expression of ciliary neurotrophic factor in rat Schwann cells. *Neuroscience Letters*, **339**, 13–16

- Jové M., Laguna J. C., and Vázquez-Carrera M., 2005. Agonist-induced activation releases peroxisome proliferator-activated receptor β/δ from its inhibition by palmitate-induced nuclear factor- κ B in skeletal muscle cells. *Biochimica et Biophysica Acta*, **1734**, 52–61
- Kern J., Schrage K., Koopmans G. C., Joosten E. A., McCaffery P., and Mey J., 2007. Characterization of retinaldehyde dehydrogenase-2 induction in NG2-positive glia after spinal cord contusion injury. *International Journal of Developmental Neuroscience*, **25**, 7–16
- Kielian T. and D. Drew P., 2003. Effects of peroxisome proliferator-activated receptor- γ agonists on central nervous system inflammation. *Journal of Neuroscience Research* **71**, 315–325
- Kliewer S. A., Forman B. M., Blumberg B., et al., 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 7355–7359
- Kota B. P., Huang T. h.-W., and Roufogalis B. D., 2005. An overview on biological mechanisms of PPARs. *Pharmacological Research*, **51**, 85–94
- Lane M. A. and Bailey S. J., 2005. Role of retinoid signalling in the adult brain. *Progress in Neurobiology*, **75**, 275–293
- Lee S., Kim W., Moon S.-O., et al., 2006. Rosiglitazone ameliorates cisplatin-induced renal injury in mice. *Nephrology Dialysis Transplantation*, **21**, 2096–2105
- Li A. C. and Palinski W., 2006. Peroxisome proliferator-activated receptors: how their effects on macrophages can lead to the development of a new drug therapy against atherosclerosis," *Annual Review of Pharmacology and Toxicology*, **46**, 1–39
- Liberto C.M., Albrecht P. J., Herx L.M., Yong V.W., and Levison S.W., 2004. Pro-regenerative properties of cytokine-activated astrocytes. *Journal of Neurochemistry* **89**, 1092–1100
- Liu J.-P., Laufer E., and Jessell T.M., 2001. Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron*, **32**, 997–1012
- LoVerme J., Russo R., La Rana G., et al., 2006. Rapid broad-spectrum analgesia through activation of peroxisome proliferator-activated receptor- α . *Journal of Pharmacology and Experimental Therapeutics*, **319**, 1051–1061
- Luna-Medina R., Cortes-Canteli M., Alonso M., Santos A., Martínez A., and Perez-Castillo A., 2005. Regulation of inflammatory response in neural cells *in vitro* by thiazolidinone derivatives through peroxisome proliferator-activated receptor γ activation. *Journal of Biological Chemistry*, **280**, 21453–21462
- Makwana M. and Raivich G., 2005. Molecular mechanisms in successful peripheral regeneration. *FEBS Journal* **272**, 2628–2638
- Maden M., Keen G., and Jones G. E., 1998. Retinoic acid as a chemotactic molecule in neuronal development. *International Journal of Developmental Neuroscience*, **16**, 317–322
- Mey J. and Thanos S., 1991. Ontogenetic changes in the regenerative ability of chick retinal ganglion cells as revealed by organ explants. *Cell and Tissue Research*, **264**, 347–355
- Mey J. and Rombach N., 1999. Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells *in vitro*. *NeuroReport*, **10**, 3573–3577
- Mey J., Morassutti D. J., Brook G., et al., 2005. Retinoic acid synthesis by a population of NG2-positive cells in the injured spinal cord. *European Journal of Neuroscience*, **21**, 1555–1568

- Mey J., 2006. New therapeutic target for CNS injury? The role of retinoic acid signaling after nerve lesions. *Journal of Neurobiology* **66**, 757–779 2006
- Moraes L. A., Piqueras L., and Bishop-Bailey D., 2006 Peroxisome proliferator-activated receptors and inflammation. *Pharmacology and Therapeutics*, **110**, 371–385
- Moreno S., Farioli-Vecchioli S., and Cer`u M. P., 2004. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. *Neuroscience*, **123**, 131–145
- Mun-Bryce S. and Rosenberg G. A., 1998. Gelatinase B modulates selective opening of the blood-brain barrier during inflammation. *American Journal of Physiology*, **274**, R1203–R1211
- Na S.-Y., Kang B. Y., Chung S. W., *et al.*, 1999. Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NF κ B. *Journal of Biological Chemistry*, **274**, 7674–7680
- Nakajima A., Wada K., Miki h., *et al.*, 2001 Endogenous PPAR γ mediates anti-inflammatory activity in murine ischemiareperfusion injury. *Gastroenterology*, **120**, 460–469
- Nguyen J., Dumont J., and Bauvois B., 2006 Comparative effects of interferon-gamma and all-*trans* retinoic acid on secreted and surface-associated matrix metalloproteinase-9 expression of human monocytes. *Cellular and Molecular Biology*, **52**, 51–58
- Noble L. J., Donovan F., Igarashi T., Goussev S., and Werb Z., 2002 Matrix metalloproteinases limit functional recovery after spinal cord injury by modulation of early vascular events. *Journal of Neuroscience*, **22**, 7526–7535
- Nozaki Y., Yamagata T., Sugiyama M., Ikoma S., Kinoshita K., and Funauchi M., 2006. Anti-inflammatory effect of all-*trans*retinoic acid in inflammatory arthritis. *Clinical Immunology*, **119**, 272–279
- Okamoto h., Iwamoto T., Kotake S., Momohara S., Yamanaka h., and Kamatani N., 2005. Inhibition ofNK- κ B signaling by fenofibrate, a peroxisome proliferator-activated receptor- α ligand, presents a therapeutic strategy for rheumatoid arthritis. *Clinical and Experimental Rheumatology*, **23**, 323–330
- Orfanos C. E. and Bauer R., 1983. Evidence for anti-inflammatory activities of oral synthetic retinoids: experimental findings and clinical experience. *British Journal of Dermatology*, **109**, 55–60
- Park S.-W., Yi J.-H., Miranpuri G., *et al.*, 2007. Thiazolidinedione class of peroxisome proliferator-activated receptor γ agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *Journal of Pharmacology and Experimental Therapeutics*, **320**, 1002–1012
- Petkovich M., Brand N. J., Krust A., and Chambon P., 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature*, **330**, 444–450
- Petrova T. V., Akama K. T., and Van Eldik L. J., 1999. Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy- Δ 12,14-prostaglandin J2. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 4668–4673
- Popovich P. G., Stuckman S., Gienapp I. E., and Whitacre C. C., 2001. Alterations in immune cell phenotype and function after experimental spinal cord injury. *Journal of Neurotrauma*, **18**, 957–966
- Poynter M. E. and Daynes R. A., 1998. Peroxisome proliferatoractivated receptor α activation modulates cellular redox status, represses nuclear factor- κ B signaling, and reduces inflammatory cytokine production in aging. *Journal of Biological Chemistry*, **273**, 32833–32841

- Quinn S. D. P. and De Boni U., 1991. Enhanced neuronal regeneration by retinoic acid of murine dorsal root ganglia and of fetal murine and human spinal cord *in vitro*. *In Vitro Cellular and Developmental Biology - Animal*, **27**, 55–62
- Raivich G., Bohatschek M., Kloss C. U. A., Werner A., Jones L. L., and Kreutzberg G. W., 1999. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Research Reviews*, **30**, 77–105
- Ricote M., Li A. C., Willson T. M., Kelly C. J., and Glass C. K., 1998. The peroxisome proliferator-activated receptor- γ is a negative regulator of macrophage activation. *Nature*, vol. **391**, 79–82
- Rodriguez-Tebar A. and Rohrer h., 1991. Retinoic acid induces NGF-dependent survival response and high-affinity NGF receptors in immature chick sympathetic neurons. *Development*, **112**, 813–820
- Romero-Sandoval E. A., Molina C., Alique M., Moreno- Manzano V., Lucio F. J., and Herrero J. F., 2006. Vitamin A active metabolite, all-*trans* retinoic acid, induces spinal cord sensitization. I. Effects after oral administration. *British Journal of Pharmacology*, **149**, 56–64
- Schrage K., Koopmans G., Joosten E. A. J., and Mey J., 2006. Macrophages and neurons are targets of retinoic acid signalling after spinal cord contusion injury. *European Journal of Neuroscience*, **23**, 285–295
- Silver J. and Miller J. h., 2004. Regeneration beyond the glial scar, *Nature Reviews Neuroscience* **5**, 146–156
- So P.-L., Yip P. K., Bunting S., *et al.*, 2006. Interactions between retinoic acid, nerve growth factor and sonic hedgehog signalling pathways in neurite outgrowth. *Developmental Biology*, **298**, 167–175
- Sockanathan S., Perlmann T., and Jessell T. M., 2003. Retinoid receptor signaling in postmitotic motor neurons regulates rostrocaudal positional identity and axonal projection pattern. *Neuron*, **40**, 97–111
- Spencer N. F. L., Poynter M. E., Im S.-Y., and Daynes R. A., 1997. Constitutive activation of NF- κ B in an animal model of aging. *International Immunology*, **9**, 1581–1588
- Storer P. D., Xu J., Chavis J., and Drew P. D., 2005. Peroxisome proliferator-activated receptor- γ agonists inhibit the activation of microglia and astrocytes: implications for multiple sclerosis. *Journal of Neuroimmunology*, **161**, pp. 113–122
- Sundararajan S., Gamboa J. L., Victor N. A., Wanderi E. W., Lust W. D., and Landreth G. E., 2005. Peroxisome proliferator-activated receptor- γ ligands reduce inflammation and infarction size in transient focal ischemia. *Neuroscience*, **130**, 685–696
- Sung B., Park S., Yu B. P., and Chung h. Y., 2006. Amelioration of age-related inflammation and oxidative stress by PPAR γ activator: suppression of NF- κ B by 2,4-thiazolidinedione. *Experimental Gerontology*, **41**, 590–599
- Taylor B. K., Dadia N., Yang C. B., Krishnan S., and Badr M., 2002. Peroxisome proliferator-activated receptor agonists inhibit inflammatory edema and hyperalgesia. *Inflammation*, **26**, 121–127
- Taha M. O., Rosseto M., Fraga M. M., *et al.*, 2004. Effect of retinoic acid on tibial nerve regeneration after anastomosis in rats: histological and functional analyses. *Transplantation Proceedings*, **36**, 404–408
- Vermot J., Schuhbaur B., Le Mouellie h., *et al.*, 2005. Retinaldehyde dehydrogenase 2 and Hoxc8 are required in the murine brachial spinal cord for the specification on Lim1+ motoneurons and the correct distribution of Islet1+ motoneurons. *Development*, **132**, 1611–1621
- von Knethen A. and Brüne B., 2001. Delayed activation of PPAR γ by LPS and IFN- γ attenuates the oxidative burst in macrophages. *FASEB Journal*, **15**, 535–544

- Wada K., Nakajima A., Takahashi h., *et al.*, 2004. Protective effect of endogenous PPAR γ against acute gastric mucosal lesions associated with ischemia-reperfusion. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, **287**, G452–G458
- Wong L.-F., Yip P. K., Battaglia A., *et al.*, 2006. Retinoic acid receptor β 2 promotes functional regeneration of sensory axons in the spinal cord. *Nature Neuroscience*, **9**, 243–250
- Wuarin L., Sidell N., and de Vellis J., 1990. Retinoids increase perinatal spinal cord neuronal survival and astroglial differentiation. *International Journal of Developmental Neuroscience*, **8**, 317–326
- Wuarin L. and Sidell N., 1991. Differential susceptibilities of spinal cord neurons to retinoic acid-induced survival and differentiation. *Developmental Biology*, **144**, 429–435
- Wyatt S., Andres R., Rohrer h., and Davies A. M., 1999. Regulation of neurotrophin receptor expression by retinoic acid in mouse sympathetic neuroblasts. *Journal of Neuroscience*, **19**, 1062–1071
- Xu J., Storer P. D., Chavis J. A., Racke M. K., and Drew P. D., 2005. Agonists for the peroxisome proliferator-activated receptor- α and the retinoid X receptor inhibit inflammatory responses of microglia. *Journal of Neuroscience Research*, **81**, 403–411
- Xu J. and Drew P. D., 2006. 9-*cis*-retinoic acid suppresses inflammatory responses of microglia and astrocytes. *Journal of Neuroimmunology*, **171**, 135–144
- Xu J., Chavis J. A., Racke M. K., and Drew P. D., 2006. Peroxisome proliferator-activated receptor- α and retinoid X receptor agonists inhibit inflammatory responses of astrocytes. *Journal of Neuroimmunology*, **176**, 95–105
- Yamamoto M., McCaffery P., and Dräger U. C., 1996. Influence of the choroid plexus on cerebellar development: analysis of retinoic acid synthesis. *Developmental Brain Research*, **93**, 182–190
- Yip P. K., Wong L.-F., Pattinson D., *et al.*, 2006. Lentiviral vector expressing retinoic acid receptor β 2 promotes recovery of function after corticospinal tract injury in the adult rat spinal cord. *Human Molecular Genetics*, **15**, 3107–3118
- Zhelyaznik N., Schrage K., McCaffery P., and Mey J., 2003. Activation of retinoic acid signalling after sciatic nerve injury: upregulation of cellular retinoid binding proteins. *European Journal of Neuroscience*, **18**, 1033–1040
- Zhelyaznik N. and Mey J., 2006. Regulation of retinoic acid receptors α , β and retinoid X receptor α after sciatic nerve injury. *Neuroscience*, **141**, 1761–1774
- Zhu L., Bisgaier C. L., Aviram M., and Newton R. S., 1999. 9-*cis* retinoic acid induces monocyte chemoattractant protein-1 secretion in human monocytic THP-1 cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **19**, 2105–2111

Inflammatory chemokine release of astrocytes *in vitro* is reduced by all-*trans* retinoic acid

4

S.G.A. van Neerven^{1,2}, T. Regen³, D. Wolf¹, A. Nemes¹,
S. Johann⁴, C. Beyer⁴, U. Hanisch³ and J. Mey^{1,2}

¹Institute of Biology II, RWTH Aachen University,
RWTH Aachen, Germany

²EURON European Graduate School of Neuroscience,
Maastricht, The Netherlands

³Institute of Neuropathology, Medical Faculty, University of Göttingen,
Göttingen, Germany

⁴Institute of Neuroanatomy, Medical Faculty, RWTH Aachen University,
Germany

Journal of Neurochemistry, 2010; 114(5): 1511–26

Abstract

The production of chemokines by astrocytes constitutes an important component of neuroinflammatory processes in the brain. As the transcriptional activator retinoic acid (RA), used for chemotherapy and dermatological applications, exerts anti-inflammatory effects on monocytes and lymphocytes, we have tested whether the physiologically occurring isomer, *all-trans* RA, affects chemokine expression by astrocytes. Under control conditions, primary cultures of murine cortical astrocytes expressed no or very low levels of CCL and CXCL chemokines. After treatment with bacterial lipopolysaccharides to simulate inflammation *in vitro*, we detected a strong increase in the release of CCL2 (to > 4 ng/mL in cell culture supernatant), CCL3 (> 20 ng/mL), CCL5 (> 25 ng/mL), CXCL1 (> 30 ng/mL) and CXCL2 (> 20 ng/mL). Although simultaneous exposure to RA did not significantly affect this response, 12 h pretreatment with 0.1 μ M *all-trans* RA strongly suppressed mRNA expression and protein release of all chemokines. The anti-inflammatory activity of RA engaged RA and retinoid X receptors and correlated with a decreased expression of the lipopolysaccharides co-receptor CD14. A minor reduction of nuclear NF- κ B was observed but not significant, activation of Jun amino-terminal kinase, p38 and signal transducer and activator of transcription 3 were not altered by RA. The results suggest that retinoids should be further investigated as candidates for the treatment of neuroinflammation.

Keywords: CCL, CXCL, glia, inflammation, lipopolysaccharide, retinoids

Introduction

Inflammatory reactions drive neurodegenerative processes that result from ischemia, brain injury or during neurological disorders. Although for many years microglia have been in the focus of neuroimmunological research, accumulating evidence points to a crucial role of astrocytes in this context (Farina *et al.* 2007). To limit neuronal cell death under pathological conditions, it is therefore important to understand and control astroglial inflammatory reactions. In the present study, we have explored whether the transcriptional activator retinoic acid (RA) modulates one important aspect of inflammation, the production of chemokines.

Astrocytes, which are the most abundant glia in the vertebrate CNS, can be stimulated to produce a wide variety of inflammatory mediators including cytokines, chemokines and prostaglandins (Farina *et al.* 2007; Johann *et al.* 2008). As astrocytes are also involved in protective mechanisms (Liberto *et al.* 2004; Yiu and He 2006; Herrmann *et al.* 2008), a precise regulation of their response may determine the outcome of CNS inflammation. To study this aspect *in vitro*, cells are typically stimulated with bacterial endotoxins, most frequently by using *Escherichia coli* lipopoly-saccharides (LPS). After binding to CD14 and toll-like receptor (TLR)4, LPS activate several intracellular signalling cascades eventually targeting transcription factors NF- κ B, AP-1 and signal transducer and activator of transcription (STAT)3, which in turn activate a large number of pro-inflammatory genes (Li and Stark 2002; Herrmann *et al.* 2008; Okun *et al.* 2008).

An important aspect of the inflammatory cascade is the production of chemokines, because they induce the invasion of lymphocytes and monocytes through the blood–brain barrier into the brain (Fig. 1; Baggiolini 1998). These small, secreted proteins are classified based on the position of conserved cysteine residues into C, CC, CXC and CX3C subfamilies. Their G-protein coupled receptors can activate several downstream signaling cascades. Blocking of chemokine activity has been shown to reduce damage of the blood brain barrier and to be neuroprotective after ischemia in rats (Anthony *et al.* 1998). Astrocytes which express functional chemokine receptors themselves (Dorf *et al.* 2000), produce chemokines that are responsible for neutrophil, T-cell and monocyte infiltration of the injured CNS (Babcock *et al.* 2003; Ambrosini and Aloisi 2004).

In the present study, we investigated CCL2 (monocyte chemoattractive protein, MCP-1), CCL3 (macrophage inflammatory protein, MIP-1 α), and CCL5 (regulated upon activation normal T-cell expressed and secreted, RANTES). These factors are chemoattractants for monocytes and leukocytes and were found to be elevated after trauma, ischemia, in multiple sclerosis, experimental autoimmune encephalomyelitis, after HIV, bacterial infection, and in brain tumors (Ambrosini and Aloisi 2004). In addition, we measured the neutrophil chemoattractants CXCL1 (KC, the mouse homologue of Gro- α) and CXCL2 (MIP-2) produced by astrocytes and up-regulated after experimental autoimmune

encephalomyelitis and infection. These chemokines maintain and exacerbate the inflammatory reaction in astrocytes by an autoregulatory mechanisms involving mRNA stabilization (Luo *et al.* 2000; Ambrosini and Aloisi 2004).

Recently, the possible pharmacological function of retinoids as modulators of neuroinflammation has gained scientific interest (Xu and Drew 2006; Zhang-Gandhi and Drew 2007; Kampmann *et al.* 2008). The transcriptional activator RA is involved in a wide range of biological processes including glial and neuronal survival, axonal regeneration (Wong *et al.* 2006; Yip *et al.* 2006) and has been implicated in neurological and psychiatric disorders (Malaspina and Michael-Titus 2008; van Neerven *et al.* 2008). Endogenously, RA is synthesized from blood-derived vitamin A and released by cells in a paracrine and auto-crine fashion. Taken up by target cells, RA binds to RA receptors (RAR α , - β , - γ) that heterodimerize with retinoid X receptors (RXR α , - β , - γ) to form a ligand activated transcriptional complex (Bastien and Rochette-Egly 2004). All-trans RA and 9-cis RA bind to the RAR family, whereas only 9-cis RA is a high affinity ligand for the RXR. In addition to the direct transcriptional control, many target genes are regulated indirectly, and several non-genomic actions of RA are known (Na *et al.* 1999; Choi *et al.* 2005; Blomhoff and Blomhoff 2006). The observation that spinal cord injury was followed by RA synthesis and nuclear translocation

Fig. 1. Retinoic acid in the context of chemokine signaling. Following tissue damage, Chemokines are transported through the endothelium into the blood, where they induce integrin expression in lymphocytes, neutrophils and macrophages, causing these cells to infiltrate into the CNS through the blood-brain barrier. We hypothesize that retinoic acid (RA) interferes with this process. microglia and macrophages secrete pro-inflammatory cytokines and chemokines.

of retinoid receptors in glia cells led us to postulate that RA is an endogenous modulator of neuroinflammation (Mey *et al.* 2005; Schrage *et al.* 2006). In the present study, we have explored the potential of RA to modulate the production of chemokines. Therefore, primary astrocyte cultures were challenged with LPS and treated with all-*trans* RA to investigate its effect on expression and secretion of proinflammatory chemokines.

Material and methods

Astrocyte primary cultures

Primary cultures of astrocytes were prepared from the mesencephalon and cerebral cortices of 1- to 2-day-old balb/c mice (Charles River). After dissection and removal of the meninges, both brain regions were dissociated mechanically in 0.25% Trypsin-EDTA solution (Sigma Aldrich, München, Germany), passed through a 50 μ m nylon mesh, and centrifuged at 130 g for 10 min. Cells were resuspended and further cultivated in medium containing DMEM including 20% heat-inactivated fetal calf serum, 0.5% penicillin/streptavidin and 0.1% fungizone (all from Invitrogen, Karlsruhe, Germany) and subsequently seeded in T75 flasks coated with poly-L-lysine (200 μ g/mL; Sigma Aldrich). Cells were grown for 5 days at 37° C in a humidified incubator with 5% CO₂, and medium was changed every 2–3 days. When reaching confluence, cells were passaged three times to remove microglia.

Purity of the cultures was checked with immunocytochemical staining using antibodies against glial fibrillary acidic protein to mark astrocytes (rabbit anti-mouse polyclonal antibody: DAKO 1/2000) and ED1 to detect activated microglia (mouse monoclonal, Serotec, MCA341R, 1/100). Microglia cells were also visualized with tomato lectin, 10 μ g/mL (Vector Laboratories, Burlingame, CA, USA; FL-1201). Results were evaluated with a Zeiss Axiophot epifluorescence microscope coupled to an online digital camera (Axiovision software; Carl Zeiss, Göttingen, Deutschland).

Treatment of cell cultures

After the third passage, astrocytes were seeded into poly-L-lysine coated 6-wells plates, containing approximately 400.000 cells per well. Before treatment, astrocytes were kept for 48 h in serum-free neurobasal medium supplemented with 1% glutamine, 0.2% B27 supplement (both from Gibco, Invitrogen, Karlsruhe, Germany, Invitrogen), 0.5% penicillin/streptavidin and 0.1% fungizone. To induce an inflammatory reaction, 100 ng/mL E. coli LPS (Sigma) were applied to the culture medium.

All-*trans* RA (Sigma) was dissolved at 0.1 M in DMSO. To prevent oxidation, air above the aliquots was replaced with nitrogen, and stock aliquots were stored at -80° C.

All handling with RA occurred under conditions of amber lighting (Na⁺ lamp). For use, RA was further diluted to 1, 0.1 and 0.01 mM in 100% ethanol and added to the cell culture medium, reaching final concentrations of 1, 0.1 or 0.01 μ M RA.

The effect of LPS and the influence of RA on chemokine release were monitored for time intervals of 30 min, 1 h, 6 h, 12 h, 24 h, 48 h and 72 h. In addition, the effect of RA pre-treatment on LPS induced chemokine release and expression was measured: As pre-treatment, concentrations of 0.01, 0.1 or 1 μ M RA, or 100% ethanol solvent only were administered 12 h prior to LPS treatment. Subsequently, 100 ng/mL LPS was added, RA replaced with medium change, and cells were incubated for an additional period of 12 h for measuring chemokine expression or additional 3 h for measuring activation of transcription factors. To verify whether RAR/RXR were involved in the molecular signal transduction of RA, astrocytes were treated with the RAR pan-antagonist LE540 or the RXR pan-antagonist PA452 (kindly provided by Drs Kagechika and Shudo, R&R Inc., Tokyo, Japan) in a concentration of 1 μ M, 5 min before RA administration (0.1 μ M).

In additional control experiments, cultures were treated with clodronate (dichloro-phosphono-methyl phosphonic acid; Calbiochem/ Merck Chemicals, Darmstadt, Germany) to eliminate residual microglial cells. After the third passage, cells were left to adhere in normal culture medium for 2 days. Subsequently, the medium was renewed with medium containing 200 μ g/mL clodronate. Cells were incubated for 48 h at 37° C, 5% CO₂, and placed over night on a shaker at 250–300 rpm to remove possible microglia. The next morning astrocytes were washed with phosphate-buffered saline, received normal culture medium, and standard experiments were carried out. As a positive control for the physiological response to LPS, these cells were also simultaneously treated with 100 nM dexamethasone.

Cytotoxicity test: lactate dehydrogenase release

In addition to immunocytochemical observations, described below, the cytotoxicity of treatment conditions was measured using lactate dehydrogenase (LDH) release into the medium (Roche Cytotoxicity Detection Kit; Roche Diagnostics, Mannheim, Germany). LDH release was normalized with respect to maximal LDH release after cell lysis.

Quantitative RT-PCR

Isolation of total RNA was performed with the PeqGOLD extraction kit (Peqlab, Erlangen, Germany); RNA concentration and purity were assessed photometrically (OD₂₆₀, OD₂₆₀/OD₂₈₀ ratio). Total RNA was reverse transcribed into cDNA using Qiagen Omniscript RT kit (Qiagen, Hilden, Germany) and Oligo (dT) 12–18 primers (Invitrogen) with calculated concentrations of 50 ng/ μ L RNA extract per reaction.

Samples were diluted 1/10 after reverse transcription and then used for amplification with the MyiQ5 real-time PCR detection system (Bio-Rad, Munich, Germany). Reactions were carried out in a 96-well reaction module, containing 10 μ L total reaction volume per well. Samples contained 5 μ L of the QuantiTect SYBR Green PCR kit (Qiagen; including Sybr Green I, Hot-StarTaq DNA polymerase, reaction buffer and MgCl_2), 2 μ L nuclease-free water, 2 μ L cDNA sample and 0.5 μ L of each primer (sense and anti-sense each 10 μ M stock solutions). The protocol consisted of 15 min enzyme activation at 95° C, followed by 40 cycles of 15 s denaturation at 94° C, 20 s annealing at the primer-specific temperature, 20 s amplification at 72° C, and 5 s fluorescence measurements at 74° C. Primer sequences and annealing temperatures are listed in Table 1. Expression of the housekeeping gene hypoxanthine–guanine phosphoribosyl-transferase was measured to normalize gene expression. Melting curve analysis (from 65° C to 95° C, in 0.1° C/s) and agarose gel electrophoresis of the PCR products were routinely performed. Relative mRNA concentrations were determined using crossing point analysis. Concentration series (dilutions 1/4, 1/8 ... 1/64) of sample pools, which were included in all runs, showed PCR efficiencies between 90% and 100%, and the $\Delta\Delta\text{Ct}$ method was therefore used for quantification. All samples and concentration series were measured in duplicates.

Tab. 1. Primers used in this study: Melting points of chemokine and LPS receptor PCR products were empirically determined following qRT-PCR (retinoid receptor expression was detected but not quantified).

Name	Primer sequences (sense, antisense)	Annealing T	Product size Product melting T
HPRT	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	59° C	249 bp 79.2° C
CCL2	AGCACCAGCCAACTCTCACT GCTGCTGGTGATCCTCTTGT	62° C	219 bp 84.6° C
CCL3	CCTCTGTCACCTGCTCAACA GATGAATTGGCGTGGAATCT	60° C	163 bp 82.5° C
CCL5	CCCTCACCATCATCCTCACT GAGCACTTGCTGCTGGTGTA	62° C	154 bp 85.9° C
CXCL1	GCTGGGATTACCTCAAGAA CTTTTGGACAATTTTCTGAACCA	60° C	150 bp 82.9° C
CXCL2	AGTGAAGTGGCTGTCAATG CTTCAGGGTCAAGGCAAACT	62° C	154 bp 83.3° C
RAR α	TCTCCCTGGACATTGACCTC GTGCTTGCTCAGGCGTGTA	55° C	196 bp
RAR β	ACAAGTCATCGGGCTACCAC CTGTGCATTCTGCTTTGAA	55° C	249 bp
RAR γ	AGGTCACCAGAAATCGATGC CTGGCAGAGTGAGGGAAAAG	55° C	212 bp
RXR α	CTCACTGGGACATTGTGGTG CTCCCCAACACAGGACAGAT	55° C	150 bp
RXR β	TTAGCAGGGTTCGTTTGGTC GCCAAATGAGAAGGAAGCAG	55° C	175 bp
RXR γ	TGTGGTCAACAGTGTGAGCA TGAAGAAGCCTTGCAACCT	55° C	188 bp
Tgm-2	CATGGTCAACTGCAATGATG GGGCGGAGTTGTAGTTGGT	59° C	236 bp 88.3° C
TLR-2	CGGACTGTTTCCTTCTGACC GCCACCCAAGATCCAGAAGAG	60° C	185 bp 82.4° C
TLR-4	TTCTTCTCCTGCCTGACACC TGTCATCAG GGACTTTGCTG	60° C	106 bp 80.5° C
CD-14	CATGGAGCGTGTGCTTGG CGCCGTACAATCCACATCT	60° C	173 bp 85.2° C
SOCS-3	CCTTTGACAAGCGACTCTC GCCAGCATAAAACCCTTCA	59° C	85.2° C

ELISA

Cell culture supernatants were collected after different periods of incubation, using the same treatment protocols as for quantitative RT-PCR. The release of chemokines into the supernatant was measured with ELISA. CXCL1 (KC), CXCL2 (MIP-2), CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES), CCL11 (Eotaxin), CCL17 (thymus and activation-regulated chemokine) and CCL22 (macrophage-derived chemokine) levels were determined using DuoSet ELISA Development Kits (R&D Systems, Wiesbaden, Germany). All ELISA procedures were performed according to the manufacturer's instructions and absorbance was measured at 450 nm with subtraction of absorbance at 540 nm using a microplate reader (Bio-Rad). For calibration, standard concentration series of all chemokines were measured routinely.

Western blotting

Activation of signal transduction cascades was investigated with immunoblotting. Cells were grown in Petri dishes, treated as previously described and harvested by trypsinization, then centrifuged, pellets washed in phosphate-buffered saline and diluted in nuclear extraction buffer, which was supplemented with 10 μ g/mL aprotinin, 10 μ M leupeptin, 0.1 mM phenylmethylsulfonyl fluoride and 200 μ M NaVO₃ to prevent protein degradation. Cytosolic and nuclear extracts were isolated with NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Perbio Science, Bonn, Germany) according to the manufacturer's instructions. As described previously (Kuenzel *et al.* 2009), sodium dodecyl sulfate–polyacrylamide gel electrophoresis were run with 10% poly acrylamide gels and 10 μ g protein per slot, based on protein measurements with the bicinchoninic acid assay. Proteins were transferred onto nitrocellulose membranes, checked for protein content with Ponceau-S staining, and investigated using primary antibodies, directed against p-STAT3 (#9131), STAT3 (#9132), p-Jun amino-terminal kinase (p-JNK) (#46685), p-MAPK(p38) (#9215), NF- κ B p65 (#4764) and lamin (#2032) all primary antibodies were from Cell Signaling/New England Biolabs (Frankfurt/M, Germany) and diluted 1/1000. The antibodies against CD14 (sc-9150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (A1066; Sigma) were diluted 1/333 and 1/500, respectively. In these experiments, 15 μ g sample protein were used per lane.

Immunoreactive signals were visualized with peroxidaseconjugated secondary antibodies (goat anti-rabbit; Sigma, 1/5000) and the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Freiburg i. Brsg., Germany) according to standard protocol. For semiquantitative evaluation of protein expression, X-ray films were exposed 30 s to 20 min to determine the linear range of the signal, and signal intensities were evaluated with a digital image analysis system (LTF, Wasserburg, Germany). Concentrations of phosphorylated STAT3, c-Jun and p38 were normalized to total STAT3, of CD14 to actin, and NF- κ B in nuclear extracts was normalized to lamin, as detected in the same blots after stripping.

Statistical analysis

The effects of RA on LPS-stimulated astrocytes in the preincubation experiments and time-course experiments were measured for each condition in 6–9 cultures prepared from 2 to 3 different litters. Data were analyzed using one-way ANOVA and post hoc Dunnett's test. In control experiments using clodronate or inhibitors of retinoid receptors, pre-selected comparisons were made using t-test. Any differences in number of independent experiments, or tests are indicated in the figure legends. All statistical analyses were carried out with the logarithms of data, in RT-PCR experiments with Ct values, which showed normal distribution and similar variances. Gene expression results were normalized to levels under LPS stimulation. In the graphs, results are presented as mean $\pm$ standard error of the mean (SEM).

Results

Chemokine release in primary cultures of mouse astrocytes

In the present study, cell culture experiments were performed to test the hypothesis that RA reduces the release of chemokines by murine cortical astrocytes. The applied protocol yielded primary cultures of > 95% astrocytes (Johann *et al.* 2008). Less than 2% of the cells were microglia, identified with tomato lectin or ED1 immunocytochemistry, and no oligodendrocytes or neurons were identified in the cultures. To elicit an inflammatory response, cells were stimulated for 30 min to 72 h with 100 ng/mL LPS. Treatment with LPS or 0.01, 0.1 and 1 μ M all-*trans* RA did not affect cell viability.

In addition to the chemokines whose induction is described below, we measured protein concentrations of CCL11 (eotaxin) and CCL17 (thymus and activation-regulated chemokine). The assay for CCL11 was calibrated with a concentration range of 16–500 pg/mL, and contents in astrocyte supernatants were below the detection limit under all conditions. Culture supernatants contained low concentrations of CCL17 (168 ± 25 pg/mL; mean $\pm$ SEM, $n = 25$; assay standards 125–2000 pg/mL), but there was no effect of LPS or RA treatment on the release of this chemokine. In contrast to CCL11 and CCL17, LPS treatment caused a highly significant increase in protein release of CCL2 (MCP-1), CCL3 (MIP-1 α), CCL5 (RANTES), CXCL1 (KC) and CXCL2 (MIP-2). Data for astrocytes from mouse cerebral cortex are presented in Figs 2 and 3. LPS-effects of a similar magnitude on all five cytokines were observed with astrocytes from mouse midbrain (data not shown). Retinoic acid reduced the release of CCL chemokines into the culture medium.

Retinoic acid reduced the release of CCL chemokines into the culture medium

In the first set of experiments, astrocytes were incubated for various time periods with LPS, *all-trans* RA, or simultaneously with LPS plus RA, and protein concentrations were measured in cell culture supernatants. Inflammatory stimulation with LPS caused a maximum rise of the three CCL chemokines after 48 h with a significant increase observed first after 12 (CCL2, CCL3) or 24 h (CCL5). Compared with the low levels in control cultures, we found induction higher than 10- (to over 4 ng/mL CCL2 released into the culture medium) or 100-fold (> 20 ng/mL CCL3; > 25 ng/mL CCL5). Simultaneous incubation with 0.01, 0.1 or 1 μ M RA had no effect on the pattern or magnitude of chemokine release (Fig. 2a, c and e). In a second set of experiments, however, cells were pre-treated 12 h with RA and then exposed for another 12 h to LPS plus RA. In this case, RA led to a highly significant reduction of the LPS-induced release of CCL2 (to 35% of the level measured after LPS treatment alone), of CCL3 (to 28%) and of CCL5 (to 8%) at the highest concentration of RA (Fig. 2b, d and f; ANOVA was highly significant for all three chemokines, $p < 0.001$). An RA concentration of 10 nM was similarly effective as 1 μ M. The LPS-induced synthesis of CCL3 was transient, as shown by a highly significant reduction (to 30%) of the chemokine concentration when cells received LPS-free medium for another 12 h. Under this condition, protein concentration of CCL2 was lower than in the LPS-treated group but not significantly so, and CCL5 release remained high 12 h after withdrawal of the inflammatory stimulus. Retinoic acid reduced the release of CXCL chemokines into the culture medium.

Retinoic acid reduced the release of CXCL chemokines into the culture medium

Similar experiments and measurements were performed for CXCL1 and CXCL2, which gave comparable results. Again, although RA alone did not affect the synthesis of the chemokines, LPS exposure resulted in an increased release after 12 h reaching a maximum after 48 h (80-fold, to more than 30 ng/mL for CXCL1 and 60-fold, 20 ng/mL for CXCL2). As for the CCL chemokines, no reduction of this effect was measured when astrocytes were simultaneously treated with RA, whereas pre-treatment with 1 μ M *all-trans* RA reduced the amount of cytokine release of CXCL1 to 16% and of CXCL2 to 20% with similar effects of 0.01 and 0.1 μ M RA (Fig. 3; ANOVA $p < 0.001$). This was highly significant for all concentrations of RA. The recovery experiment showed a strong decline in production of CXCL2 (to 19%), whereas CXCL1 continued to be released 12 h after removal of LPS.

Fig. 2. Effect of lipopolysaccharide (LPS) and retinoic acid (RA) on the release of CCL chemokines by astrocytes.

Following the different treatment protocols, chemokine concentrations were measured in cell culture supernatants with ELISA. (a, b) CCL2 (MCP-1), (c, d) CCL3 (MIP-1 α), (e, f) CCL5 (RANTES). In (a), (c) and (e), cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/mL and the three concentrations of all-*trans* RA. In (b), (d) and (f), cultures were pre-treated 12 h with RA followed by 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. In recovery experiments (L) LPS-free medium was given for 12 h after the LPS stimulation. Seven to nine cultures from three different litters were analyzed, error bars indicate SEM. Following ANOVA (in b, d, f), levels after exposure to LPS-only or RA-only were compared with controls (###*p* < 0.001), and RA + LPS conditions were compared with LPS only (**p* < 0.05, ***p* < 0.01, ****p* < 0.001; Dunn's test).

Retinoic acid reduced the mRNA expression of CCL and CXCL chemokines by cortical astrocytes

As 12 h RA pre-treatment of the cells mitigated chemokine release while simultaneous exposure to RA and LPS had no significant effect, it was tested whether RA pre-treatment affected chemokine expression at the mRNA level in the astrocyte cultures (quantitative RT-PCR; Fig. 4). In accordance with the ELISA data, transcript concentrations of all five chemokines were strongly increased after LPS treatment. In the absence of LPS, very low expression of CCL2, CCL3 and CCL5 was measured (Fig. 5), and CXCL1 and CXCL2 transcripts were not detected at all (Fig. 6). RA significantly reduced the elevation of chemokine transcripts, indicating an inhibitory effect on gene regulation or RNA stability (Figs 5 and 6). The RA modulation of the inflammatory response lowered transcript concentrations to 30% (CCL2), 29% (CCL3), 9% (CCL5), 16% (CXCL1) and 25% (CXCL2). These are data for the lowest dose of 0.01 μ M all-*trans* RA, with similar efficacy of the higher concentrations.

After replacement of culture medium and cultivation for 12 h in the absence of LPS the altered mRNA expression showed a pattern that corresponded well to the data on protein release. Expression of CCL2 was reduced (to 37%) but did not return to basal levels in control cultures. CCL3 and CXCL2 showed a highly significant down-regulation after LPS removal (to 8% and 5%, respectively), whereas CCL5 and CXCL1 continued to be expressed (no significant change compared with the LPS condition). As shown with ELISA measurements before, treatment with all-*trans* RA alone did not induce gene expression of chemokines.

Immunohistochemical staining of LPS-treated cultures revealed less than 1% tomato lectin and less than 2% ED1- positive cells in the cultures (Fig. 7a). Nitric oxide production, a characteristic of microglia cells (Saura 2007), was not induced by LPS at all. Nevertheless, even a small proportion of microglia could affect the astroglial response or be responsible for part of the chemokine production. To assess their contribution to the observed effects of LPS and RA, cultures were treated 2 days with 200 μ g/mL clodronate, which is used to deplete macrophages and other monocytes. Following this procedure, the cytokine induction of CCL2 and CXCL2 was still observed, and the inhibitory effect of RA also persisted (Fig. 7b and c). The anti-inflammatory steroid dexamethasone blocked the effect of LPS completely. Although chemokine expression and their regulation remained in the complete absence of microglia, clodronate treatment also affected the astrocytes (e.g. lower rate of proliferation). The up-regulation of chemokines was not as strong as observed without clodronate.

Involvement of retinoid receptors in RA-dependent effects

Although several direct and indirect modes of action are being discussed for the various effects of RA, in most cases the involvement of nuclear retinoid receptors is postulated (Blomhoff and Blomhoff 2006). This seems also to be the case for the chemokine regula-

Fig. 3. Effect of lipopolysaccharide (LPS) and retinoic acid (RA) on the release of CXCL chemokines by astrocytes.

Chemokine concentrations were measured in cell culture supernatants with ELISA. (a, b) CXCL1 (KC), (c, d) CXCL2 (MIP-2). In (a) and (c), cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/mL and the three concentrations of all-*trans* RA. In (b) and (d), cultures were pre-treated 12 h with RA followed by 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. In recovery experiments (L) LPS-free medium was given for 12 h after the LPS stimulation. Seven to nine cultures from three different litters were analyzed, error bars indicate SEM. Following ANOVA (in b, d), levels after exposure to LPS only or RA only were compared with controls (###p < 0.001), and RA + LPS conditions were compared with LPS only (**p < 0.01, ***p < 0.001; Dunnett's test).

tion in astrocytes, as analyzed here. Using RT-PCR, we demonstrated the expression of all six retinoid receptors RAR α , RAR β , RAR γ , RXR α , RXR β and RXR γ in the astrocyte primary cultures. With polyclonal antibodies, RAR and RXR immunoreactivity was detected in the cultured astrocytes (Fig. 8).

The question whether these receptors were necessary for the anti-inflammatory effect of RA was addressed by using pan-antagonists against RAR (LE540) and RXR (PA452). To test the effectiveness of the inhibitors, we measured expression of transglutaminase-2 (Tgm-2), an enzyme which is induced by RA in other cell types (Melino *et al.* 1997). As ex-

Fig. 4. Quantitative RT-PCR measurements of chemokine mRNA concentrations. RNA extracts of cultures were evaluated with conventional and quantitative RT-PCR. (a, b) All chemokine amplification products had the expected molecular sizes (see Table 1). (c, d) Melting curve analysis after every quantitative PCR reaction confirmed the absence of non-specific amplicons. Relative PCR efficiencies were determined with dilution series of a cDNA pool, duplicates, $r^2 > 0.95$, efficiency $> 90\%$ in all cases.

pected, RA treatment caused a two- to threefold increase in transcript levels of Tgm-2 also in astrocytes. This effect was inhibited by LE540 and PA452 (Fig. 9a). Transglutaminase-2 has also been reported to increase after various inflammatory stimuli, and accordingly LPS induced its expression. In the astrocytes, the effects of RA and LPS were additive (Fig. 9b). Incubation with either LE540 or PA452 abolished the RA-dependent up-regulation of Tgm-2 but did not reduce the additional effect of LPS (Fig. 9a and b). In the presence of the RAR inhibitor, the antagonistic effect of RA on the LPS-induced expression of CCL2 and CXCL2 disappeared completely. The same was observed for the RXR inhibitor and CCL2; however, in the presence of $1 \mu\text{M}$ PA452 treatment with $0.1 \mu\text{M}$ all-*trans* RA still reduced expression of CXCL-2 (Fig. 9c and d), though not significantly.

Molecular mechanisms of the RA/LPS interaction

Measurements of protein release as well as of gene expression demonstrated that the effectiveness of RA treatment depended on RA incubation before the cells were exposed to LPS. A negative effect of RA on the basal expression of LPS receptors could explain this observation. Quantitative RT-PCR experiments showed indeed that RA lowered expres-

Fig. 5. Effect of lipopolysaccharide (LPS) and retinoic acid (RA) on mRNA expression of CCL chemokines by astrocytes. Following the different treatment protocols, chemokine transcript levels were measured in cell extracts with quantitative RT-PCR: (a) CCL2 (MCP-1), (b) CCL3 (MIP-1 α), (c) CCL5 (RANTES). Cultures were pre-treated 12 h with RA, followed by 12 h simultaneous exposure to LPS and RA. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. In recovery experiments (L) LPS-free medium was given for 12 h after the LPS stimulation. Six cultures from two different litters were analyzed, error bars indicate SEM. Following ANOVA, mRNA levels after exposure to LPS-only or RA-only were compared with controls (### $p < 0.001$), and RA + LPS conditions were compared with LPS only (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Dunnett's test).

Fig. 6. Effect of lipopolysaccharide (LPS) and retinoic acid (RA) on mRNA expression of CXCL chemokines by astrocytes. Chemokine transcript levels were measured in cell extracts with quantitative RT-PCR. (a) CXCL1 (KC), (b) CXCL2 (MIP-2). Cultures were pre-treated 12 h with RA followed by 12 h simultaneous exposure to LPS and RA. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. In recovery experiments (L) LPS-free medium was given for 12 h after the LPS stimulation. Six cultures from two different litters were analyzed, error bars indicate SEM. Following ANOVA, mRNA levels after exposure to LPS-only or RA-only were compared with controls (### $p < 0.001$), and RA + LPS conditions were compared with LPS only (* $p < 0.05$, *** $p < 0.001$; Dunnett's test).

sion of the LPS co-receptor CD14 to 52% ($p < 0.05$). Transcript levels of the second receptor TLR4, and a related receptor TLR2 were also reduced (to 51% and 34%, respectively), but this was significant only in the case of TLR2 ($p < 0.05$, $n = 10$ independent experiments; Fig. 10a). LPS treatment, while not affecting TLR4, caused a strong increase in the expression TLR2 and CD14. Similar to the observations with chemokines, 12 h pre-treatment with all-*trans* RA abrogated this response (Fig. 10b). Quantification of CD14 protein expression with immunoblotting confirmed the increase after LPS treatment (1.56-fold compared with the control condition) and its partial reduction by RA (1.36-fold), whereas a reduction of low base levels of CD14 was not observed when RA was applied in the

Fig. 7. Retinoic acid (RA) reduces lipopolysaccharide (LPS)-induced expression of CCL2 and CXCL2 in clodronate treated astrocyte cultures. (a) Immunohistochemical staining of astrocyte primary cultures with antibodies against GFAP, ED-1 and with fluorescence-labeled tomato lectin; same magnification in the photographs, scale bar = 200 μ m. Less than 2% of all cells (4',6-diamidino-2-phenylindole dihydrochloride) showed ED-1 or tomato lectin labeling. (b, c) Following clodronate treatment, microglia-depleted cultures were treated as before and qRT-PCR for CCL2 (b) and CXCL-2 (c) were repeated. Statistical comparisons (t-test) were made between LPS treatment and controls ($\#p < 0.05$) and between RA + LPS and LPS treatment ($*p < 0.05$).

absence of LPS (Fig. 10c). Finally, we investigated whether RA treatment had an influence on signal transduction mechanisms that are activated directly or indirectly by TLR4/CD14 ligands (Fig. 11).

With immunoblotting and specific antibodies, the phosphorylation of STAT3, JNK and p38 MAPK was determined. In astrocyte nuclear extracts, immunoreactivity of the transcription factor NF- κ B was measured. Phosphorylated STAT3 and JNK and nuclear concentrations of NF- κ B increased in response to LPS; phosphorylation of p38 was not changed. Under the same conditions where we were able to reduce chemokine expression, that is, 12 h pre-treatment with 0.1 μ M all-*trans* RA, the activation of STAT3, JNK and NF- κ B was not significantly affected, although the tendency of a decrease in nuclear NF- κ B immunoreactivity appeared. Both isomers, 9-*cis* and all-*trans* RA have been reported

Fig. 8. Retinoid receptor expression in astrocyte primary cultures. Immunofluorescence staining showed (a) positive staining of retinoic acid receptor (RARα in cell nuclei and cytosol, (b) strong RARβ immunoreactivity mostly in the cell nuclei, (c) nuclear and perinuclear RXRα signal, (d) strong cytosolic staining of RXRβ, and (e) weak RXRγ immunoreactivity. No reliable results were obtained with RARγ antibodies; same magnification in all photographs. (f) RT-PCR revealed mRNA expression of all six retinoid receptors in mouse cortical astrocytes.

to cause an increase in suppressor of cytokine signalling (SOCS)3, a suppressor of the Jak/STAT pathway (Choi *et al.* 2005). In the primary cultures of murine cortical astrocytes investigated here, this effect was not observed (Fig. 12; 0.5–48 h, ANOVA not significant).

Discussion

To limit neuronal cell death under pathological circumstances, it is important to understand and control the inflammatory reaction of microglia and astrocytes. In this study, we demonstrate that all-*trans* RA reduces chemokine expression in astrocytes. The inflammatory condition was simulated by cultivating murine astrocyte primary cultures with 100 ng/mL LPS, which caused a strong increase in mRNA expression and protein release of CCL2, CCL3, CCL5, CXCL1 and CXCL2. Chemokine release into the medium peaked after 24–48 h. RA, which alone did not alter chemokine production, was able to reduce this inflammatory reaction by 70–90%, depending on the chemokine. In all cases, the influence was observed with respect to mRNA and protein levels, but only when the cells had been pre-treated 12 h before LPS stimulation, suggesting an indirect effect on the glial inflammatory response. Experiments with antagonists against RAR and RXR indicated that both classes of retinoid receptors were involved in the regulation. We observed an RA-dependent decrease in the expression of the LPS co-receptor CD14, which may partly explain the decreased inflammatory response of astrocytes in the presence of RA. A reduced nuclear localization of NF- κ B was observed, but this was not significant. As RA treatment also did not affect phosphorylation of STAT3, JNK, p38, or expression of SOCS3, the molecular cross-talk between LPS and RA signal transduction remains to be elucidated.

All-*trans* RA reduces the astroglial synthesis of chemokines

Although several studies have demonstrated a regulatory function of RA with respect to chemokine and cytokine production by lymphocytes, monocytes and dendritic cells (Gross *et al.* 1993; Mucida *et al.* 2007; Choi *et al.* 2009; for review: van Neerven and Mey 2007), retinoids are still somewhat neglected in the discussion of anti-inflammatory strategies for the nervous system (see, e.g. the otherwise excellent review by Donnelly and Popovich 2008). In the context of neuroinflammation, RA clearly mitigates the deleterious activity of microglia (Dheen *et al.* 2005; Xu *et al.* 2005; Xu and Drew 2006). Many pathological features associated with microglial reactions involve the activation of astrocytes, and their response is also subject to modulation by RA (Xu and Drew 2006; Xu *et al.* 2006). In these cases, ligand binding (of 9-*cis* RA) to RXR may have been involved, especially because anti-inflammatory effects, that is, reduction of NO, interleukin (IL-1 β , IL-6, CCL2, could be achieved with liver X receptor/RXR activation without involvement of RAR

Fig. 9. Retinoic acid receptor (RAR) inhibitor reduced the RA-effect on chemokine expression. Experiments were carried out as described for Figs 5 and 6, except that with 0.1 μ M all-*trans* RA cells were simultaneously given 1 μ M RAR inhibitor LE540 (LE) or RXR inhibitor PA452 (PA). Transcript levels in cell extracts were measured with qRT-PCR: (a) transglutaminase-2 (Tgm-2) was induced by RA-RAR/ RXR signaling and inhibited by LE and PA. Data were normalized to control condition; (b) effects of LPS, LPS + RA and inhibitors on Tgm-2, data normalized to LPS treatment; (c) CCL2; (d) CXCL2, normalization as in (b). Three cultures were subjected to every condition, error bars indicate SEM, *,# $p < 0.05$, t-test.

(Zhang-Gandhi and Drew 2007). However, physiologically occurring all-*trans* RA, which binds to RAR only, is also effective. In astrocytes this isomer inhibited the interferon- γ -induced signaling pathways and at the same time caused an up-regulation of the cytokine inhibitor SOCS3 (Choi *et al.* 2005). Our data demonstrate a general effect of all-*trans* RA to block LPS induced activation of inflammatory mediators in astrocytes [chemokines in the present study; IL-1 β , IL-6, tumor necrosis factor α , IL12p40, lipocalin-2 in a related study (van Neerven *et al.*, 2010). A caveat applies to all experiments using specific isomers of RA, including the present ones, because spontaneous or enzymatic isomerization of retinoids is possible, even when cultures are protected from light. With HPLC analyses of cell culture supernatants we observed that up to 7% of all-*trans* or 9-*cis* RA were detected in the medium after incubation of oligodendrocytes with the other RA isomer (Mey and Hammelmann 2000).

Fig. 10. Effect of retinoic acid (RA) treatment on transcript expression of toll-like receptor (TLR)2, TLR4 and lipopolysaccharide (LPS) co-receptor CD14. Experiments were carried out as described for Figs 5 and 6, mRNA levels in cell extracts were measured with qRT-PCR: (a) Effect of treatment with 0.1 μ M all-trans RA only, t-tests, * $p < 0.05$; (b) influence of RA on the effect of LPS; comparison LPS vs control: ### $p < 0.001$; RA + LPS vs. LPS: * $p < 0.05$, ** $p < 0.01$ (t-tests; $n = 10$ cultures for every condition). (c) Protein expression of CD14 was checked with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting; the graph shows colorimetric readings of immunoreactivity, normalized to actin. This experiment was repeated once with a similar result.

Relevance of RA chemokine interaction for neuroinflammation

In previous studies, anti-inflammatory activity of retinoids was investigated with respect to the release of cytokines, prostaglandins and NO, although to our knowledge only regulation of one chemokine (CCL2/MCP-1) has been studied in astrocytes (Xu and Drew 2006). In light of the relevance of astrocytes in the neuroinflammatory process, the effect of RA on these signals deserves closer attention. Chemokines induce leukocyte migration into the CNS under inflammatory conditions. Several routes of invasion across the blood–brain barrier or into the cerebrospinal fluid have been characterized (Ubogu *et al.* 2006), and most chemokine receptors are stimulated by more than one ligand. The chemokines found in the present study to be released by astrocytes activate mononuclear cells, eosinophils, basophils (predominantly CCL), neutrophils, T- and B-lymphocytes and natural killer cells (mostly the CXCL) (Pease and Williams 2006; Rostène *et al.* 2007). Extravasation of monocytes and macrophages into the brain parenchyma is induced primarily by CCL2 and CX3CL1 (Ubogu *et al.* 2006; Rostène *et al.* 2007). The pathological infiltration of neutrophils, while having a protective role in case of bacterial infection, contributes substantially to secondary neurodegeneration that occurs after spinal cord injury and cerebral ischemia. Subsequently, invading macrophages and lymphocytes have destructive but also beneficial aspects, and their activation may therefore be controlled to therapeutic benefit (Donnelly and Popovich 2008). Although RA appears to have little effect on the production of neurotrophic molecules by astrocytes (S. van Neerven, A. Nemes, J. Mey, unpublished data) the suppression of NO and pro-inflammatory cytokines makes its use promising to limit damage caused by inflammation and autoimmunity (Xu and Drew 2006).

Astrocytes do not only secrete chemokines (Ambrosini *et al.* 2005; this study) but express several chemokine receptors themselves, which may be implicated in the induction of gliosis (Dorf *et al.* 2000; Kiyota *et al.* 2009). Given that chemotaxis is a common denominator of these signals in the immune system it is tempting to assume that glial migration to a site of neural tissue destruction also constitutes a major function of chemokines after CNS injury or ischemia (Hattermann *et al.* 2008). As they are major culprits in inflammatory diseases, considerable effort has been directed towards the development of specific CCR and CXCR antagonists, albeit with limited success so far (Pease and Williams 2006). In this context, the discovery that RA reduces production of chemokines by stimulated glia cells is important. Certainly, confirmation with inflammation models *in vivo* is now necessary, especially since the retinoid effect on chemokines is cell type specific: In acute promyelocytic leukemia derived leukemia cells, inflammatory chemokines, e.g. CCL3, CCL4, CCL22 and CCL24, were induced by RA (Shibakura *et al.* 2005; Luesing *et al.* 2009), and during development RA contributes to the differentiation of lymph nodes by way of inducing CXCL13 in stromal organizer cells. In contrast, when T-cells were exposed to all-*trans* RA and LPS, CCL22 and CXCL10 were markedly reduced (Tsai *et al.* 2008).

Fig. 11. Effect of retinoic acid (RA) on phosphorylation of signal transducer and activator of transcription (STAT)3, Jun amino-terminal kinase (JNK), MAPK, and nuclear translocation of nuclear factor κ B (NF- κ B). Following experiments, carried out as described before, protein extracts of astrocytes were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting; (a) representative blots, phosphorylated transcription factor immunoreactivities (IR) were normalized to non-phosphorylated STAT3 IR, NF- κ B IR in nuclear extracts was normalized to lamin IR. (b–e) Quantification of IR signals, $n = 5$ independent experiments, error bars indicate SEM.

Mechanisms of anti-inflammatory activity of RA

For the inhibition of gene expression by RA, some mechanisms have been identified in some cell types, but it is not well understood in others. It has been known for some time, that RA inhibits AP-1 binding to the 12-O-tetradecanoylphorbol 13-acetate-response element, which activates gene transcription in cell proliferation, inflammation and stress responses (Hanazawa *et al.* 1994; Konta *et al.* 2001). Several non-receptor-mediated mechanisms of RA activity are known, including direct binding to cRaf and protein kinases C (Hoyos *et al.* 2000; Radomska-Pandya *et al.* 2000; Liou *et al.* 2005). Yet, in most instances of RA application, retinoid receptor-mediated gene regulation is the decisive mechanism (for review: Blomhoff and Blomhoff 2006; van Neerven and Mey 2007; Malaspina and Michael-Titus 2008). For instance, RAR α has been shown to be involved in T-cell regulation (Dawson *et al.* 2008). Astrocyte primary cultures of human (Chattopadhyay and Brown 2001) or murine origin (this study) do possess RAR and RXR. *In vivo* an increase in RXR β immunoreactivity is most conspicuous in activated astrocytes (Schrage *et al.* 2006; Jokic *et al.* 2007). As discussed above, the efficacy of peroxisome proliferator-activated receptor agonists (van Neerven and Mey 2007; van Neerven *et al.* 2008) or LXR agonists (Zhang-Gandhi and Drew 2007) indicates that RXR activation in the absence of RAR may elicit an anti-inflammatory effect. In the presence of an RXR ligand, in this case 9-*cis* RA, a mutual inhibition between RXR and NF- κ B was observed with expected suppression of the inflammatory cytokine IL-12 (Na *et al.* 1999). Although mechanisms not involving RAR cannot be ruled out to explain some inhibitory effects of RA on the astroglial response to LPS, the present experiments with RAR and RXR inhibitors imply that both families of nuclear receptors were involved in the partial suppression of chemokine induction.

Pre-incubation of the cell cultures with RA proved necessary (chemokines; present data) or far more effective than simultaneous treatment (cytokines; van Neerven *et al.*, submitted) in reducing the LPS effects. This is in accordance with previous publications: with a short pre-incubation time of 1 h the release of tumor necrosis factor α but not of CCL2 was reduced in the experiments reported by Xu and Drew (2006), and pre-treatment was necessary to block the Jak/STAT pathway by interferon γ treatment of astrocytes (Choi *et al.* 2005). Thus, it is likely that RA interferes with LPS signaling in an indirect manner which depends on canonical RAR/RXR signaling and may involve transcriptional regulation of genes other than the investigated chemokines and cytokines themselves. As we tested the potential benefit of RA using LPS as an inflammatory stimulus, it is possible that RA reduced the basal expression of LPS receptors, TLR-4 and CD14. Astrocytes have been reported to possess these receptors (Bowman *et al.* 2003; Bsibsi *et al.* 2007), and it was shown that RA reduced the expression of CD14 and a related receptor, TLR-2, in monocytes (Liu *et al.* 2005). Indeed, we observed a significant reduction in expression of CD14. In addition, after LPS treatment a strong increase of CD14 and TLR-2 occurred,

which, similar to the chemokine response, was partly suppressed by RA. Since this latter phenomenon is to be considered as another secondary consequence of RA treatment it does not provide a mechanistic explanation for the observed effects.

After binding to CD14 and TLR4, LPS activate several intracellular signalling cascades eventually targeting transcription factors including NF- κ B, AP-1 and STAT3, which in turn induce a number of inflammatory genes. Several studies demonstrated that RA interfered with some of these intracellular pathways. A central role is taken by the transcription factor NF- κ B, whose nuclear translocation can be inhibited by all-*trans* RA in microglia (Dheen *et al.* 2005) and monocyte precursor cells (Austena *et al.* 2008). The latter study also demonstrated the necessity of new protein synthesis, histone deacetylation and involvement of STAT1 activation. In T-cells, all-*trans* RA suppressed chemokine expression at least in part via the cRaf-MAPK pathway (Tsai *et al.* 2008), and in astrocytes phosphorylation of janus kinases and consequently of STAT1 and STAT3 were reduced whereas expression of the cytokine inhibitor SOCS3 increased (Choi *et al.* 2005). Neuroprotection by RA in hippocampal slice cultures correlated with reduced phosphorylation of JNK and p38 (Shinozaki *et al.* 2007).

In the present study, we tried to identify an intracellular pathway engaged in the interaction between RA signalling and chemokine induction. However, although LPS induced phosphorylation of JNK and STAT3 as well as the nuclear translocation of NF- κ B were readily detected, 0.1 μ M all-*trans* RA did not significantly affect these signals in the absence

Fig. 12. Retinoic acid (RA) did not influence expression of suppressor of cytokine signaling (SOCS)3. Astrocytes were exposed to 0.1 μ M alltrans RA or vehicle 0.1% ethanol (control) for the time indicated below the abscissa. Transcript levels in cell extracts were measured with qRT-PCR and normalized to the control condition. Three different cell cultures were tested for every condition and time period, error bars indicate SEM.

or presence of LPS. No strong changes in the phosphorylation status of p38 occurred, and a significant increase in expression of SOCS after RA treatment was not observed either. Thus, although a strong anti-inflammatory influence of RA-RAR/RXR signaling on astrocytes was found with respect to the production of several chemokines and pattern recognition receptors, the responsible molecular mechanisms remain to be investigated.

Conclusion

Under inflammatory conditions, astrocytes are induced to release chemokines including CCL2, CCL3, CCL5, CXCL1 and CXCL2, which in the CNS attract and activate monocytes and neutrophils across the blood–brain barrier. These interactions have been implicated in a wide variety of neuropathological disorders with an inflammatory component. We report that all-*trans* RA reduces the LPS-induced expression of these chemokines by astrocytes *in vitro*. Targeting the astroglial response by RA may therefore be a promising approach to study its anti-inflammatory efficacy in animal models of spinal cord injury, ischemia and neurodegenerative diseases.

Acknowledgements

The authors thank Marie Pradella and Stefanie Kurth for technical assistance with cell cultures and quantitative RT-PCR experiments. SvN received a Marie-Curie Fellowship of the European Union (EURON). We thank Drs Shudo and Kagechika for kindly letting us use their RAR and RXR antagonists.

References

- Ambrosini E. and Aloisi F. (2004) Chemokines and glial cells: a complex network in the central nervous system. *Neurochem. Res.* **29**, 1017–1038
- Ambrosini E., Remoli M. E., Giacomini E., Rosicarelli B., Serafini B., Lande R., Aloisi F. and Coccia E. M. (2005) Astrocytes produce dendritic cell-attracting chemokines *in vitro* and in multiple sclerosis lesions. *J. Neuropathol. Exp. Neurol.* **64**, 706–715
- Anthony D., Dempster R., Fearn S., Clements J., Wells G., Perry V. h. and Walker K. (1998) CXC chemokines generate age-related increases in neutrophil-mediated brain inflammation and blood–brain barrier breakdown. *Curr. Biol.* **8**, 923–926.
- Austena L. M., Carlsen h., Hollung K., Blomhoff h. K. and Blomhoff R. (2008) Retinoic acid dampens LPS-induced NF-kappaB activity: results from human monoblasts and *in vivo* imaging of NF-kappaB reporter mice. *J. Nutr. Biochem.* **20**, 726–734
- Babcock A. A., Kuziel W. A., Rivest S. and Owens T. (2003) Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. *J. Neurosci.* **23**, 7922–7930
- Baggiolini M. (1998) Chemokines and leukocyte traffic. *Nature* **392**, 565–568
- Bastien J. and Rochette-Egly C. (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1–16
- Blomhoff R. and Blomhoff h. K. (2006) Overview of retinoid metabolism and function. *J. Neurobiol.* **66**, 606–630
- Bowman C. C., Rasley A., Tranguich S. L. and Marriott I. (2003) Cultured astrocytes express toll-like receptors for bacterial products. *Glia* **43**, 281–291
- Bsibsi M., Bajramovic J. J., Van Duijvenvoorden E., Persoon C., Ravid R., Van Noort J. M. and Vogt M. h. (2007) Identification of soluble CD14 as an endogenous agonist for Toll-like receptor 2 on human astrocytes by genome-scale functional screening of glial cell derived proteins. *Glia* **55**, 473–482
- Chattopadhyay N. and Brown E. M. (2001) Retinoic acid receptors are expressed in human primary astrocytes and their agonists inhibit parathyroid hormone-related peptide expression and secretion. *Mol. Brain Res.* **92**, 172–176
- Choi W.-H., Ji K.-A., Jeon S.-B., Yang M.-S., Kim h., min K., Shong M., Jou I. and Joe E.-H. (2005) Anti-inflammatory roles of retinoic acid in rat brain astrocytes: suppression of interferon-c-induced JAK/STAT phosphorylation. *Biochem. Biophys. Res. Com.* **329**, 125–131
- Choi B.-K., Kim J.-H., Jung J.-S., Lee Y.-S., Han M.-E., Baek S.-Y., Kim B.-S., Kim J.-B. and Oh S.-O. (2009) Reduction of ischemia-induced cerebral injury by all-trans retinoic acid. *Exp. Brain Res.* **193**, 581–589
- Dawson H. D., Collins G., Pyle R., Key M. and Taub D. D. (2008) Retinoic acid receptor-alpha mediates human T-cell activation and Th2 cytokine production. *BMC Immunol.* **9**, doi: 10.1186/1471-2172-9-16
- Dheen S. T., Jun Y., Yan Z., Tay S. S. W. and Ling E. A. (2005) Retinoic acid inhibits expression of TNF α and iNOS in activated rat microglia. *Glia* **50**, 21–31
- Donnelly D. J. and Popovich P. G. (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp. Neurol.* **209**, 378–388

- Dorf M. E., Berman M. A., Tanabe S., Heesen M. and Luo Y. (2000) Astrocytes express functional chemokine receptors. *J. Neuroimmunol.* **111**, 109–121
- Farina C., Aloisi F. and Meinl E. (2007) Astrocytes are active players in cerebral innate immunity. *Trends Immunol.* **28**, 138–145
- Gross V., Villiger P. M., Zhang B. and Lotz M. (1993) Retinoic acid inhibits interleukin-1-induced cytokine synthesis in human monocytes. *J. Leukocyte Biol.* **54**, 125–132
- Hanazawa S., Tageshita A. and Kitano S. (1994) Retinoic acid suppression of c-fos gene inhibits expression of tumor necrosis factor- α -induced monocyte chemoattractant JE/MCP-1 in clonal osteoblastic MC3T3-E1 cells. *J. Biol. Chem.* **269**, 21379–21384
- Hattermann K., Ludwig A., Gieselmann V., Held-Feindt J. and Mentlein R. (2008) The chemokine CXCL16 induces migration and invasion of glial precursor cells via its receptor CXCR6. *Mol. Cell. Neurosci.* **39**, 133–141
- Herrmann J. E., Imura T., Song B., Qi J., Ao Y., Nguyen T. K., Korsak R. A., Takeda K., Akira S. and Sofroniew M. V. (2008) STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. *J. Neurosci.* **28**, 7231–7243
- Hoyos B., Imam A., Chua R., Swenson C., Tong G. X., Levi E., Noy N. and Hammerling U. (2000) The cystein-rich regions of the regulatory domains of Raf and protein kinase C as retinoid receptors. *J. Exp. Med.* **192**, 835–845
- Johann S., Kampmann E., Denecke B., Arnold S., Kipp M., Mey J. and Beyer C. (2008) Expression of enzymes involved in the prostanoid metabolism by cortical astrocytes after LPS-induced inflammation. *J. Mol. Neurosci.* **34**, 177–185
- Jokic N., Ling Y. Y., Ward R. E., Michael-Titus A. T., Priestley J. V. and Malaspina A. (2007) Retinoid receptors in chronic degeneration of the spinal cord: observations in a rat model of amyotrophic lateral sclerosis. *J. Neurochem.* **103**, 1821–1833
- Kampmann E., Johann S., van Neerven S., Beyer C. and Mey J. (2008) Anti inflammatory effect of retinoic acid on prostaglandin synthesis in cultured cortical astrocytes. *J. Neurochem.* **106**, 320–332
- Kiyota T., Yamamoto M., Schroder B., Jacobsen M. T., Swan R. J., Lambert M. P., Klein W. L., Gendelman h. E., Ransohoff R. M. and Ikezu T. (2009) AAV1/2 mediated CNS gene delivery of dominant-negative CCL2 mutant suppresses gliosis, beta-amyloidosis, and learning impairment of APP/PS1 mice. *Mol. Ther.* **17**, 803–809
- Konta T., Xu Q., Furusu A., Nakayama K. and Kitamura M. (2001) Selective roles of retinoic acid receptor and retinoid x receptor in the suppression of apoptosis by all-*trans* retinoic acid. *J. Biol. Chem.* **276**, 12697–12701
- Kuenzel T., Wirth M. J., Luksch h., Wagner h. and Mey J. (2009) Increase of Kv3.1b expression in avian auditory brainstem neurons correlates with synaptogenesis *in vivo* and *in vitro*. *Brain Res.* **1302**, 64–75
- Li X. and Stark G. R. (2002) NF κ B-dependent signaling pathways. *Exp. Hematol.* **30**, 285–296
- Liberto C. M., Albrecht P. J., Herx L. M., Yong V. W. and Levison S. W. (2004) Pro-regenerative properties of cytokine-activated astrocytes. *J. Neurochem.* **89**, 1092–1100

- Liou J. C., Ho S. Y., Shen M.-R., Liao Y.-P., Chiu W.-T. and Kang K.-H. (2005) A rapid nongenomic pathway facilitates the synaptic transmission induced by retinoic acid at the developing synapse. *J. Neurosci.* **24**, 8562–8576
- Liu P. T., Krutzik S. R., Kim J. and Modlin R. L. (2005) Cutting edge: all-*trans* retinoic acid down-regulates TLR2 expression and function. *J. Immunol.* **174**, 2467–2470
- Luesing M., Pennings J. L., Wissink W. M., Linssen P. C., Muus P., Pfundt R., de Witte T. J., van der Reijden B. A. and Jansen J. h. (2009) Chemokine induction by all-*trans* retinoic acid and arsenic trioxide in acute promyelocytic leukemia: triggering the differentiation syndrome. *Blood* **114**, 5512–5521
- Luo Y., Fischer F. R., Hancock W. W. and Dorf M. E. (2000) Macrophage inflammatory protein-2 and KC induce chemokine production by mouse astrocytes. *J. Immunol.* **165**, 4015–4023
- Malaspina A. and Michael-Titus A. T. (2008) Is the modulation of retinoid and retinoid-associated signaling a future therapeutic strategy in neurological trauma and neurodegeneration? *J. Neurochem.* **104**, 584–595
- Melino G., Draoui M., Bellincampi L., Bernassola F., Bernardini S., Piacentini M., Reichert U. and Cohen P. (1997) Retinoic acid receptors alpha and gamma mediate the induction of “tissue” transglutaminase activity and apoptosis in human neuroblastoma cells. *Exp. Cell Res.* **235**, 55–61
- Mey J. and Hammelmann S. (2000) OLN-93 oligodendrocytes synthesize all-*trans* retinoic acid *in vitro*. *Cell Tissue Res.* **302**, 49–58
- Mey J., Morassutti D., Brook G. A., Liu R.-H., Zhang Y.-P., Koopmans G. and McCaffery P. (2005) Retinoic acid synthesis by a population of NG-2 positive cells in the injured spinal cord. *Eur. J. Neurosci.* **21**, 1555–1568
- Mucida D., Park Y., Kim G., Turovskaya O., Scott I., Kronenberg M. and Cheroutre h. (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260
- Na S. Y., Kang B. Y., Chung S. W., Han S. J., Ma X., Trinchieri G., Im S. Y., Lee J. W. and Kim T. S. (1999) Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFkappaB. *J. Biol. Chem.* **274**, 7674–7680
- van Neerven S. and Mey J. (2007) RAR/RXR and PPAR/RXR signaling in spinal cord injury. *PPAR Res.* doi: 10.1155/2007/29275
- van Neerven S., Kampmann E. and Mey J. (2008) RAR/RXR and PPAR/RXR signaling in neurological and psychiatric diseases. *Prog. Neurobiol.* **85**, 433–451
- Okun E., Griffioen K. J., Lathia J. D., Tang S. C., Mattson M. P. and Arumugam T. V. (2008) Toll-like receptors in neurodegeneration. *Brain Res. Rev.* **59**, 278–192
- Pease J. E. and Williams T. J. (2006) The attraction of chemokines as a target for specific anti-inflammatory therapy. *Br. J. Pharmacol.* **147**(Suppl. 1), S212–S221
- Radomska-Pandya A., Chen G., Czernik P. J., Little J. M., Samokyszyn V. M., Carter C. A. and Nowak G. (2000) Direct interaction of all-*trans*-retinoic acid with protein kinase C (PKC). *J. Biol. Chem.* **275**, 22324–22330
- Rostene W., Kitabgi P. and Parsadaniantz S. M. (2007) Chemokines: a new class of neuromodulators. *Nat. Rev. Neurosci.* **8**, 895–904

- Saura J. (2007) Microglial cells in astroglial cultures: a cautionary note. *J. Neuroinflammation* **4**, 26. doi: 10.1186/1742-2094-4-26
- Schrage K., Koopmans G., Joosten E. A. and Mey J. (2006) Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. *Eur. J. Neurosci.* **23**, 285–295
- Shibakura M., Niiya K., Niiya M., Asaumi N., Yoshida C., Nakata Y. and Tanimoto M. (2005) Induction of CXC and CC chemokines by all-*trans* retinoic acid in acute promyelocytic leukemia cells. *Leuk. Res.* **29**, 755–759
- Shinozaki Y., Sato Y., Koizumi S., Ohno Y., Nagao T. and Inoue K. (2007) Retinoids acting through retinoid receptors protect hippocampal neurons from oxygen-glucose deprivation-mediated cell death by inhibition of JNK and p38 mitogen-activated protein kinase. *Neuroscience* **147**, 153–163
- Tsai Y. C., Chang h. W., Chang T. T., Lee M. S., Chu Y. T. and Hung C. h. (2008) Effects of all-*trans* retinoic acid on Th1- and Th2-related chemokines production in monocytes. *Inflammation* **31**, 428–433.
- Ubogu E. E., Cossoy M. B. and Ransohoff R. M. (2006) The expression and function of chemokines involved in CNS inflammation. *Trends Pharmacol. Sci.* **27**, 48–55
- Wong L.-F., Yip P. K., Battaglia A. *et al.* (2006) Retinoic acid receptor b2 promotes functional regeneration of sensory axons in the spinal cord. *Nat. Neurosci.* **9**, 243–250
- Xu J. and Drew P. D. (2006) 9-*cis*-Retinoic acid suppresses inflammatory responses of microglia and astrocytes. *J. Neuroimmunol.* **171**, 135–144
- Xu J., Storer P. D., Chavis J. A., Racke M. K. and Drew P. D. (2005) Agonists for the peroxisome proliferator-activated receptor- α and the retinoid X receptor inhibit inflammatory responses in microglia. *J. Neurosci. Res.* **81**, 403–411
- Xu J., Chavis J. A., Racke M. K. and Drew P. D. (2006) Peroxisome proliferator activated receptor- α and retinoic X receptor agonists inhibit inflammatory responses in astrocytes. *J. Neuroimmunol.* **176**, 95–105
- Yip P. K., Wong L. F., Pattinson D., Battaglia A., Grist J., Bradbury E. J., Maden M., McMahon S. B. and Mazarakis N. D. (2006) Lentiviral vector expressing retinoid acid receptor b2 promotes recovery of function after corticospinal tract injury in the adult rat spinal cord. *Hum. Mol. Gen.* **15**, 3107–3118
- Yiu G. and He Z. (2006) Glial inhibition of CNS axon regeneration. *Nat. Rev. Neurosci.* **7**, 617–627
- Zhang-Gandhi C. X. and Drew P. D. (2007) Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. *J. Neuroimmunol.* **183**, 50–59

Inflammatory cytokine release of astrocytes *in vitro* is reduced by all-*trans* retinoic acid

5

S.G.A. van Neerven^{1,2}, A. Nemes¹, P. Imholz¹, T. Regen³,
B. Denecke⁴, S. Johann⁵, C. Beyer⁵, U. Hanisch³ and J. Mey^{1,2}

¹Institute of Biology II, RWTH Aachen University,
RWTH Aachen, Germany

²EURON European Graduate School of Neuroscience,
Maastricht, The Netherlands

³Institute of Neuropathology, Medical Faculty, University of Göttingen,
Göttingen, Germany

⁴Interdisziplinäres Zentrum für klinische Forschung (IKZF "Biomat"),
Medical Faculty, RWTH Aachen University, Germany

⁵Institute of Neuroanatomy, Medical Faculty, RWTH Aachen University,
Germany

Abstract

In the central nervous system inflammation is mediated by microglia and astrocytes. To investigate its regulation, murine astrocyte cultures were treated with bacterial lipopolysaccharides (LPS) and analyzed with Affymetrix gene array, qRT-PCR and ELISA. Cells responded to LPS with a strong upregulation of proinflammatory cytokines and chemokines. Treatment with the transcriptional activator retinoic acid (RA) suppressed mRNA expression and protein release of several important cytokines (IL-1 β 4%, IL-6 21%, TNF α 30%, IL-12p40 42%, and IL-12p35/p40 27%; $p < 0.01$). The data are consistent with the hypothesis that all-*trans* RA takes part in endogenous anti-inflammatory feedback loops in the CNS.

Keywords: Inflammation, Glia, Mouse, Retinoids, Cytokine, LPS

Introduction

Spinal cord and brain lesions, ischemia or neurodegenerative diseases are accompanied by inflammatory reactions involving the activation and proliferation of microglia and astrocytes, and when the blood–brain barrier is disrupted, immune cells from the periphery participate in the inflammatory reaction. In the intercellular communication of this inflammatory process cytokines and chemokines play a prominent role (Benveniste, 1998). Especially the cytokines, IL-1 β , TNF α , and IL-6, which are quickly released after a pathological event, are essential for the initiation and maintenance of the immune response (Donnelly and Popovich, 2008). The inhibition of these cytokines has been shown to be neuroprotective in different experimental models of brain injury (Knobloch *et al.*, 1999; Mulcahy *et al.*, 2003; Hailer, 2008). In contrast, several studies showed that the inhibition of cytokine signaling can also aggravate injury because some cytokines, for instance IL-6, TGF β and TNF α , are involved in repair mechanisms (McCoy and Tansey, 2008; Benveniste, 1998). Different receptor combinations and interaction with other signaling pathways may explain opposing effects of inflammatory mediators in different contexts.

The reaction of different cytokine releasing cells contributes to the specific cocktail of signal activity at any time and place. While for a long time it was supposed that microglia were the only immune competent cells of the CNS it is now clear that cytokine signaling provides mutual communication between astrocytes and microglia and that this occurs not only during CNS inflammation but also under normal physiological conditions (Dong and Benveniste, 2001). Astrocytes produce a wide variety of inflammatory mediators including cytokines, chemokines (Farina *et al.*, 2007; van Neerven *et al.*, 2010) and prostaglandins (Kampmann *et al.*, 2008). In addition, astrocytes are also involved in anti-inflammatory mechanisms. These include scar formation, which limits the area of secondary degeneration, and the production of neurotrophic factors (Liberto *et al.*, 2004). The dual role of astrocytes implicates that a precise regulation of their response may determine the outcome of CNS inflammation. To be able to therapeutically modulate the glial inflammatory response, it is important to understand the molecular mechanisms that regulate the astroglial contribution to neuroinflammation. To study this condition *in vitro*, astrocytes can be stimulated with bacterial endotoxins like *Escherichia coli* lipopolysaccharides (LPS). These bind to CD14 and TLR4 and activate a large number of inflammatory genes (Li and Stark, 2002; Okun *et al.*, 2008).

Ligands of nuclear receptors, such as RAR, RXR, and PPAR have been noticed as regulators of this response. Glia cells of the CNS are affected by the RAR and RXR agonist 9-*cis* retinoic acid (Xu *et al.*, 2005; Xu and Drew, 2006). Retinoic acid (RA) is a vitamin A derivative, whose anti-inflammatory effects were originally discovered in skin diseases. It has then been shown to influence the production of cytokines, chemokines and prostaglandins in several cell types of the immune system (for review: van Neerven and Mey, 2007). While most reports indicate an anti-inflammatory influence of RA (Austenaa *et al.*, 2008;

Na *et al.*, 1999; Mucida *et al.*, 2007; Zimmerman *et al.*, 2006; Xu and Drew, 2006) it is important to note that RA can have opposite effects on different cell types. For instance, in combination with LPS treatment, it was found to increase the production of PGE2 in neuroblastoma cells (Alique *et al.*, 2007), decrease it in astrocytes (Kampmann *et al.*, 2008), and raise systemic prostaglandin levels *in vivo* (Devaux *et al.*, 2001).

Retinoic acid is locally synthesized and released in a paracrine and autocrine fashion. Taken up by target cells, it binds to retinoic acid receptors (RAR α , - β , - γ) that heterodimerize with retinoid X receptors (RXR α , - β , - γ) to form a ligand activated transcriptional complex (Bastien and Rochette-Egly, 2004). All-*trans* RA and 9-*cis* RA bind to the RAR family, whereas only 9-*cis* RA is a high affinity ligand for the RXRs. Of these isomers only the all-*trans* form has been detected *in vivo* (Kane *et al.*, 2008).

Experiments with rats and mice demonstrate that RA treatment *in vivo* is beneficial in the cases of spinal cord injury (Takenaga *et al.*, 2009; Agudo *et al.*, 2010) and cerebral ischemia (Harvey *et al.*, 2004; Sato *et al.*, 2008). Although in these cases direct RA signaling towards RAR/RXR expressing neurons may play a major role, indirect, anti-inflammatory effects are probably involved. After experimental ischemia, for instance, all-*trans* RA reduced infarct size by the inhibition of proinflammatory c/EBP β mediated COX2 induction and reduced immunoreactivity of IL-1 β in microglia (Choi *et al.*, 2009; Shen *et al.*, 2009). As these studies indicate that RA application may be beneficial in various cases of neurodegeneration, it is important to determine its effect on the inflammatory reaction of glial cells. In the present study we therefore tested how RA affects cytokine expression by cortical astrocytes.

Material and Methods

Astrocyte primary cultures

Primary cultures of astrocytes were prepared from cerebral cortices of 1 day old Balb/c mice (Charles River, Sulzfeld, Germany). After dissection and careful removal of the meninges, both hemispheres were dissociated mechanically in 0.25% Trypsin-EDTA solution (Sigma-Aldrich, Schnellendorf, Germany), passed through a 50 μ m nylon mesh, and centrifuged at 130 $\times$ g for 10 min. Cells were resuspended and further cultivated in medium containing Dulbecco's modified Eagle medium including 20% heat-inactivated fetal calf serum, 0.5% penicillin/streptavidin and 0.1% fungizone (all from Invitrogen, Karlsruhe, Germany) and subsequently seeded in T75 flasks coated with poly-L-lysine (200 μ g/ml, Sigma-Aldrich). Cells were grown for 5 days at 37° C in a humidified incubator with 5% CO2, and medium was changed every 2–3 days. When reaching confluence, cells were passaged three times to remove microglia.

Treatment of cell cultures

After the third passage, astrocytes were seeded into poly-L-lysine coated 6-wells plates containing approximately 400,000 cells/well. Before treatment, astrocytes were kept for 48 h in serum-free neurobasal medium supplemented with 1% glutamine, 0.2% B27 supplement (both from Gibco, Invitrogen), 0.5% penicillin/streptavidin and 0.1% fungizone. To induce an inflammatory reaction 100 ng/ml *E. coli* LPS (Sigma-Aldrich) were applied to the culture medium.

All-*trans* retinoic acid (RA; Sigma-Aldrich) was dissolved at 0.1M in DMSO. To prevent oxidation, air above the aliquots was replaced with nitrogen, and subsequently stock solutions were stored at -75° C. All handling with RA occurred under conditions of amber lighting (Na⁺ lamp). Solutions were always made fresh and therefore stock aliquots were further diluted prior to the experiment to 1 mM, 0.1 mM and 0.01 mM RA in 100% ethanol. These dilutions were then added to the cell culture medium, reaching final concentrations of 1, 0.1, or 0.01 μ M RA.

The effect of LPS and the influence of RA on cytokine release were monitored for time intervals of 30 min, 1 h, 6 h, 12 h, 24 h, 48 h and 72 h. In addition, the effect of RA pretreatment on LPS-induced cytokine release and expression was measured: as pretreatment, concentrations of 0.01, 0.1 or 1 μ M RA, or 1% ethanol only were administered 12 h prior to LPS treatment. Subsequently, the medium was replaced, 100 ng/ml LPS with fresh RA was added, and cells were incubated for an additional period of 12 h.

In a control experiment the LPS response was blocked with 100 nM of the anti-inflammatory steroid dexamethasone. In additional controls, cultures were treated with clodronate (dichloro-phosphono-methyl phosphonic acid; Calbiochem/Merck Chemicals, Germany) to eliminate possible microglial cells. After the third passage cells were left to adhere in normal culture medium for two days, subsequently the medium was renewed with medium containing 200 μ g/ml clodronate. Cells were incubated for 48 h at 37° C, 5% CO₂, and placed overnight on a shaker at 250–300 rpm to remove possible microglia. The next morning astrocytes were washed with PBS, received normal culture medium, and standard experiments were carried out.

Cytotoxicity test: lactate dehydrogenase release

In addition to immunocytochemical observations, the cytotoxicity of treatment conditions was measured using lactate dehydrogenase (LDH) release into the medium (Roche, Mannheim, Germany; Cytotoxicity Detection Kit). LDH release was normalized with respect to maximal LDH release after cell lysis.

Affymetrix GeneChip array

Total RNA extraction and the production of biotinylated complementary probes were carried out as described in the Affymetrix GeneChip™ Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). We used 3.6 µg of total RNA per sample to synthesize cDNA and biotinylated RNA probes (GeneChip Expression 3' Amplification One-Cycle Target Labeling and Control Reagents Lot). Each sample was hybridized to an Affymetrix GeneChip Mouse Genome 430 2.0 array, which represents approximately 34,000 genes, and arrays were scanned with the GeneChip Scanner 3000 (Affymetrix). Two independent experiments with and without LPS stimulation, 100 nM for 12 h, were carried out under identical conditions. Data analysis was performed with Bioconductor-1 packages, R2 background correction and normalization with GCRMA algorithm. Affymetrix Microarray Suite 5.0 software was used to make detective calls for every probe set (Archer and Reese, 2010). For every gene the difference of expression in LPS vs. control condition was determined in the two independent experiments. The two data sets were then compared and an average was calculated when changes occurred in the same direction.

Quantitative RT-PCR

Isolation of total RNA was performed with the PeqGOLD extraction kit (Peqlab Biotechnology, Erlangen, Germany). Subsequently, RNA concentration and purity were assessed photometrically (OD260, OD260/OD280 ratio). Total RNA was reverse transcribed into cDNA using Qiagen Omniscript RT kit and random hexanucleotide primers (Invitrogen) with calculated concentrations of 25 ng/µl RNA extract per reaction. Samples were diluted 1/10 after reverse transcription, and were used for amplification with the MyiQ5 Real-Time PCR Detection System (Biorad, Munich, Germany). Reactions were carried out in a 96-well reaction module, containing 10 µl total reaction volume per well. Samples contained 5 µl of the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany; including Sybr Green I, Hot-StarTaq DNA polymerase, reaction buffer and MgCl₂), 2 µl nuclease-free water, 2 µl cDNA sample and 0.5 µl of each primer (sense and anti-sense each 10 µM stock solutions). The protocol consisted of a 15 min enzyme activation at 95° C, followed by 40 cycles of 15 s denaturation at 94° C, 20 s annealing at the primer specific temperature, 20 s amplification at 72° C, and 5 s fluorescence measurement at 74° C. Primer sequences and annealing temperatures are listed in Table 1. Expression of the housekeeping gene HPRT was measured to normalize gene expression. In previous experiments we compared the expression of HPRT, actin and GAPDH in astrocytes. Since HPRT showed the lowest variability this was chosen as a control gene. Melting curve analysis (from 65° C to 95° C, in 0.1° C/s) and agarose gel electrophoresis of the PCR products were routinely performed. Relative mRNA concentrations were determined using crossing point analysis of log/linear plots of fluorescence/cycle number. To determine the PCR efficiency, concentration

series (dilutions 1/2, 1/4, ... 1/128) of sample pools were performed once for every primer pair. Since PCR with all primer pairs showed efficiencies above 90% the $\Delta\Delta C_t$ method was used to analyze data. All samples and standards were measured in duplicates.

Enzyme-linked immunosorbent assays (ELISA)

Cell culture supernatants were collected after different periods of incubation. The release of chemokines IL-1 β , IL-6, TNF α , IL12p40 homodimers, IL12p40/IL12p35 and IL12p40/IL12p70 heterodimers into the supernatant was measured using DuoSet ELISA Development Kits (R&D Systems, Wiesbaden, Germany). All ELISA procedures were performed according to the manufacturer's instructions and absorbance was measured at 450 nm with subtraction of absorbance at 540 nm using a microplate reader (BioRad). For calibration, standard concentration series of all cytokines were measured routinely.

Levels of the NO derivative nitrite were determined photometrically in the culture medium using the Griess reaction, and the assay was calibrated with NaNO₂ concentration series.

Immunocytochemistry

After removal of the medium, cells were fixed (4% PFA) for standard immunocytochemical procedure. Cell type composition of the cultures was checked with primary antibodies against glial fibrillary acidic protein (GFAP; rabbit anti-mouse polyclonal antibody: DAKO, Hamburg, Germany, 1/2000) and ED1 (mouse monoclonal antibody: Serotec, Düsseldorf, Germany, MCA341R, 1/100). Polyclonal antibodies against retinoid receptors were obtained from Santa Cruz Biotechnology (sc-551, sc-552, sc-7387, sc-553, sc-831, and sc-555; all diluted 1/100). As secondary antibodies, Alexa Fluor 546 goat anti-rabbit IgG (MoBiTec, Göttingen, Germany, A-11010) or Alexa Fluor 488 goat anti-mouse IgG (MoBiTec, A-11001, both 1/500) were used. In addition, cell nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 1/1000). Microglia cells were also visualized with tomato lectin 10 μ g/ml (Vector Laboratories/AXXORA, Lörrach, Germany, FL-1201). Results were evaluated with a Carl Zeiss Axiophot epifluorescence microscope coupled to an online digital camera (SPOT imaging software).

Tab. 1. Primers used in this study: Melting points were empirically determined following qRT-PCR (retinoid receptor expression was detected but not quantified).

Name	Primer sequences (sense, antisense)	Annealing T	Product size Melting T product
HPRT	GCTGGTGAAAAGGACCTCT CACAGGACTAGAACACCTGC	62° C	248 bp 79.2° C
IL-1β	CAGCTCATATGGGTCCGACA CTGTGCTTTCCCGTGGACC	62° C	200 bp 81.2° C
IL-6	GTTCTCTGGGAAATCGTGGA CTCTGAAGGACTCTGGCTTTG	59° C	260 bp 81.0° C
IL-12p40	TGACACGCCTGAAGAAGATG CCGGAGTAATTGGTGCTTC	59° C	255 bp 94.8° C
TNFα	ATGGCCTCCCTCTCATCAGT CTCTCCACTTGGTGGTTTG	59° C	113 bp 83.5° C
LCN-2	GCCAGTTCACTCTGGGAAAT GCTCCTTGTTCTTCCATACA	59° C	162 bp 82.5° C
Tgm-2	CATGGTCAACTGCAATGATG GGGCGGAGTTGTAGTTGGT	59° C	236 bp 88.3° C

Statistical analysis

The effects of RA on LPS-stimulated astrocytes in the preincubation and timeline experiments were measured for each condition in 6–12 cultures prepared from at least two different litters. Data from the pre-incubation experiments were analyzed using Oneway ANOVA, post-hoc Dunnett's test and from the timeline experiments by two-way ANOVA with post-hoc Bonferroni–Holm corrected t-test. All statistical analyses were carried out with the logarithms of data, which showed normal distribution and similar variances. Gene expression results were normalized to levels under LPS stimulation. In the graphs, results are presented as means±standard error of the mean (SEM).

Results

Cytokine release in primary cultures of mouse astrocytes

Cell culture experiments were performed to investigate the inflammatory reaction of astrocytes and its modulation by RA. Since glial inflammatory reactions are important in the progression of neurodegenerative diseases and cerebral ischemia, astrocytes were isolated from mouse cerebral cortex. The applied protocol yielded primary cultures of GFAP-positive astrocytes containing less than 2% microglia, as identified with tomato lectin or

Fig. 1. Viability of the primary cultures. Primary astrocyte cultures from mouse cerebral cortex were characterized with antibodies against cell type-specific markers. (a) Double immunofluorescence staining for GFAP, a marker of activated astrocytes, and (b) tomato lectin, a marker of microglia; same cells were photographed in both panels. Cultures contained less than 2% microglia. (c) Ratios of dying cells were assessed by measuring lactate dehydrogenase activity in the culture-medium. Treatment with LPS and RA had no significant effect on cell viability (ANOVA).

ED1 immunocytochemistry, and no oligodendrocytes or neurons were present in the cultures (Fig. 1a, b). To elicit an inflammatory response, cells were stimulated for 30 min to 72 h with 100 ng/ml LPS. Treatment with LPS or all-*trans* RA did not affect cell viability. No increased leakage of lactate dehydrogenase was detected in the experimental conditions (Fig. 1c). A prominent response of microglia to LPS is the production of nitric oxide, which is not observed in pure astrocyte cultures (Saura, 2007). In the primary cultures described here no induction of NO synthesis by LPS was detected (Supplementary Fig. S1).

Otherwise the LPS stimulation of cortical astrocytes had a profound effect on the expression of genes that are characteristic for immune competent cells. Specifically, we found a more than 40-fold increase in transcript concentrations of 21 cytokines and chemokines (Table 2).

Among the pro-inflammatory cytokines the strongest upregulation was observed for IL-6, IL12p40, IL-1β and TNFα. We corroborated this effect with quantitative RT-PCR

Table. 2. LPS induced cytokine and chemokine gene expression in cortical astrocytes.. Data are mean values of two independent gene chip experiments. Under the experimental condition cells were treated 12 h with 100 ng/ml LPS.

Affymetrix probe set	Gene symbol	LPS vs C induction	Abbreviation	Name
1450297_at	Il6	3493	IL-6	interleukin 6
1427429_at	Csf2	564	GM-CSF	Granulocyte macrophage colony stimulating factor
1449984_at	Cxcl2	534	CXCL-2	chemokine (C-X-C motif) ligand 2
1422029_at	Ccl20	510	CCL-20	chemokine (C-C motif) ligand 20
1419530_at 1449497_at	Il12b	383	IL-12p40	interleukin 12p40 subunit
1449399_a_at	Il1b	338	IL-1 β	interleukin 1 beta
1421473_at	Il1a	264	IL-1 α	interleukin 1 alpha
1417574_at	Cxcl12	179	CXCL-12	chemokine (C-X-C motif) ligand 12
1419698_at 1419697_at	Cxcl11	150	CXCL-11	chemokine (C-X-C motif) ligand 11
1418126_at	Ccl5	142	CCL-5	chemokine (C-C motif) ligand 5
1418652_at	Cxcl9	130	CXCL-9	chemokine (C-X-C motif) ligand 9
1421228_at	Ccl7	130	CCL-7	chemokine (C-C motif) ligand 7
1419607_at	Tnf	125	TNF α	tumor necrosis factor alpha
1421578_at	Ccl4	110	CCL-4	chemokine (C-C motif) ligand 4
1419427_at	Csf3	107	G-CSF	granulocyte colony stimulating factor 3
1448898_at 1417936_at	Ccl9	70	CCL-9	chemokine (C-C motif) ligand 9
1419561_at	Ccl3	67	CCL-3	chemokine (C-C motif) ligand 3
1427736_a_at	Ccr12	61	CCR-2	chemokine (C-C motif) receptor like 2
1441855_x_at	Cxcl1	44	CXCL-1	chemokine (C-X- C motif) ligand 1
1419282_at	Ccl12	43	CCL-12	chemokine (C-C motif) ligand 12
1420380_at	Ccl2	41	CCL-2	chemokine (C-C motif) ligand 2

(Supplementary Fig. S2a–c). Time line experiments performed with IL-1 β revealed increased expression of this cytokine already after one hour of LPS treatment; expression reached a 180-fold increase at 12 h and then gradually declined again (Fig. 2a). Under control conditions, i.e. addition of vehicle or 0.1 μ M RA, IL-1 β expression remained very low at all times. In accordance with the gene chip data the other cytokines were also strongly upregulated after a 12 h LPS exposure (Fig. 2b). Extreme ratios of increase for, e.g. IL-6 and IL-12 (Table 2, Fig. 2b) are due to the fact that transcript levels in control samples were close to zero. Recently, lipocalin-2 (LCN-2) has been identified as an autocrine mediator of astrogliosis (Lee *et al.*, 2009). The expression of this factor was therefore also investigated, and it was found to increase by a factor of >20 compared to the control condition (qRT-PCR). In the gene chip experiment, LCN-2 was found to be upregulated 4-fold.

Based on this very robust inflammatory reaction of the astrocytes, we asked whether RA would modify their response. Since RA affects gene expression via binding to nuclear receptors, we confirmed with immunocytochemistry that the cells had retinoic acid and retinoid X receptors (Fig. 3; their mRNA expression is illustrated in Supplementary Fig. S2d). In a parallel paper we are describing the effect of RA on chemokine expression (see Table 2) and experiments to understand the possible molecular mechanism (van Neerven *et al.*, 2010).

Retinoic acid reduced cytokine mRNA expression by cortical astrocytes

A previous investigation of prostaglandin synthesis revealed that a 12 h RA pretreatment of the cells mitigated the expression of cyclooxygenase-2 (Kampmann *et al.*, 2008). In a first set of tests the same pretreatment protocol was used with astrocyte primary cultures, and the transcript concentrations of IL-1 β , IL-6, TNF α and LCN-2 were assessed using quantitative RT-PCR. Normalized to the expression levels after LPS only, RA caused a dose dependent reduction of the cytokine and LCN-2 response (Fig. 4). After a 12 h exposure to 0.1 μ M all-*trans* RA followed by a 12 h 100 ng/ml LPS plus RA the upregulation of IL-1 β decreased to 51%, IL-6 to 29%, TNF α to 59% and LCN-2 to 48%. All these effects were statistically significant or highly significant. RA alone did not affect the low base levels of expression (Fig. 4).

Since microglia constitutes the classical immune competent cell population of the CNS and since similar data have been reported for microglia cultures (Xu *et al.*, 2005), we wanted to exclude that residual microglia cells might be responsible for the observed LPS effects or its modulation by RA. For this purpose astrocyte cultures were treated two days with 200 μ g/ml clodronate, which is used to deplete macrophages and other monocytes. Following this procedure, some LPS/RA experiments were repeated (Fig. 5). The induction of IL-1 β and TNF α was still observed in the clodronate treated cultures, and the inhibitory effect of RA also persisted. In additional control experiments, cells were incubated with the anti-inflammatory steroid dexamethasone. This blocked

Fig. 2. Effect of LPS on the expression of pro-inflammatory cytokines by astrocytes. Transcript levels of cytokines and lipocalin-2 were measured in cell extracts with quantitative RT-PCR: (a) treatment for 1–72 h with solvent (0.1% ethanol, i.e. control condition), 0.1 μ M all-trans RA or 100 ng/ml LPS; expression of IL-1 β . (b) LPS-induced upregulation of pro-inflammatory cytokines after 12 h of treatment with RA or LPS, normalized to the control condition, 3–6 cultures from two different litters were analyzed, and error bars indicate SEM. Following ANOVA, mRNA levels after exposure to LPS or RA were compared to controls with Dunnett's test (* <0.05 and ***<0.001). Expression of LCN-2 and inflammatory cytokines was increased while that of TGF β was not. Note the logarithmic scale of the ordinate.

the effect of LPS completely. While we can conclude that the inflammatory reactions reported here are indeed produced by isolated cortical astrocytes, it became also apparent that clodronate treatment not only eliminated residual microglia but affected the astrocytes, which showed a lower rate of proliferation, as determined by cell counting. Also, the upregulation of TNF α was not as strong as in the cultures without clodronate: in standard astrocyte preparations 12 h of LPS treatment raised TNF α expression by a factor of more than 20 (Fig. 2b), whereas in the clodronate treated cultures the increase was only about 3-fold (for IL-1 β the upregulation was similar in both preparations). Therefore,

Fig. 3. Retinoid receptor immunoreactivity in astrocyte primary cultures from mouse cerebral cortex. Immunofluorescence staining showed (a) positive staining of RAR α in cell nuclei and cytosol, (b) strong RAR β immunoreactivity mostly in the cell nuclei, (c) nuclear and perinuclear RXR α signal, and (d) strong cytosolic staining of RXR β ; same magnification in all photographs. RT-PCR data are shown in Supplementary Fig. S2.

a small percentage of microglia present in the cultures without clodronate treatment about 1% of the cells were positive for tomato lectin, may have contributed to the inflammatory response.

Retinoic acid reduced the release of cytokines into the culture medium

After brain injury or stroke the reactive secretion of some inflammatory cytokines occurs very fast, i.e. within a few hours. For clinical applications anticipatory treatment with RA is impossible. To assess whether RA might still reduce cytokine production when given only simultaneously with LPS, astrocytes were exposed to 0.01, 0.1 or 1 μ M all-*trans* RA together with LPS for 0.5 h to 72 h. Subsequently, the protein concentrations were mea-

Fig. 4. RA reduced the LPS effect on cytokine expression by astrocytes. Cytokine transcript levels were measured in cell extracts with quantitative RT-PCR. (a) IL-1 β , (b) TNF α , (c) IL-6, and (d) LCN-2. Cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to LPS and RA. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. Six cultures from two different litters were analyzed, and error bars indicate SEM. Following ANOVA, mRNA levels after exposure to LPS only or RA only were compared to controls (#<0.05 and ###<0.001), and RA+LPS conditions were compared to LPS only (*<0.05 and **<0.01; Dunnett's test).

sured in the cell culture supernatants. As predicted from the RT-PCR results we found very low levels of all cytokines in the absence of LPS. Inflammatory stimulation caused a massive increase in the release of all tested cytokines with a maximum after 24–72 h after exposure (Figs. 6 and 7).

Compared with the low levels in control cultures we found induction to higher than 100-fold for IL-1 β (>150 pg/ml, maximum concentration measured in the culture medium), IL-12p40 (>25 ng/ml), and IL-12p35/IL-12p40 (>2 ng/ml) and more than 1000-fold for IL-6 (>3 ng/ml) and TNF α (>3 ng/ml). Simultaneous incubation with 0.01, 0.1 or 1 μ M RA had an effect on the release of IL-1 β , with increasing concentration also on IL-12, but not on the other cytokines (Figs. 6a, c, e and 7a, c). Elevated cytokine levels at isolated time points after RA treatment were not significant and therefore attributed to random variability of the different cultures.

In a second set of experiments, equal to the gene expression analysis, cells were pretreated 12 h with RA and then exposed for another 12 h to LPS plus RA. In this case RA lead to a highly significant reduction of the LPS-induced release of IL-1 β (to 5% of the level measured after LPS treatment alone), IL-6 (to 21%), TNF α (to 30%), IL-12p40 (to 42%) and IL-12p35/IL-12p40 (to 14%) using 0.1 μ M of RA (Figs. 6b, d, f and 7b, d; ANOVA was highly significant for all cytokines). For IL-6 the highest dose of 1 μ M RA caused the strongest reduction, otherwise a retinoic acid concentration of 10 nM was similarly effective as 1 μ M. The LPS-induced synthesis of the cytokines was transient, as shown by a highly significant reduction of their concentrations in the medium when this was collected after cells had received LPS free medium for another 12h (marked (L) in Fig. 6b, d, f).

In a previous investigation we observed marked differences in the inflammatory response between cortical and midbrain astrocytes and their regulation by steroid hormones (Kipp *et al.*, 2007). The experiments for ELISA analysis were therefore carried out in parallel with astrocyte primary cultures prepared from midbrain. The results were very similar to those reported for cortical astrocytes. They are presented in the Supplementary figures (Figs. S3 and S4).

Discussion

A gene chip expression screen revealed that murine cortical astrocytes responded to stimulation with bacterial LPS with a very strong upregulation of a number of cytokine and chemokine genes. Quantitative RT-PCR and ELISA measurements confirmed the increase of IL-1 β , IL-6, TNF α , IL-12 and other pro-inflammatory factors. The purpose of this study was to investigate the relevance of all-*trans* RA as a possible modulator of cytokine release under pathological conditions because we proposed previously (Mey *et al.*, 2007) that interactions between RA- and cytokine signaling may constitute an anti-inflammatory feedback loop in the nervous system. In accordance with this hypothesis, RA treatment of the astrocyte cultures reduced the LPS-induced release of all cytokines listed previously. On the protein level, the observed influence was even stronger than on the level of mRNA expression, suggesting the involvement of post-transcriptional regulation. Indirect mechanisms are likely because the antagonistic effect of RA on IL-1 β and IL-12 release was much stronger when RA treatment began 12 h before LPS stimulation than with simultaneous treatment, and for IL-6 and TNF α the pre-treatment was required to achieve an anti-inflammatory effect with RA.

Fig. 5. Inflammatory cytokine expression in clodronate treated astrocytes. To eliminate possible residual monocytes in the cultures these were treated for 48 h with clodronate, and then experiments were performed as described in Fig. 4. In additional controls, the LPS effect was blocked with the anti-inflammatory steroid dexamethasone. Results of quantitative RT-PCR are shown in (a) for IL-1 β and (b) TNF α . Six cultures from two different litters were analyzed, and error bars indicate SEM. Following ANOVA, mRNA levels after exposure to LPS only or RA only were compared to controls (###<0.001), and RA+LPS conditions were compared to LPS only (*<0.05 and ***<0.001; Dunnett's test).

The role of astrocytes and astrocyte/microglia interactions in neuroinflammation

More than any other type of glia astrocytes are engaged in multiple functions both in the healthy brain and under neuropathological conditions. After traumatic lesions or ischemia their signals are neuroprotective but can also exacerbate neuronal cell death and prevent regeneration (Farina *et al.*, 2007; Liberto *et al.*, 2004).

While it is known that LPS induces the production of IL-1 β , IL-6 and TNF α in astrocytes (Benveniste, 1998; Lieberman *et al.*, 1989), the major release of cytokines and

Fig. 6. Effect of LPS and RA on the release of cytokines by astrocytes from cerebral cortex. Cytokine concentrations were measured in cell culture supernatants with ELISA. (a–b) IL-1 β , (c–d) IL-6, and (e–f) TNF α . In a, c, and e cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/ml LPS and the three concentrations of all-*trans* RA. In b, d, and f cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. In recovery experiments (L) LPS free medium was given for 12 h after the LPS stimulation. Seven to 9 cultures from three different litters were analyzed, and error bars indicate SEM. Following ANOVA (in b, d, and f), levels after exposure to LPS only or RA only were compared to controls (###<0.001), and RA+LPS conditions were compared to LPS only (*<0.05, **<0.01, and ***<0.001; Dunnett's test).

Fig. 7. Effect of LPS and RA on the release of cytokines by astrocytes from cerebral cortex. Cytokine concentrations were measured in cell culture supernatants with ELISA. (a–b) IL-12p40 homodimers and (c–d) IL-12p40/p35 heterodimers. In a and c cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/ml LPS and the three concentrations of all-*trans* RA. In b and d cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μM all-*trans* RA. Recovery experiments (L) used a 12 h LPS free medium after the LPS stimulation. Release of IL-12p70 was not detected under any condition. Seven to 9 cultures from three different litters were analyzed, and error bars indicate SEM. Following ANOVA (in b and d), levels after exposure to LPS only or RA only were compared to controls (###<0.001), and RA+LPS conditions were compared to LPS only (*<0.05, **<0.01, and ***<0.001; Dunnett's test).

chemokines is characteristic of microglia, invading macrophages and neutrophils, the protagonists of inflammation when the blood–brain barrier has been breached (Hailer, 2008; Raivich *et al.*, 1999; Hanisch and Kettenmann, 2007; Donnelly and Popovich, 2008). Accumulating evidence from cell cultures emphasizes the importance of astroglia in this context because they are not only capable of synthesizing sundry pro-inflammatory cytokines (Benveniste, 1998) but prostaglandins (Johann *et al.*, 2008) and chemokines (Pang *et al.*, 2001) as well. The present results confirm and extend the concept that immune competent astrocytes form an integral component of the neuroinflammatory network.

Our experiments were performed with astrocyte primary cultures prepared according to an established protocol (Pawlak and Beyer, 2005). Based on cytochemical staining against ED-1 and tomato lectin the cultures contained about 1% microglial cells and must therefore be considered “highly enriched” but not “pure” astrocyte cultures (Saura, 2007). Nevertheless, since clodronate treatment to deplete monocytes confirmed the regulatory effects on cytokines, we conclude that the factors investigated here were synthesized by astrocytes and that RA reduced the inflammatory response of these cells. One signal that is characteristically released by microglia but not in pure astrocyte cultures is NO (Saura, 2007). Nitric oxide measurements from our primary cultures showed that LPS did not induce NO release at all, supporting the validity of the present results with respect to astrocyte physiology. Very similar data were obtained with highly enriched astrocyte cultures produced from murine midbrain.

On the other hand, it was observed that the magnitude of the inflammatory response and its modulation by retinoids was smaller in clodronate treated “pure” astrocyte cultures. This may indicate that even a small population of contaminating microglia contributed to cytokine production or indirectly influenced the astrocytes. However, clodronate treatment affected the surviving astrocytes also, and possibly this may have affected their inflammatory response as well (Dehghani *et al.*, 2004).

RA modulates the astroglial inflammatory responses

Several data point at a regulatory function of RA with respect to chemokine and cytokine production by lymphocytes (Mucida *et al.*, 2007), monocytes (Gross *et al.*, 1993), chondrocytes (Hung *et al.*, 2008) and dendritic cells (Zapata-Gonzalez *et al.*, 2007). In the context of neuroinflammation RA was shown to mitigate the activity of microglia cells (Choi *et al.*, 2009; Dheen *et al.*, 2005; Xu and Drew, 2006). Most pathological features associated with microglial reactions involve the activation of astrocytes, which are influenced by RA as well. The RXR agonist 9-*cis* RA suppressed the production of TNF α that was triggered by LPS in astrocyte primary cultures (Xu and Drew, 2006). Although in this study the release of IL-1 β , IL-6 and MCP-1 (CCL2) were not affected by 9-*cis* RA, other experiments revealed a synergistic effect of 9-*cis* RA and a PPAR α agonist with respect to the reduction of these inflammatory mediators (Xu *et al.*, 2005). In these cases ligand binding to RXR may have been involved, especially since anti-inflammatory effects, e.g. reduction of NO, IL-1 β , IL-6, CCL2, can be caused by LXR/RXR or PPAR/RXR activation without involvement of RAR (Zhang-Gandhi and Drew, 2007; Zapata-Gonzalez *et al.*, 2008). However, we show here that all-*trans* RA (binding to RAR only) is effective in astrocytes. It was published previously that this isomer inhibited the IFN γ -induced phosphorylation of janus kinases and consequently of STAT1 and STAT3. The cytokine inhibitor SOCS3, on the other hand, was upregulated by all-*trans* RA (Choi *et al.*, 2005).

Recently, the upregulation of cyclooxygenase 2 and associated production of prostaglandin-E2 by astroglia has been shown to be reduced by all-*trans* RA (Kampmann *et al.*, 2008). Experiments by Környei *et al.* (2007) even indicate that the astrocytes themselves may produce RA.

Is the cross talk between RA and cytokine signaling a physiological component of neuroinflammation *in vivo*?

As the present study demonstrates that RA is a modulator of the inflammatory response of astrocytes, two questions demand attention: 1) Do these regulatory interactions represent physiological processes *in vivo*? 2) Even if this is not the case, can RA be used as a therapeutic tool to interfere with neuroinflammation?

At this point we have only begun to unravel the physiological relevance of RA signaling for astrocytic activity *in vivo*. In a rat model of amyotrophic lateral sclerosis, degeneration of motoneurons was associated with localized RXR β immunoreactivity in the activated astrocytes (Jokic *et al.*, 2007). Following spinal cord contusion injury, RA was shown to be synthesized locally in the meninges, pericytes and in NG2 positive glia close to the site of injury (Mey *et al.*, 2005). The subsequent findings that nuclear translocation of retinoid receptors occurs in macrophages when the inflammatory reactions subside in the spinal cord (Schrage *et al.*, 2006) or the sciatic nerve (Zhelyaznik and Mey, 2006) gave rise to the hypothesis that RA signaling is part of an anti-inflammatory feedback loop in the nervous system (Mey, 2006; Mey *et al.*, 2007). Although this concept is indirectly supported by the present data, empirical confirmation *in vivo* is still missing. While anti-inflammatory effects of 9-*cis* RA have been well documented *in vitro* (Xu and Drew, 2006; Xu *et al.*, 2005), the physiological involvement of this isomer *in vivo* remains doubtful. Using a highly sensitive mass spectrometric assay, Kane and coworkers analyzed tissue from various brain regions of mice. All-*trans*-, 9,13-di-*cis*- and 13-*cis*-RA isomers were found in the hippocampus, cerebral cortex, olfactory bulb, thalamus, cerebellum and striatum at concentrations ranging from 16 to 80 pmol/g depending on the region, whereas 9-*cis* RA was never detected (Kane *et al.*, 2008). This, in accordance with several previous, less sensitive measurements (Mey *et al.*, 1997), suggests that 9-*cis* RA does not act as a natural ligand for retinoid receptors. When RA was found to reduce the lesion size after brain ischemia, Harvey and coworkers reported 9-*cis* but not all-*trans* RA to be effective (Harvey *et al.*, 2004), whereas other studies found positive effects of all-*trans* but not 9-*cis* RA (Sato *et al.*, 2008; Komatsu *et al.*, 2007). RXRs are known to heterodimerize also with other members of the nuclear hormone receptor superfamily, including peroxisome proliferator-activated receptors (PPAR), vitamin D receptor, thyroid hormone receptor (TR), or liver X receptor (LXR). Since several of these nuclear receptors were shown to have anti-inflammatory properties themselves, the RXR family is in a pivotal position for the development of therapeutic approaches (Zhang-Gandhi and Drew, 2007; Genovese *et al.*, 2005).

Beneficial effects of RA after cerebral ischemia or traumatic insults

Can retinoids be employed therapeutically to limit neuroinflammation? It is interesting to note the efficacy of RA in animal models of CNS pathology, whose results may partly be explained by interactions with inflammatory signals. One important finding, confirmed in several studies, is that systemic RA application reduces ischemic lesions in the cerebral cortex (Choi *et al.*, 2009; Harvey *et al.*, 2004; Sato *et al.*, 2008). Cytokines and chemokines are likely to be involved here (Dénes *et al.*, 2008; Dimitrijevic *et al.*, 2006; Shinozaki *et al.*, 2007). Although a number of convincing studies demonstrated that RA exerts anti-inflammatory effects on microglia even with respect to these cells its potential is still largely neglected in therapeutic considerations (Hailer, 2008; Donnelly and Popovich, 2008).

In the framework of a more neurobiological approach, it has been hypothesized for some time that RA has neurotrophic properties, which could be engaged for nerve regeneration (Corcoran and Maden, 1999; Mey and Rombach, 1999; Malaspina and Michael-Titus, 2008). Maden, McMahon, Mazarakis and collaborators conducted the first thorough experiments to manipulate RA signaling after spinal cord injury *in vivo*. Their studies show that transfection of RAR β , whose activation was neurotrophic *in vitro* (Corcoran *et al.*, 2000), promoted axonal growth of sensory axons into the CNS (Wong *et al.*, 2006) and even regeneration of descending fibers within the spinal cord (Yip *et al.*, 2006). Corresponding behavioral benefits were also reported. Next, Takenaga *et al.* (2009) tested systemic application of the RAR α/β agonist Am80 in the rat model of spinal cord contusion. This treatment reduced the area of tissue degeneration and also caused a significant improvement of motor functions, indicative of neuronal regeneration within the CNS (Takenaga *et al.*, 2009). Based on the promising data with RAR β transfection Corcoran and coworkers have now applied an RAR β agonist with mini osmotic pumps to the lateral ventricle. Rats received a dorsal column lesion at the cervical level of the spinal cord. The retinoid treatment promoted axonal regeneration in the descending corticospinal tract and was associated with functional recovery of forelimb grid walk and beam walk (Agudo *et al.*, 2010). Thus, the evidence is conclusive that RA *in vivo* has a positive influence on the outcome of at least two pathological conditions, ischemia and spinal cord injury. There is still a lot to learn about the underlying mechanisms of these effects. While synergistic interactions between RA and neurotrophins are implied in several studies (Corcoran and Maden, 1999; Mey and Rombach, 1999), the modulation of cytokine release by RA constitutes another possible neuroprotective mechanism.

Conclusion

Stimulation with bacterial LPS induced mouse cortical astrocytes to release cytokines IL-1 β , IL-6, IL-12, and TNF α . These factors are involved in cellular degeneration under neuroinflammatory conditions which are associated with pathologies such as ischemia, CNS injury and neurodegenerative disorders. We found that pretreatment of astrocyte primary cultures with all-*trans* RA interfered with the LPS-induced mRNA expression and protein release of these cytokines. Targeting the inflammatory astroglial response with retinoids may therefore be a promising therapeutic approach, which can now be tested in animal models.

Acknowledgements

The authors thank Stefanie Kurth for valuable help with RT-PCR experiments. Jeyathevy Sivalingam detected retinoid receptors with immunocytochemistry. SvN received a Marie-Curie Fellowship of the European Union (EURON).

References

- Agudo, M., Yip, P., Davies, M., Bradbury, E., Doherty, P., McMahon, S., Maden, M., Corcoran, J.P., 2010. A retinoic acid receptor beta agonist (CD2019) overcomes inhibition of axonal outgrowth via phosphoinositide 3-kinase signalling in the injured adult spinal cord. *Neurobiol. Dis.* **37**, 147–155
- Alique, M., Herrero, J.F., Lucio-Cazana, F.J., 2007. All-*trans* retinoic acid induces COX-2 and prostaglandin E2 synthesis in SH-SY5Y human neuroblastoma cells: involvement of retinoic acid receptors and extra-cellular-regulated kinase 1/2. *J.Neuroinflammation* doi:10.1186/1742–2094–4–1
- Archer, K.J., Reese, S.E., 2010. Detection call algorithms for high-throughput gene expression microarray data. *Brief Bioinform.* **11**, 244–252
- Austenaa, L.M., Carlsen, h., Hollung, K., Blomhoff, h.K., Blomhoff, R., 2008. Retinoic acid dampens LPS-induced NF-kappaB activity: results from human monoblasts and *in vivo* imaging of NF-kappaB reporter mice. *J. Nutr. Biochem.* **20**, 726–734
- Bastien, J., Rochette-Egly, C., 2004. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1–16
- Benveniste, E.N., 1998. Cytokine actions in the central nervous system. *Cytokine Growth Factor Rev.* **9**, 259–275
- Choi, W.H., Ji, K.A., Jeon, S.B., Yang, M.S., Kim, h., min, K., Shong, M., Jou, I., Joe, E.H., 2005. Anti-inflammatory roles of retinoic acid in rat brain astrocytes: suppression of interferon- γ -induced JAK/STAT phosphorylation. *Biochem. Biophys. Res. Commun.* **329**, 125–131
- Choi, B.K., Kim, J.H., Jung, J.S., Lee, Y.S., Han, M.E., Baek, S.Y., Kim, B.S., Kim, J.B., Oh, S.O., 2009. Reduction of ischemia-induced cerebral injury by all-*trans* retinoic acid. *Exp. Brain Res.* **193**, 581–589
- Corcoran, J., Maden, M., 1999. Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nat. Neurosci.* **2**, 307–308
- Corcoran, J., Shroot, B., Pizzey, J., Maden, M., 2000. The role of retinoic acid receptors in neurite outgrowth from different populations of embryonic mouse dorsal root ganglia. *J. Cell Sci.* **113**, 2567–2574
- Dehghani, F., Conrad, A., Kohl, A., Korf, h.W., Hailer, N.P., 2004. Clodronate inhibits the secretion of proinflammatory cytokines and NO by isolated microglial cells and reduces the number of proliferating glial cells in excitotoxically injured organotypic hippocampal slice cultures. *Exp. Neurol.* **189**, 241–251
- Dénes, A., Ferenczi, S., Halasz, J., Kornyei, Z., Kovacs, K.J., 2008. Role of CX3CR1 (fractalkine receptor) in brain damage and inflammation induced by focal cerebral ischemia in mouse. *J. Cereb. Blood Flow Metab.* **28**, 1707–1721
- Devaux, Y., Seguin, C., Grosjean, S., de Talance, N., Schwartz, M., Burlet, A., Zannad, F., Meistelman, C., Mertes, P.M., Ungureanu-Longrois, D., 2001. Retinoic acid and lipopolysaccharide act synergistically to increase prostanoid concentrations in rats *in vivo*. *J. Nutr.* **131**, 2628–2635
- Dheen, S.T., Jun, Y., Yan, Z., Tay, S.S.W., Ling, E.A., 2005. Retinoic acid inhibits expression of TNF α and iNOS in activated rat microglia. *Glia* **50**, 21–31
- Dimitrijevic, O.B., Stamatovic, S.M., Keep, R.F., Andjelkovic, A.V., 2006. Effects of the chemokine CCL2 on blood–brain barrier permeability during ischemia–reperfusion injury. *J. Cereb. Blood Flow Metab.* **26**, 797–810

- Dong, Y., Benveniste, E.N., 2001. Immune function of astrocytes. *Glia* **36**, 180–190.
- Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp. Neurol.* **209**, 378–388.
- Farina, C., Aloisi, F., Meinl, E., 2007. Astrocytes are active players in cerebral innate immunity. *Trends Immunol.* **28**, 138–145.
- Genovese, T., Mazzon, E., Di Paola, R., Cannavò, G., Muià, C., Bramanti, P., Cuzzocrea, S., 2005. Role of endogenous ligands for the peroxisome proliferators activated receptors alpha in the secondary damage in experimental spinal cord trauma. *Exp. Neurol.* **194**, 267–278.
- Gross, V., Villiger, P.M., Zhang, B., Lotz, M., 1993. Retinoic acid inhibits interleukin-1-induced cytokine synthesis in human monocytes. *J. Leukocyte Biol.* **54**, 125–132.
- Hailer, N.P., 2008. Immunosuppression after traumatic or ischemic CNS damage: it is neuroprotective and illuminates the role of microglial cells. *Prog. Neurobiol.* **84**, 211–233.
- Hanisch, U.K., Kettenmann, h., 2007. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat. Neurosci.* **10**, 1387–1394.
- Harvey, B.K., Shen, h., Chen, G.J., Yoshida, Y., Wang, Y., 2004. Midkine and retinoic acid reduce cerebral infarction induced by middle cerebral artery ligation in rats. *Neurosci. Lett.* **369**, 138–141.
- Hung, L.F., Lai, J.H., Lin, L.C., Wang, S.J., Hou, T.Y., Chang, D.M., Liang, C.C., Ho, L.J., 2008. Retinoid acid inhibits IL-1-induced iNOS, COX-2 and chemokine production in human chondrocytes. *Immunol. Invest.* **37**, 675–693.
- Johann, S., Kampmann, E., Denecke, B., Arnold, S., Kipp, M., Mey, J., Beyer, C., 2008. Expression of enzymes involved in the prostanoid metabolism by cortical astrocytes after LPS-induced inflammation. *J. Mol. Neurosci.* **34**, 177–185.
- Jokic, N., Ling, Y.Y., Ward, R.E., Michael-Titus, A.T., Priestley, J.V., Malaspina, A., 2007. Retinoid receptors in chronic degeneration of the spinal cord: observations in a rat model of amyotrophic lateral sclerosis. *J. Neurochem.* **103**, 1821–1833.
- Kampmann, E., Johann, S., van Neerven, S., Beyer, C., Mey, J., 2008. Anti-inflammatory effect of retinoic acid on prostaglandin synthesis in cultured cortical astrocytes. *J. Neurochem.* **106**, 320–332.
- Kane, M.A., Folias, A.E., Wang, C., Napoli, J.L., 2008. Quantitative profiling of endogenous retinoic acid *in vivo* and *in vitro* by tandem mass spectrometry. *Anal. Chem.* **80**, 1702–1708.
- Kipp, M., Karakaya, S., Johann, S., Kampmann, E., Mey, J., Beyer, C., 2007. Oestrogen and progesterone reduce lipopolysaccharide-induced expression of tumor necrosis factor- α and interleukin-18 in midbrain astrocytes. *J. Neuroendocrinol.* **19**, 1–4.
- Knobloch, S.M., Fan, L., Faden, A.I., 1999. Early neuronal expression of tumor necrosis factor- α after experimental brain injury contributes to neurological impairment. *J. Neuroimmunol.* **95**, 115–125.
- Komatsu, T., Hirano, T., Songkram, C., Kawachi, E., Kagechika, h., 2007. Novel thyroid hormone receptor antagonists with an N-alkylated diphenylamine skeleton. *Bioorg. Med. Chem.* **15**, 3115–3126.
- Környei, Z., Gócza, E., Rühl, R., Orsolits, B., Vörös, E., Szabó, B., Vágovits, B., Madarász, E., 2007. Astroglia-derived retinoic acid is a key factor in glia-induced neurogenesis. *FASEB J.* **21**, 2496–2509.
- Lee, S., Park, J.Y., Lee, W.H., Kim, h., Park, h.C., Mori, K., Suk, K., 2009. Lipocalin-2 is an autocrine mediator of reactive astrocytosis. *J. Neurosci.* **29**, 234–249.

- Li, X., Stark, G.R., 2002. NFkappaB-dependent signaling pathways. *Exp. Hematol.* **30**, 285–296
- Liberto, C.M., Albrecht, P.J., Herx, L.M., Yong, V.W., Levison, S.W., 2004. Pro regenerative properties of cytokine-activated astrocytes. *J. Neurochem.* **89**, 1092–1100
- Lieberman, A.P., Pitha, P.M., Shin, h.S., Shin, M.L., 1989. Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. *Proc. Natl Acad. Sci. USA* **86**, 6348–6352
- Malaspina, A., Michael-Titus, A.T., 2008. Is the modulation of retinoid and retinoid-associated signaling a future therapeutic strategy in neurological trauma and neurodegeneration? *J. Neurochem.* **104**, 584–595
- McCoy, M.K., Tansey, M.G., 2008. TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. *J. Neuroinflammation* **5**, 45. doi:10.1186/1742–2094–5–45
- Mey, J., 2006. A new target for CNS injury? The role of retinoic acid signaling after nerve lesions. *J. Neurobiol.* **66**, 757–779
- Mey, J., Rombach, N., 1999. Retinoic acid increases BDNF-dependent regeneration of chick retinal ganglion cells *in vitro*. *NeuroReport* **10**, 3573–3577
- Mey, J., McCaffery, P., Dräger, U.C., 1997. Retinoid acid synthesis in the developing chick retina. *J. Neurosci.* **17**, 7441–7449
- Mey, J., Morassutti, D., Brook, G.A., Liu, R.H., Zhang, Y.P., Koopmans, G., McCaffery, P., 2005. Retinoic acid synthesis by a population of NG-2 positive cells in the injured spinal cord. *Eur. J. Neurosci.* **21**, 1555–1568
- Mey, J., Schrage, K., Wessels, I., Vollpracht-Crijns, I., 2007. Effects of inflammatory cytokines IL-1 β , IL-6 and TNF α on the intracellular localization of retinoid receptors in Schwann cells. *Glia* **55**, 152–164
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., Cheroutre, h., 2007. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260
- Mulcahy, N.J., Ross, J., Rothwell, N.J., Loddick, S.A., 2003. Delayed administration of interleukin-1 receptor antagonist protects against transient cerebral ischaemia in the rat. *Br. J. Pharmacol.* **140**, 471–476
- Na, S.Y., Kang, B.Y., Chung, S.W., Han, S.J., Ma, X., Trinchieri, G., Im, S.Y., Lee, J.W., Kim, T.S., 1999. Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFkappaB. *J. Biol. Chem.* **274**, 7674–7680
- Okun, E., Griffioen, K.J., Lathia, J.D., Tang, S.C., Mattson, M.P., Arumugam, T.V., 2008. Toll-like receptors in neurodegeneration. *Brain Res. Rev.* **59**, 278–292
- Pang, Y., Cai, Z., Rhodes, P.G., 2001. Analysis of genes differentially expressed in astrocytes stimulated with lipopolysaccharide using cDNA arrays. *Brain Res.* **914**, 15–22
- Pawlak, J., Beyer, C., 2005. Developmental expression of MNAR mRNA in the mouse brain. *Cell Tissue Res.* **320**, 545–549
- Raivich, G., Bohatschek, M., Kloss, C.U.A., Werner, A., Jones, L.L., Kreutzberg, G.W., 1999. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res. Rev.* **30**, 77–105
- Sato, Y., Meller, R., Yang, T., Taki, W., Simon, R.P., 2008. Stereo-selective neuroprotection against stroke with vitamin A derivatives. *Brain Res.* **1241**, 188–192
- Saura, J., 2007. Microglial cells in astroglial cultures: a cautionary note. *J. Neuroinflammation* **4**, 26. doi:10.1186/1742–2094–4–26

- Schrage, K., Koopmans, G., Joosten, E.A., Mey, J., 2006. Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. *Eur. J. Neurosci.* **23**, 285–295
- Shen, h., Luo, Y., Kuo, C.C., Deng, X., Chang, C.F., Harvey, B.K., Hoffer, B.J., Wang, Y., 2009. 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone morphogenetic protein. *J. Neurosci. Res.* **87**, 545–555
- Shinozaki, Y., Sato, Y., Koizumi, S., Ohno, Y., Nagao, T., Inoue, K., 2007. Retinoids acting through retinoid receptors protect hippocampal neurons from oxygen–glucose deprivation-mediated cell death by inhibition of JNK and p38 mitogen-activated protein kinase. *Neuroscience* **147**, 153–163
- Takenaga, M., Ohta, Y., Tokura, Y., Hamaguchi, A., Shudo, K., Okano, h., Igariashi, R., 2009. The effect of Am-80, a synthetic retinoid, on spinal cord injury-induced motor dysfunction in rats. *Biol. Pharm. Bull.* **32**, 225–231
- van Neerven, S., Mey, J., 2007. RAR/RXR and PPAR/RXR signaling in spinal cord injury. *PPAR Res.* doi:10.1155/2007/29275
- van Neerven, S., Regen, T., Wolf, D., Nemes, A., Johann, S., Beyer, C., Hanisch, U.K., Mey, J., 2010. Inflammatory chemokine release of astrocytes *in vitro* is reduced by all-trans Retinoic acid. *J. Neurochem.* **114**, 1511–1526
- Wong, L.F., Yip, P.K., Battaglia, A., Grist, J., Corcoran, J., Maden, M., Azzouz, M., Kingsman, S.M., Kingsman, A.J., Mazarakis, N.D., McMahon, S.B., 2006. Retinoic acid receptor β 2 promotes functional regeneration of sensory axons in the spinal cord. *Nat. Neurosci.* **9**, 243–250
- Xu, J., Drew, P.D., 2006. 9-cis-Retinoic acid suppresses inflammatory responses of microglia and astrocytes. *J. Neuroimmunol.* **171**, 135–144
- Xu, J., Storer, P.D., Chavis, J.A., Racke, M.K., Drew, P.D., 2005. Agonists for the peroxisome proliferator-activated receptor-alpha and the retinoid X receptor inhibit inflammatory responses of microglia. *J. Neurosci. Res.* **81**, 403–11
- Yip, P.K., Wong, L.F., Pattinson, D., Battaglia, A., Grist, J., Bradbury, E.J., Maden, M., McMahon, S.B., Mazarakis, N.D., 2006. Lentiviral vector expressing retinoid acid receptor β 2 promotes recovery of function after corticospinal tract injury in the adult rat spinal cord. *Hum. Mol. Gen.* **15**, 3107–3118
- Zapata-Gonzalez, F., Rueda, F., Petriz, J., Domingo, P., Villarroja, F., de, M.A., Domingo, J.C., 2007. 9-cis-Retinoic acid (9cRA), a retinoid X receptor (RXR) ligand, exerts immunosuppressive effects on dendritic cells by RXR-dependent activation: inhibition of peroxisome proliferator-activated receptor gamma blocks some of the 9cRA activities, and precludes them to mature phenotype development. *J. Immunol.* **178**, 6130–6139.
- Zapata-Gonzalez, F., Rueda, F., Petriz, J., Domingo, P., Villarroja, F., Diaz-Delfin, J., de Madariaga, M.A., Domingo, J.C., 2008. Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly through PPAR(gamma): RXR heterodimers: comparison with other polyunsaturated fatty acids. *J. Leukoc. Biol.* **84**, 1172–1182
- Zhang-Gandhi, C.X., Drew, P.D., 2007. Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. *J. Neuroimmunol.* **183**, 50–59
- Zhelyaznik, N., Mey, J., 2006. Expression of retinoic acid receptors and retinoid X receptors after sciatic nerve injury. *Neuroscience* **141**, 1761–1774
- Zimmerman, T.L., Thevananther, S., Ghose, R., Burns, A.R., Karpen, S.J., 2006. Nuclear export of RXR α in response to IL-1 β -mediated cell signaling: roles for JNK and Ser260. *J. Biol. Chem.* **281**, 15434–15440

Supplementary figures

Fig. S1. Effect of LPS and RA on the release of NO from primary cultures of murine cortical astrocytes. Levels of the NO derivative nitrite were determined photometrically in the culture medium by the Griess reaction, and the assay was calibrated with NaNO₂ concentration series. (a) Cultures were exposed simultaneously but for different periods of time (0.5–48 h) to 100 ng/ml LPS and the three concentrations of all-*trans* RA. Nitric oxide release from 3 cultures per time point and condition was measured. (b) Cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. Nine to 12 cultures were analyzed for every condition, and error bars indicate SEM.

Fig. S2. Quantitative RT-PCR measurements of cytokine mRNA concentrations. RNA extracts of cultures were evaluated with conventional and quantitative RT-PCR. (a–b) Melting curve analyses were carried out after every quantitative PCR reaction and Ct values were used only in the absence of non-specific amplicons. Relative PCR efficiencies were determined with dilution series of cDNA pool, in duplicates, $r_2 > 0.95$ and PCR efficiency $> 90\%$ in all cases. (c) All cytokine amplification products had the expected molecular sizes, and no unexpected bands appeared (see Table 1). Only extremely low levels of IL-1 β , IL-6 and IL-12p40 were detected in control samples (C). (d) Gene expression of all six retinoid receptors in mouse cortical astrocytes as shown with RT-PCR; primer sequences have been published recently (van Neerven, *et al.*, 2010).

Fig. S3. Effect of LPS and RA on the release of cytokines by midbrain astrocytes. Cytokine concentrations were measured in cell culture supernatants with ELISA. (a–b) IL-1 β , (c–d) IL-6, and (e–f) TNF α . In a, c, and e cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/ml LPS and the three concentrations of all-*trans* RA. In b, d, and f cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. Seven to 9 cultures from three different litters were analyzed, and error bars indicate SEM. Following ANOVA (in b, d, and f), levels after exposure to LPS only or RA only were compared to controls (### < 0.001), and RA+LPS conditions were compared to LPS only (* < 0.05, ** < 0.01, and *** < 0.001; Dunnett's test).

Fig. S4. Effect of LPS and RA on the release of cytokines by midbrain astrocytes. Cytokine concentrations were measured in cell culture supernatants with ELISA. (a–b) IL-12p40 homodimers and (c–d) IL-12p40/p35 heterodimers. In a and c cultures were exposed simultaneously but for different periods of time (0.5–72 h) to 100 ng/ml LPS and the three concentrations of all-*trans* RA. In b and d cultures were pretreated 12 h with RA followed by a 12 h simultaneous exposure to RA and LPS. Under control conditions, astrocytes received only vehicle, LPS or 0.1 μ M all-*trans* RA. Release of IL-12p70 was not detected under any condition. Seven to 9 cultures from three different litters were analyzed, and error bars indicate SEM. Following ANOVA (in b and d), levels after exposure to LPS only or RA only were compared to controls (###<0.001), and RA+LPS conditions were compared to LPS only (***<0.001; Dunnett's test).

**Systemic but not local
administration of retinoic acid
reduces early transcript levels
of pro-inflammatory cytokines after
experimental spinal cord injury**

**S.G.A. van Neerven^{1,2,3,4}, J. Mey^{3,4}, E.A. Joosten^{1,4},
H.W. Steinbusch^{2,4}, M. van Kleef¹,
M.A.E. Marcus¹, R. Deumens^{1,4,5}**

¹Department of Anesthesiology, School for Mental Health
and Neuroscience, Maastricht University Medical Center,
Maastricht, The Netherlands

²Department of Neuroscience, School for Mental Health
and Neuroscience, Maastricht University, Maastricht, The Netherlands

³Institute of Biology II, RWTH Aachen University, Aachen, Germany

⁴EURON, European Graduate School of Neuroscience,
Maastricht, The Netherlands

⁵Institute of Neuropathology, Medical Faculty,
RWTH Aachen University, Aachen, Germany

Abstract

Early rises of pro-inflammatory cytokines play a key role in tissue damage and has detrimental consequences for functional outcome after spinal cord injury (SCI). All-*trans* retinoic acid (RA) has been shown to be a therapeutic agent reducing cytokine expression *in vitro*, but its use may be limited due to adverse side effects, associated with systemic delivery. Local delivery of RA may circumvent adverse side effects, but may simultaneously reduce the therapeutic benefits of the therapy. Here, we investigated whether local or systemic RA treatment differentially affected pro inflammatory cytokine expression early after rat SCI. Pro-inflammatory cytokines IL-1 β , IL-6 and TNF α were investigated at 6 h after moderate contusion injury of the thoracic (T9) spinal cord, when mRNA levels are known to peak. Rats were either treated with intrathecal RA (0, 2.5, 10, or 100 ng) or received an intraperitoneal injection of RA (15 mg/kg bodyweight). Surprisingly intrathecal RA up to amounts of 100 ng did not attenuate SCI-induced increases in gene-expression of pro-inflammatory cytokines. In contrast, intraperitoneal RA rendered a 60%, 35% and 58% reduction of IL-1 β , IL-6 and TNF α mRNA levels, respectively. Although local doses higher than 100 ng RA may reduce pro-inflammatory cytokine gene expression, such doses precipitate and possibly increase risks of adverse side effects. We conclude that in contrast to systemic delivery, intrathecal administration of RA up to doses of 100 ng is ineffective in reducing early pro-inflammatory cytokine gene-expression. Future studies are required to investigate the effects of single intraperitoneal RA treatment on long-term SCI outcome.

Keywords: inflammation, cytokines,
nuclear hormone receptors

Introduction

Early neuroinflammation is a key event that exacerbates neurotrauma and outlines the persistent and debilitating consequences of spinal cord injury (SCI) including motor deficits and neuropathic pain. Pharmacological attenuation of early levels of pro-inflammatory cytokines is, therefore, thought to render neuroprotection and subsequently improved functional outcome (Donnelly & Popovich 2008; Marchand *et al.* 2009; Genovese *et al.* 2008).

Nuclear hormone receptors, including glucocorticoid receptor, the liver X receptors (LXR), peroxisome proliferator activated receptors (PPAR) and retinoid receptors (RAR/RXR) have been described as potential immunomodulatory drug targets for neurodegenerative diseases including SCI (van Neerven *et al.* 2008). The glucocorticoid methyl-prednisolone (MP) currently is the only recognized treatment options for SCI patients and is effective in reducing early pro-inflammatory cytokine levels (Tsai *et al.* 2008). However, agonists for PPAR and LXR have also been shown to attenuate pro-inflammatory cytokine levels following SCI (Park *et al.* 2007; Paterniti *et al.* 2010). We recently added all-*trans*-retinoic acid (RA), the active metabolite of Vitamin A and natural ligand of nuclear hormone receptors RAR/RXR to this list by showing that RA strongly reduced pro-inflammatory cytokine and chemokine expression in *in vitro* models of central nervous system inflammation (van Neerven *et al.* 2010). Although ligands of nuclear hormone receptors are potentially very interesting therapeutic agents in early treatment of SCI, they have often been associated with adverse side effects (Hanson & Leachman 2001; Suh *et al.* 1992; Shah & Mudaliar 2010).

Adverse side effects can be omitted when drugs are administered locally, rather than systemically, although the route of drug administration may, obviously, influence the drug's therapeutic efficacy. We here investigated whether the route of RA administration (local versus systemic) differentially affected the early spinal rises of pro-inflammatory cytokine transcripts which are fundamental to neuroinflammatory responses following SCI.

Material and Methods

Procedures were approved by the ethical committee of Maastricht University and carried out according to the recommendations of the European Commission (European Communities Council Directive of 24 November 1986; 86/609/EEC). Twelve weeks old female Sprague Dawley rats, weighting approximately 250 g, were housed under standard housing conditions, including 12:12 hrs light/dark regime and *ad libitum* access to water and standard laboratory chow.

Intrathecal (i.t.) catheters were implanted at five days before SCI via an incision in the atlanto-occipital membrane and tunnelling of the catheter up to the eighth thoracic

spinal level (T8). SCI was inflicted using the New York University Impactor (MASCIS device) by a 10-g weight drop from a distance of 12.5 mm onto the exposed dorsal surface of the T9 spinal cord as described previously (Gruner 1992). Post-operative care included subcutaneous administration of 5 mL saline to compensate for fluid loss during surgery and 5 mg/kg enrofloxacin (Baytril) as anti-biotic treatment.

Preparation of RA (Sigma) involved dilution to a stock solution of 100 mg/mL in 100% DMSO. To prevent oxidation, air above the aliquots was replaced with nitrogen, and stock aliquots were stored at -80°C . All handling with RA occurred under conditions of amber lighting (Na⁺ lamp). For i.t. injections, RA stock solution was further diluted in 100% ethanol to a concentration of 1 mg/mL and subsequently in physiological salt solution, reaching final amounts of 2.5 ng, 10 ng and 100 ng in a volume of maximally 10 μL . Vehicle solutions consisted of 1% ethanol and 0.01% DMSO in saline. For intraperitoneal (i.p.) injections, RA stock solution was used and given at 15 mg/kg bodyweight. This dose was selected on the basis of two criteria: (1) i.p. injection of 5 mg/kg bodyweight RA was able to reduce IL-1 β expression in an experimental model of cerebral infarction (Choi *et al.* 2009), but did not reduce cytokine levels in our model (data not shown); (2) previous work showed that after systemic administration the RA levels ending up in the spinal cord are only a third of those in the brain (Werner & Deluca 2002);

In previous *in vitro* investigations we found that the anti-inflammatory effects of RA were enhanced when RA was given prior to and immediately after an inflammatory trigger (van Neerven *et al.* 2010). For this reason, i.t. drug application to SCI animals was done at 48 h before, 24 h before and at the time of SCI. Animals either received vehicle solution (n = 6), 2.5 ng RA (n = 5), 10 ng RA (n = 5), or 100 ng RA (n = 3). Naïve animals (n = 6) were used as controls. Although RA has been reported to pass the blood-brain barrier (Luo *et al.* 2004), RA signalling at the level of the spinal cord following i.p. delivery was validated by investigating RA-target gene (RAR β ; (Balmer, 2002)) expression in naïve animals treated with RA (n = 4) or vehicle (n = 2) using a single i.p. injection. The effects of systemic RA administration on SCI-induced cytokines were then investigated using RA-treated SCI animals (n = 6) and vehicle-treated SCI animals (n = 6).

Animals were sacrificed at 6 h following the last RA treatment by an overdose of pentobarbital (180 mg/kg bodyweight i.p.) and were transcardially perfused with ice-cold saline. This time point of sacrifice was selected on the basis of previous work showing that SCI-induced levels in pro-inflammatory cytokine transcripts are strongly increased at this time point (Streit *et al.*, 1998; Donnelly & Popovich 2008). After saline perfusion, meninges were carefully removed and a 1 cm-long spinal segment containing the centre of the lesion was snap-frozen in liquid nitrogen. Tissues were kept at -80°C until used for further processing.

Isolation of total RNA was performed with the phenol-chloroform method and therefore tissues were homogenized in Trizol (*Invitrogen*) using glass beads. RNA concentration and purity were assessed photometrically (OD₂₆₀, OD₂₆₀/OD₂₈₀ ratio). Total RNA

was reverse transcribed into cDNA using *Biorad iScript cDNA kit* with calculated concentrations of 50 ng/ μ L RNA extract per reaction. Samples were diluted 1/10 after reverse transcription and then used for amplification with the *Roche LightCycler® 480 Real-Time PCR System*. Reactions were carried out in a 96-well reaction module, containing 20 μ L total reaction volume per well. Samples contained 10 μ L of the *Roche SYBR Green PCR kit*, 7 μ L nuclease-free water, 2 μ L of the oligo mix containing 5 μ M of each sense and anti-sense primer and 1 μ L cDNA sample. The PCR protocol consisted of 5m enzyme activation at 95° C, followed by 45 cycles of 20 s denaturation at 95° C, 30 s annealing at the primers specific temperature and 23 s elongation and fluorescence measurement at 72° C. Expression of the housekeeping gene HPRT or GAPDH was used to normalize gene-expression. Melt-ing curve analysis and agarose gel electrophoresis of the PCR products were routinely performed. Primer sequences and annealing temperatures are listed in Tab. 1. Relative mRNA concentrations were determined using crossing point analysis of log/linear plots of fluorescence/cycle number. To determine the PCR efficiency, concentration series (dilutions 1/2, 1/4, ...1/128) of sample pools were performed once for every primer pair. All primer pairs showed efficiencies around 2.0 and standard curves had slopes between 3.1–3.6.

Statistical analyses were performed with GraphPad Prism 5 software. Differences in RAR β expression between RA-treated and vehicle-treated animals were compared using a non-paired t-test. Data were normally distributed. Other data were analyzed using one-way ANOVA and *post-hoc* Tukey's correction for multiple comparisons. The graphs represent means with standard error of the mean (SEM). *p*-Values of less than 0.05 were considered as statistically significant.

Tab. 1. Primers used in this study

Name	Primer sequences 5' $\rightarrow$ 3' sense antisense	Annealing temperature	Product size	Product Tm
GAPDH	TGCCAAGTATGATGACATCAAGAAG AGCCCAGGATGCCCTTTAGT	55	80	86
HPRT	GCAGACTTTGCTTTCCTTGG CCGCTGTCTTTTAGGCTTTG	60	239	80
IL-1 β	CACCTTCTTTTCCTTCATCTTTG GTCGTTGCTTGTCTCTCCTTGTA	55	241	82
IL-6	AAGTTTCTCTCCGAAGAGACTTCCAG AGGCAAATTCCTGTTATATCCAGTT	55	326	81
TNF α	CCCAGACCCTCACACTCAGAT TTGTCCCTTGAAGAGAACCTG	55	215	85
RAR β	GGTACCACTATGGCGTCAG TGCTCTGGGGTATACCTGG	60	554	89

Results

Intrathecal RA application

To determine the effect of drug administration route on therapeutic reduction of early SCI-induced pro-inflammatory cytokine levels, animals received RA locally via an i.t. catheter, which had been implanted 5 days before injury, or animals received RA systemically via an i.p. injection. In animals with i.t. catheters, SCI induced a strong and statistically significant increase of pro-inflammatory cytokine expression at the lesion site 6 h after SCI (Fig. 1b-d). Transcripts of IL-1 β , IL-6 and TNF α were found to be significantly increased by SCI at 6 h. Rostral and caudal from the lesion site ($\pm$ 1.5 cm from the lesion epicenter), transcript levels were comparable to those of un-injured animals (data not shown). Local repetitive treatment with 2.5, 10 or 100 ng RA did not reduce these SCI-induced increases in pro-inflammatory cytokine mRNA expression. After SCI, expression of the RA-driven gene RAR β was unchanged by intrathecal administration of 100 ng RA, indicative of no RA signaling when applied in the highest dose tested in this study (Fig. 1a).

Intraperitoneal RA application

In a second treatment setup, a single high dose of RA was given systemically via i.p. injection. Expression of RAR β was used as a positive control for RA signalling and was measured in un-injured animals after a single injection of RA (15 mg/kg bodyweight). The RAR β gene has been shown to be regulated by RA transcriptional active signalling (Balmer & Blomhoff 2002). Single injection of 15 mg/ kg bodyweight RA resulted in a 2.8-fold increased expression of RAR β at 6 h, indicative of RA signalling in the spinal cord after systemic injection (Fig. 2a left). RAR β expression was also investigated after RA treatment in SCI injured animals, where RA induced RAR β expression showed a tendency towards an elevation ($p=0.09$). The RA effect on RAR β expression was, thus, more pronounced in naïve animals than in SCI animals, which suggests that SCI triggered reduction in RA signalling (Fig. 2a right).

SCI increased transcript levels of IL-1 β , IL-6 and TNF α expression at 6 h following injury (Fig. 2b-d). A single injection of 15 mg/kg bodyweight RA immediately after SCI, reduced cytokine expression levels of IL-1 β , IL-6 and TNF α to 60%, 35% and 58% of untreated animals, respectively. This reduction was statistically significant for IL-1 β and TNF α ($p < 0.05$). Systemic injection of RA in a dose of 5 mg/kg body weight did not reduce cytokine expression levels (data not shown).

Fig. 1. Intrathecal treatment with RA after SCI. (a) After SCI, application of the highest dose of RA (100 ng) did not induce expression of the RA-driven gene RAR β . SCI induced a strong and statistically significant increase of pro-inflammatory cytokine transcript levels. (b and c) I.t. application of 2.5, 10 or 100 ng RA did not diminish this elevation of cytokine mRNA. #-symbol(s), statistically significant difference between naïve animals and SCI animals; *-symbol(s), statistically significant difference between RA treated and vehicle-treated animals, #p < 0.05, ##/**p < 0.01 and ###p < 0.001.

Discussion

Surprisingly, local administration of RA up to 100 ng was unable to reduce pro-inflammatory cytokine gene-expression. Higher local doses of RA were not tested for the reasons discussed below. We showed however that systemic administration of RA reduces early expression of pro-inflammatory cytokines IL-1 β and TNF α after experimental SCI. IL-6

Fig. 2. Intraperitoneal treatment with RA after SCI. (a) left: single systemic injection of RA induces RAR β expression, indicative of RA signaling in the spinal cord. Right: after SCI, RA-induced RAR β expression showed a tendency towards an elevation. (b–d) SCI induces a strong and highly statistical significant increase of pro-inflammatory cytokine transcript levels at the lesion site. Intraperitoneal injection of RA reduced transcript levels of all three cytokines, of which the reduction of IL-1 β and TNF α expression was statistically significant. #-symbol(s), statistically significant difference between naïve animals and SCI animals; *-symbol(s), statistically significant difference between RA treated and vehicle-treated animals, #/*p < 0.05, ##/**p < 0.01 and ###/***p < 0.001.

mRNA transcripts were also diminished by RA, but this reduction did not reach statistical significance. The anti-inflammatory effect was only noticed when RA was administered in a high systemic dose.

Local administration of RA did not reduce early cytokine expression after experimental SCI

Since local drug administration increases the specificity of treatment and lowers the therapeutic doses, possible adverse side effects are strongly reduced as compared to systemic drug administration. In this study, local (intrathecal) delivery of RA (2.5–100 ng in saline up to 10 μ L) did not attenuate the SCI-induced increases in expression of pro-inflammatory cytokines IL-1 β , IL-6 or TNF α at 6 h after SCI. Prolonging the period of pre-treatment did not influence the RA effect on these early cytokine levels (data not shown). Expression of the RA-driven gene RAR β was not changed by intrathecal treatment with 100 ng RA, indicative of no RA-signalling by the highest dose tested in this study.

These findings were rather surprising for several reasons. First, previous data from our group showed that doses of RA varying from 0.3 to 3 ng/10 μ L are anti-inflammatory and non-toxic in astrocyte cultures (Kampmann *et al.* 2008; van Neerven *et al.* 2010). In the present study, a maximum dose of 100 ng was selected in order to minimize the risk of RA precipitation in watery solutions such as saline. Soluble, and thus non-precipitated, RA may strongly benefit its therapeutic effect because soluble RA can freely diffuse throughout the i.t. space. Second, as RA is fat-soluble it is expected to easily penetrate the spinal cord following i.t. application. Nevertheless, it remains unclear whether RA in the i.t. space is able to penetrate deep into the spinal parenchyma. Neuroprotective effects of higher local amounts of RA have been reported for the brain. Treatment with a dose of 20 μ g injected into the ventricles reduced infarct volume after experimental stroke (Harvey *et al.* 2004), however this application method does not involve treatment with soluble RA and may cause precipitation of RA in the ventricles. When higher doses of intrathecal RA are tested in the future, it is therefore also important to evaluate different vehicle solutions that are more suitable for the transport of hydrophobic compounds like RA.

Systemic administration of RA reduces transcript levels of pro-inflammatory cytokines

In contrast to i.t. delivery of RA up to doses of 100 ng, a single systemic injection of 15 mg/kg body weight did reduce transcript levels of pro-inflammatory cytokines at 6 h after SCI. Both IL-1 β and TNF α expression were significantly reduced, while IL-6 was not significantly attenuated. These data indicate that systemic application is a more effective administration route for RA to achieve an anti-inflammatory effect when compared to local application after 6 h. Importantly, it indicates that RA crosses the blood-brain barrier, as reported

previously (Luo *et al.* 2004; Werner & Deluca 2002). Enhanced expression of RAR β in the present study provides further evidence that intraperitoneal injection of RA at a dose of 15 mg/kg bodyweight results in RA signalling at the level of the spinal cord. It is likely that systemic RA more easily penetrates the spinal cord parenchyma and then reduces SCI-induced transcription of pro-inflammatory cytokines.

Systemic applications are, however, associated with adverse side effects, especially when high doses of RA are applied. Acute overdose of retinoids leads to a condition called hypervitaminosis A, which occurs when liver stores are exceeded. As a consequence RA up-take by the liver is compromised leading to fatal toxic effects. This acute intoxication leads to nausea, headache, blurred vision, osteoporosis, disturbed muscle coordination and other disorders (Hathcock *et al.* 1990). Despite its adverse side effects, RA is already used in treatments of several clinical conditions. Firstly, RA is used in treatment of cancer because it inhibits proliferation of immature leukemic promyelocytes and it induces differentiation of these cells into mature granulocytes. As such, it is the foremost effective treatment of acute promyelocytic leukemia (APL). Retinoic acid syndrome (RAS) is the most frequently reported intoxication-condition in APL patients treated with RA (Patatanian & Thompson 2008). Secondly, severe acne or psoriasis has been treated for decades using systemically applied 13-*cis* RA (iso-tretinoin). These applications often include long-term intake of RA, which in some cases has been associated with major depression disorder (Hull & D'Arcy 2003). Larger patient studies or experimental studies however, could not find a clear link between retinoid use and major depression disorder (van Neerven *et al.* 2008). In the present study we focused on acute RA treatment of SCI and therefore adverse side effects typically caused by chronic treatment are unlikely to occur. Nevertheless, it has to be mentioned that RA precipitates (yellowish substances) were found in the abdominal cavity at 6 h after injection of 15 mg/kg bodyweight RA i.p. Hence, other methods to administer RA systemically, like oral treatment or intravenous injections should be considered to avoid such side effects.

Anti-inflammatory effects of RA have been described previously for blood-borne cells including macrophages, neutrophils, and T-cells (Kim *et al.* 2004; Mucida *et al.* 2007; Na *et al.* 1999; Camisa *et al.* 1982). In the last decade, RA was also found to reduce the expression of inflammatory mediators in cultures of CNS cells including microglia and astroglia triggered by lipopolysaccharide (LPS) (Dheen *et al.* 2005; van Neerven *et al.* 2010; Xu & Drew 2006; Kampmann *et al.* 2008). Recent investigations showed the potential use of RA in treatment of CNS pathologies. RA was found to reduce infarct size after cerebral ischemia (Harvey *et al.* 2004; Shen *et al.* 2009; Sato *et al.* 2008), which was accompanied by reduced IL-1 β expression (Choi *et al.* 2009). In experimental spinal cord injury, endogenous RA signalling was reported to occur after the first weeks (Schrage *et al.* 2006), which partly coincides with reduced levels of pro-inflammatory cytokines. Here we show for the first time that RA inhibits SCI-induced rises in levels of pro-inflammatory cytokine mRNA at 6 h after injury when administered systemically. Importantly, it is mainly these early ris-

es in cytokine expression which are at the basis of SCI-inflammation exacerbating tissue damage and functional loss. Indeed, a recent investigation showed systemic administration of minocycline, a potent inhibitor of cytokine production (Raghavendra *et al.* 2003), attenuated SCI-induced mechanical and thermal hypersensitivity. Importantly, reduction of hypersensitivity was observed when minocycline was administered within the first two days following SCI, but not when treatment was started at two weeks following SCI (Marchand *et al.* 2009). Future studies are now needed to investigate whether the RA-related reduction of cytokine levels at 6 h after SCI have beneficial effects on long-term SCI outcome. If so, then RA may be a highly interesting therapeutic agent in early treatment of SCI. Although adverse side effects are associated with RA, such effects may be limited by a limited use in the early phase after SCI.

Acknowledgements

The authors are grateful to the Dutch Society for Anesthesiology (NVA) for their financial support of this study via the Young Investigator Grant (to R.D.). This work was also supported by a FP6- Marie Curie Early Stage Training Site grant number MEST-CT-2005-020013 (Euron consortium) (to J.M.).

References

- Balmer, J. E. and Blomhoff, R. (2002) Gene expression regulation by retinoic acid. *J Lipid Res*, **43**, 1773–1808
- Camisa, C., Eisenstat, B., Ragaz, A. and Weissmann, G. (1982) The effects of retinoids on neutrophil functions *in vitro*. *J Am Acad Dermatol*, **6**, 620–629
- Choi, B., Kim, J., Jung, J., Lee, Y., Han, M., Baek, S., Kim, B., Kim, J. and Oh, S. (2009) - Reduction of ischemia-induced cerebral injury by all-*trans*-retinoic acid. *Exp Brain Res*, **193**, 581–589
- Dheen, S., Jun, Y., Yan, Z., Tay, S. and Ling, E. (2005) - Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat. *Glia*, **50**, 21–31
- Donnelly, D. and Popovich, P. (2008) - Inflammation and its role in neuroprotection, axonal regeneration and functional. *Exp Neurol*, **209**, 378–388
- Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Muia, C., Esposito, E., Bramanti, P. and Cuzzocrea, S. (2008) TNF-alpha blockage in a mouse model of SCI: evidence for improved outcome. *Shock*, **29**, 32–41
- Gruner, J. A. (1992) A monitored contusion model of spinal cord injury in the rat. *J Neurotrauma*, **9**, 123–126; discussion 126–128
- Hanson, N. and Leachman, S. (2001) Safety issues in isotretinoin therapy. *Semin Cutan Med Surg*, **20**, 166–183
- Harvey, B., Shen, h., Chen, G., Yoshida, Y. and Wang, Y. (2004) - Midkine and retinoic acid reduce cerebral infarction induced by middle cerebral. *Neurosci Lett*, **369**, 138–141
- Hathcock, J. N., Hattan, D. G., Jenkins, M. Y., McDonald, J. T., Sundaresan, P. R. and Wilkening, V. L. (1990) Evaluation of vitamin A toxicity. *Am J Clin Nutr*, **52**, 183–202
- Hull, P. R. and D'Arcy, C. (2003) Isotretinoin use and subsequent depression and suicide: presenting the evidence. *Am J Clin Dermatol*, **4**, 493–505
- Kampmann, E., Johann, S., van Neerven, S., Beyer, C. and Mey, J. (2008) Anti-inflammatory effect of retinoic acid on prostaglandin synthesis in cultured cortical astrocytes. *J Neurochem*, **106**, 320–332
- Kim, B. h., Kang, K. S. and Lee, Y. S. (2004) Effect of retinoids on LPS-induced COX-2 expression and COX-2 associated PGE(2) release from mouse peritoneal macrophages and TNF-alpha release from rat peripheral blood mononuclear cells. *Toxicol Lett*, **150**, 191–201
- Luo, T., Wagner, E., Grun, F. and Drager, U. C. (2004) Retinoic acid signaling in the brain marks formation of optic projections, maturation of the dorsal telencephalon, and function of limbic sites. *J Comp Neurol*, **470**, 297–316
- Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A. K., Bradbury, E. J. and McMahon, S. B. (2009) Effects of Etanercept and minocycline in a rat model of spinal cord injury. *Eur J Pain*, **13**, 673–681
- Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M. and Cheroutre, h. (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*, **317**, 256–260
- Na, S., Kang, B., Chung, S., Han, S., Ma, X., Trinchieri, G., Im, S., Lee, J. and Kim, T. (1999) - Retinoids inhibit interleukin-12 production in macrophages through physical. *J Biol Chem*, **274**, 7674–7680

- Park, S. W., Yi, J. h., Miranpuri, G., Satriotomo, I., Bowen, K., Resnick, D. K. and Vemuganti, R. (2007) Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther*, **320**, 1002–1012
- Patatanian, E. and Thompson, D. F. (2008) Retinoic acid syndrome: a review. *J Clin Pharm Ther*, **33**, 331–338
- Paterniti, I., Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Galuppo, M., Bramanti, P. and Cuzzocrea, S. (2010) Liver X receptor agonist treatment regulates inflammatory response after spinal cord trauma. *J Neurochem*, **112**, 611–624
- Raghavendra, V., Tanga, F. and DeLeo, J. A. (2003) Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy. *J Pharmacol Exp Ther*, **306**, 624–630
- Sato, Y., Meller, R., Yang, T., Taki, W. and Simon, R. (2008) Stereo-selective neuroprotection against stroke with vitamin A derivatives. *Brain Res*, **19**, 188–192
- Schrage, K., Koopmans, G., Joosten, E. A. and Mey, J. (2006) Macrophages and neurons are targets of retinoic acid signaling after spinal cord contusion injury. *Eur J Neurosci*, **23**, 285–295
- Shah, P. and Mudaliar, S. (2010) Pioglitazone: side effect and safety profile. *Expert Opin Drug Saf*, **9**, 347–354
- Shen, h., Luo, Y., Kuo, C., Deng, X., Chang, C., Harvey, B., Hoffer, B. and Wang, Y. (2009) - 9-Cis-retinoic acid reduces ischemic brain injury in rodents via bone. *J Neurosci Res*, **87**, 545–555
- Streit, W. J., Semple-Rowland, S. L., Hurley, S. D., Miller, R. C., Popovich, P. G. and Stokes, B. T. (1998) Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. *Exp Neurol*, **152**, 74–87
- Suh, D. Y., Hunt, T. K. and Spencer, E. M. (1992) Insulin-like growth factor-I reverses the impairment of wound healing induced by corticosteroids in rats. *Endocrinology*, **131**, 2399–2403
- Tsai, M. C., Wei, C. P., Lee, D. Y., Tseng, Y. T., Tsai, M. D., Shih, Y. L., Lee, Y. h., Chang, S. F. and Leu, S. J. (2008) Inflammatory mediators of cerebrospinal fluid from patients with spinal cord injury. *Surg Neurol*, **70 Suppl 1**, S1:19–24; discussion S11:24
- van Neerven, S., Kampmann, E. and Mey, J. (2008) RAR/RXR and PPAR/RXR signaling in neurological and psychiatric diseases. *Prog Neurobiol*, **85**, 433–451
- van Neerven, S., Regen, T., Wolf, D., Nemes, A., Johann, S., Beyer, C., Hanisch, U. K. and Mey, J. (2010) Inflammatory chemokine release of astrocytes *in vitro* is reduced by all-*trans* retinoic acid. *J Neurochem*
- Werner, E. A. and Deluca, h. F. (2002) Retinoic acid is detected at relatively high levels in the CNS of adult rats. *Am J Physiol Endocrinol Metab*, **282**, E672–678
- Xu, J. and Drew, P. (2006) 9-Cis-retinoic acid suppresses inflammatory responses of microglia and. *J Neuroimmunol*, **171**, 135–144

Liver X receptor activation does not attenuate early pro-inflammatory cytokine gene expression after experimental spinal cord injury

**S.G.A. van Neerven¹⁻⁴ a, R.J.P. Jaken^{1,3} a, E.A. Joosten^{1,3},
H.W.M. Steinbusch^{2,3}, M. van Kleef¹, J. Mey^{3,4}, M. Mulder⁵,
P. Martinez-Martinez^{2,3}, M. Losen^{2,3},
M. De Baets^{2,3}, M.A.E. Marcus¹, R. Deumens^{1,3}**

¹Department of Anesthesiology, Maastricht University Medical Center,
Maastricht, The Netherlands

²Department of Neuroscience, Maastricht University,
Maastricht, The Netherlands

³EURON European Graduate School of Neuroscience,
Maastricht, The Netherlands

⁴Institute of Biology II, RWTH Aachen University, Aachen, Germany

⁵Department of Internal Medicine, Erasmus Medical Center,
Rotterdam, The Netherlands

a – these authors contributed equally to the present work
and are therefore joint first-authors

Abstract

Early pro-inflammatory cytokine levels play a crucial role in initiating and maintaining spinal cord injury (SCI)-induced inflammation and thereby have detrimental consequences on functional outcome. Therapeutics that reduce such early cytokine levels hold great promise in treatment of acute SCI and the combinatorial use of such agents were reported to render synergistic therapeutic effects. Such synergism occurs with high probability when the agents have differential mechanisms-of-action. T0901317 (T09), a synthetic agonist of the liver-X-receptor (LXR) has recently been reported to potently reduce protein levels of early SCI-induced cytokine levels. We, here, show that T09 does not affect gene expression of pro-inflammatory cytokines, which are known to peak within 6 h after contusion injury to the rat spinal cord. Moreover, pro-inflammatory cytokine transcripts in LPS-stimulated astrocytes were not attenuated in the presence of T09. Our data suggest that LXR agonism does not interfere with early posttraumatic gene regulation of pro-inflammatory cytokines in the spinal cord. Hence, T09 may be a particularly interesting drug candidate to use in combination with medicinal agents acting on the transcriptional level of early SCI-induced cytokines in treatment of acute SCI.

Keywords: inflammation, cytokines, nuclear hormone receptor, T0901317, neurotrauma

Introduction

Pro-inflammatory cytokines play a crucial role in spinal cord injury (SCI)-induced inflammation and thereby contribute to the detrimental consequences of SCI including motor deficits and neuropathic pain (Popovich & Jones 2003; Fleming *et al.* 2006, Deumens *et al.* 2008; Marchand *et al.* 2009). As such, pharmacological attenuation of early pro-inflammatory cytokine expression holds great promise in acute SCI treatment. Use of the nuclear hormone receptor (NHR) agonist methylprednisolone, still belonging to the only recognized SCI treatment options (Bracken *et al.* 1990), reduces early pro-inflammatory cytokine levels in both human and experimental SCI (Fu & Saporta 2005; Tsai *et al.* 2008). Combinations of multiple therapeutic agents can be more potent in reducing early pro-inflammatory cytokine expression and SCI outcome than the use of a single one (Koopmans *et al.* 2009). Synergistic therapeutic effects are most likely obtained when the agents have different mechanisms-of-action. Reduced gene expression is the most frequently described mechanism-of-action of immune-suppressive agents (Barnes 1998; Adcock 2001; Liberman *et al.* 2007a; Liberman *et al.* 2007b; Jee *et al.* 2005). Such agents are then optimally combined with medicine activating non-transcriptional mechanisms to reduce early SCI-induced levels of pro-inflammatory cytokines.

NHR have received increasing attention as therapeutic targets in treatment of acute experimental SCI. Transcription of pro-inflammatory cytokines has been effectively attenuated by activation of NHR including peroxisome proliferator-activated receptors (PPAR), retinoic acid receptors (RAR/RXR), and the glucocorticoid receptor (Park *et al.* 2007; van Neerven & Mey 2007; van Neerven *et al.* 2008; van Neerven *et al.* 2010). Activation of the liver-X-receptor (LXR) by use of the synthetic LXR agonist T0901317 (T09) has recently been reported to reduce protein levels of pro-inflammatory cytokines at 24 h following spinal cord compression in the rat (Paterniti *et al.* 2010). Nevertheless, the mechanism-of-action of T09 remains unknown to date. We, here, investigated whether T09 acts at the transcriptional level of pro-inflammatory cytokines early after experimental SCI. Since gene expression of pro-inflammatory cytokines such as tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) peak as early as 6 h following spinal cord contusion injury in the rat (Streit *et al.* 1998; Donnelly & Popovich 2008), this time point was focused at. Moreover, the effect of T09 on TNF α , IL-1 β , and IL-6 transcripts was investigated *in vitro* using LPS-stimulated astrocytes.

Materials and Methods

Procedures were approved by the ethical committee of Maastricht University and carried out according to the recommendations of the European Commission (European Communities Council Directive of 24 November 1986; 86/609/EEC). Twelve weeks old male

Wistar rats (*Charles River, Maastricht, The Netherlands*) weighing approximately 250 g, were housed under standard housing conditions, including 12:12 h light/dark regime and *ad libitum* access to water and standard laboratory chow.

Animals were randomly divided into 6 experimental groups: 1: laminectomy (LAM) and no SCI (n = 4), 2: SCI and vehicle treatment (SCI, n = 4), 3, 4, 5: SCI plus T09 treatment in doses of 10, 20, 30 mg/kg bodyweight, respectively (SCI + T09, all groups n = 4) and 6: 20 mg/kg bodyweight T09 6 h prior to the injury, followed by SCI and T09 (also 20 mg/kg bodyweight, SCI + 20+20 T09). SCI was inflicted using the New York University Impactor (MASICS device) by a 10-g weight drop from a distance of 12.5 mm onto the exposed dorsal surface of the T9 spinal cord as described previously (Gruner 1992). T09 or vehicle solution was given intraperitoneally. Post-operative care included subcutaneous administration of 5 mL saline to compensate for fluid loss during surgery and 5 mg/kg bodyweight Baytril as anti-biotic treatment.

Immediately after SCI, animals were treated with the LXR agonist T0901317 (T09; *Cayman Chemical Europe, Estonia*) freshly diluted in 1% methylcellulose, 2% Tween-80 en 5% ethanol. Animals were sacrificed at 6 h following the last T09 treatment by an overdose of pentobarbital (180 mg/kg bodyweight i.p.) and were transcardially perfused with ice-cold saline. This time point of sacrifice was selected on the basis of previous work showing that SCI-induced levels in pro-inflammatory cytokine transcripts peak at this time point (Streit *et al.* 1998; Donnelly & Popovich 2008). After saline perfusion, meninges were carefully removed and a 1 cm-long spinal segment containing the center of the lesion was snap-frozen in liquid nitrogen. Tissues were kept at -80° C until used for further processing.

Primary cultures of astrocytes were prepared from cerebral cortices of 1 day old Balb/c mice (*Charles River, Sulzfeld, Germany*). After dissection and removal of the meninges, both hemispheres were dissociated mechanically in 0.25% Trypsin-EDTA solution (*Sigma-Aldrich, Schnelldorf, Germany*), passed through a 50 µm nylon mesh, and centrifuged at 130 × g for 10 min. Cells were resuspended and further cultivated in medium containing Dulbecco's modified Eagle medium including 20% heat-inactivated fetal calf serum, 0.5% penicillin/streptavidin and 0.1% fungizone (all from *Invitrogen, Karlsruhe, Germany*) and subsequently seeded in T75 flasks coated with poly-L-lysine (200 µg/mL, *Sigma-Aldrich, Schnelldorf, Germany*). Cells were grown for 5 days at 37° C in a humidified incubator with 5% CO₂, and medium was changed every 2–3 days. When reaching confluence, cells were passaged three times to remove microglia, yielding primary astrocyte cultures of > 95% purity as previously described (van Neerven *et al.* 2010). After the third passage, astrocytes were seeded into poly-L-lysine coated 6-wells plates at a density of approximately 500.000 cells/well. Before treatment, astrocytes were kept for 48 h in serum-free neurobasal medium (NBM) supplemented with 1% glutamine, 0.2% B27 supplement (both from *Gibco, Invitrogen, Karlsruhe, Germany*), 0.5% penicillin/streptavidin

and 0.1% fungizone. To induce an inflammatory reaction 100 ng/mL *Escherichia coli* LPS (*Sigma-Aldrich, Schnelldorf, Germany*) were applied to the culture medium. T09 was diluted in 100% DMSO in stock solutions of 10 mM, air above aliquots was removed with N₂ to prevent oxidation and stock solution was stored at -20° C until use. T09 stock solution was freshly diluted prior to the experiment in 100% ethanol to concentrations of 1 mM, 100 µM, 10 µM and 1 µM. T09 was then further diluted in NBM medium reaching a final concentration of 2 µM, 200 nM, 20 nM and 2 nM. Cells were treated according to different experimental paradigms including n = 5 wells for every treatment condition: Primary astrocytes were either exposed to LPS and T09 simultaneously for 12 h, or cells were pre-incubated with T09 at 12 h prior to the LPS induced inflammation and subsequently cells were exposed to LPS plus T09 for another 12 h. After completion of the experiment medium was collected and cytotoxicity of treatment conditions was assessed by measuring lactate dehydrogenase (LDH) release into the medium using Cytotoxicity Detection Kit (*Roche, Mannheim, Germany*). LDH release was normalized with respect to maximal LDH release (i.e. after cell lysis).

Tissues and cells were used for quantitative RT-PCR analysis and therefore isolation of total RNA was performed with the phenol-chloroform method. Tissues were homogenized in Trizol (*Invitrogen, Breda, The Netherlands*) using glass beads and for cell culture experiments, medium was aspirated and 1 mL Trizol was added to the well. RNA concentration and purity were assessed photometrically (OD260, OD260/OD280 ratio). Total RNA was reverse transcribed into cDNA using *Biorad iScript cDNA kit* with calculated concentrations of 50 ng/µL RNA extract per reaction. Samples were diluted 1/10 after reverse transcription and then used for amplification with the *Roche LightCycler® 480 Real-Time PCR System*. Reactions were carried out in a 96-well reaction module, containing 20 µL total reaction volume per well. Samples contained 10 µL of the *Roche SYBR Green PCR kit*, 7 µL nuclease-free water, 2 µL of the oligo mix containing 5 µM of each sense and anti-sense primer and 1 µL cDNA sample. The PCR protocol consisted of 5 min enzyme activation at 95° C, followed by 45 cycles of 20 s denaturation at 95° C, 30 s annealing at the primers specific temperature and 23 s elongation and fluorescence measurement at 72° C. Expression of the housekeeping gene HPRT or GAPDH was used to normalize gene expression. GAPDH was the most stable housekeeping gene in SCI experiments and HPRT after LPS treatment in cell cultures. Melting curve analysis and agarose gel electrophoresis of the PCR products were routinely performed, to determine primer specificity. Primer sequences and annealing temperatures for mouse and rat are listed in Table 1 and 2 respectively. Relative mRNA concentrations were determined using crossing point analysis of log/linear plots of fluorescence/cycle number. To determine the PCR efficiency, concentration series (dilutions 1/2, 1/4,... 1/128) of sample pools were performed once for every primer pair. All primer pairs showed efficiencies around 2.0 and standard curves had slopes between 3.1–3.6.

Table 1: Mouse primers used in this study

Name	Primer sequences 5' → 3' sense antisense	Annealing temperature	Product size Product Tm
HPRT	GCTGGTGAAGGACCTCT CACAGGACTAGAACACCTGC	62	248 79
IL-1 β	CAGCTCATATGGGTCGACA CTGTGTCTTCCCGTGGACC	59	200 82
IL-6	GTTCTCTGGGAAATCGTGA CTCTGAAGGACTCTGGCTTTG	60	260 78
TNF α	ATGGCCTCCCTCTCATCAGT CTCCTCCACTTGGTGGTTG	59	113 80

Table 2: Rat primers used in this study

Name	Primer sequences 5' → 3' sense antisense	Annealing temperature	Product size Product Tm
ABCA1	CCCAGAGCAAAAGCGACTC GGTCATCATCACTTGGTCCTTG	60	102 85
GAPDH	TGCCAAGTATGATGACATCAAGAAG AGCCCAGGATGCCCTTAGT	55	80 86
IL-1 β	CACCTTCTTTTCCTTCATCTTTG GTCGTTGCTTGTCTCTCCTTGTA	55	241 82
IL-6	AAGTTTCTCTCCGAAGAGACTTCCAG AGGCAAATTCCTGTTATATCCAGTT	55	326 81
LXR α	GCTCTGCTCATAGCCATCAG CAGGGCCTCCACATATGTGT	60	99 81
LXR β	AAGGACTTCACCTACAGCAAGGA GAGAACTCAAAGATGGGATTGATGA	60	80 78
TNF α	CCCAGACCCTCACTCAGAT TTGTCCTTGAAGAGAACCTG	55	215 85

Statistical analysis was performed using Graphpad Prism 5 software. Graphs represent means with standard error of the mean. Data showed normal distribution and were analyzed using one-way ANOVA with *post-hoc* Tukey test. *p* values < 0.05 were considered as statistically significant. # represents statistical significance between SCI and LAM (Control), whereas * indicates statistical significance between SCI and SCI+T09; #/* represents a *p* value < 0.05, ##/** *p* value < 0.01 and ###/*** *p* value < 0.001.

Results

In this study T09 was administered immediately after the lesion and pro-inflammatory cytokine mRNA expression levels were investigated at 6 h after SCI (Fig. 1). Dose-response measurements of the cholesterol transporter ABCA1, a gene known to be driven by LXR activation, demonstrated that T09 induced LXR signaling in the spinal cord (Fig. 1A). This was represented by an averaged 6-fold increase in the expression of this gene by the various doses of T09. Expression of LXR genes LXR α and LXR β was neither affected by SCI nor by SCI+T09 treatments (Fig. 1b, c). Uninjured animals expressed low levels of IL-1 β , IL-6 and TNF α transcripts. At 6 h after SCI, 10-fold, 13-fold and 2-fold increases of IL-1 β , TNF α and IL-6 mRNA expression were detected, respectively (Fig. 1d-f). T09 treatment at any concentration was unable to reduce gene expression of these cytokines. Five animals received pre-treatment with T09 6 h prior to the lesion, and were again treated with T09 immediately after the lesion. Pre-treatment did not affect SCI-induced cytokine gene expression at 6 h.

T09 was then tested for its effect on pro-inflammatory cytokine gene expression in primary astrocyte cultures (Fig. 2). Cells were treated with LPS to elicit an inflammatory reaction; doses from 2 nM to 2 μ M T09 were used in simultaneous and pre-treatment protocols. To assess toxicity of treatment conditions, LDH release into the medium was determined. Photometric measurements and comparison to total cell lysis high control (HC) revealed that concentrations of T09 varying from 2 nM – 200 nM were non-toxic to cells, whereas a concentration of 2 μ M induced cell death and was therefore not used in further experiments (Fig. 2a).

Under control conditions (untreated cells (BL) and cells that received vehicle treatment (C)) astrocytes expressed low levels of IL-1 β , IL6 and TNF α mRNA. T09 by itself did not induce pro-inflammatory cytokine gene expression, while LPS treatment induced a strong and statistically significant increase in cytokine transcript levels. This increase was almost 100-fold, 8-fold and 37-fold for IL-1 β , IL-6 and TNF α , respectively ($p < 0.01$). Simultaneous treatment of LPS and T09 for 12 h did not reduce the gene expression of these pro-inflammatory cytokines in primary astrocyte cultures. Surprisingly a concentration of 2 nM T09 increased transcript levels of IL-1 β . Also, 12 h pre-treatment with T09 followed by simultaneous treatment with LPS plus T09 for another 12 h, did not attenuate cytokine transcript levels. At a concentration of 2 nM T09, gene expression was significantly increased for cytokines IL-1 β and IL-6 (Fig. 2b-d).

Fig. 1. T09 treatment after SCI. (a) Expression of the LXR driven gene ABCA1 was strongly increased after T09 treatment, indicative of positive LXR signaling in the injured spinal cord at 6 h after systemic administration. This effect was present at all concentrations tested, although the effect was slightly reduced for the highest dose administered. (b, c). SCI or SCI+T09 treatment (20 mg/kg bodyweight) did not alter gene expression of receptors LXR α or LXR β (d-f). IL-1 β , IL-6 and TNF α mRNA expression was strongly increased at 6 h after SCI. Treatment with T09 did not reduce transcript levels of pro-inflammatory cytokines after SCI. Moreover, repetitive treatment with T09, where animals received an injection 6 h prior to SCI and immediately after the lesion, did not affect pro-inflammatory cytokine gene expression.

LAM – laminectomy only (Control), SCI – spinal cord injury, SCI+T09 – spinal cord injury + T09 treatment, SCI+T0920+20 – pre-treatment -6 h with T09 plus spinal cord injury plus T09 treatment

Fig. 2. Primary astrocytes stimulated with LPS and treated with T09. (a) LDH was determined to measure toxicity of cell treatment. High control (HC) represents total cell lysis and maximal LDH release. Only a concentration of 2 μ M T09 was toxic to primary astrocytes shown by an increase in LDH release into the medium. (b-d) Without any treatment astrocytes express very low amounts of pro-inflammatory cytokine mRNA, whereas LPS treatment induced a strong increase of IL-1 β , IL-6 and TNF α transcripts. T09 alone did not induce pro-inflammatory cytokine gene expression, but in combination with LPS, a concentration of 2 nM T09 increased pro-inflammatory cytokine transcript level of IL-1 β . This same pattern was observed in experiments including pre-incubation, where a concentration of 2 nM T09 additionally increased IL-6 expression. BL – Blanco, no treatment, C – Control, vehicle treatment, LPS – Lipopolysaccharides T09 – T09 only, simultaneous LPS+T09 – Lipopolysaccharides plus T09 treatment 12 h, pre-incubation LPS+T09 – Pre-treatment -12 h T09 plus lipopolysaccharides plus T09 12 h, HC – High control, total cell lysis

Discussion

In this study we investigated whether LXR activation leads to a reduction of pro-inflammatory cytokine gene transcription at 6 h after moderate contusion injury of the thoracic rat spinal cord. Our data show that T09 treatment does not reduce such early SCI-induced pro-inflammatory cytokine transcript levels. Moreover, T09 treatment of LPS-stimulated astrocytes did not reduce pro-inflammatory cytokine transcripts, but in a dose of 2 nM even triggered higher expression levels.

Our findings might have important implications on the use of LXR in treatment of early SCI. Early rises in pro-inflammatory cytokines proteins characterize the detrimental nature of neuroinflammatory processes induced by SCI (Popovich & Jones 2003). A large number of immune-modulatory agents have been proposed in early treatment of SCI (Bethea *et al.* 1999; Bethea & Dietrich 2002; Demjen *et al.* 2004; Baptiste & Fehlings 2006; Alexander & Popovich 2009), which in itself already carries the message that the early SCI-inflammatory response is not effectively attenuated by a single drug. As such, combinatorial treatments using multiple drugs may act synergistically to more effectively reduce pro-inflammatory cytokine proteins in the early phases following SCI (Koopmans *et al.* 2009). LXR activation by use of T09 has recently been reported to be effective in reducing pro-inflammatory cytokine levels and render improved outcome in an experimental model of stroke (Morales *et al.* 2008; Sironi *et al.* 2008; Cheng *et al.* 2010) and SCI (Paterniti *et al.* 2010). Intraperitoneal injections of T09 (20 mg/kg bodyweight) at 1 h and 6 h after rat SCI reduced TNF α and IL-1 β protein levels at 24 h after injury, increased the amount of spared tissue, reduced apoptosis and substantially improved motor outcome (Paterniti *et al.* 2010). The mechanisms-of-action by which LXR activation reduces such early SCI-induced pro-inflammatory cytokine levels remain, however, unknown. We here show that LXR activation by use of T09 up to systemic doses of 30 mg/kg bodyweight does not affect pro-inflammatory cytokine transcripts at 6 h after SCI in rats. Therefore, T09 treatment may be successfully combined with treatments acting on early SCI-induced pro-inflammatory cytokine transcripts. One particularly interesting candidate agent involves the PPAR γ agonist pioglitazone and *all-trans* retinoic acid, which were found to effectively inhibit pro-inflammatory cytokine gene expression at 6 h after SCI (Park *et al.* 2007, van Neerven *et al.*, submitted work).

Our finding that LXR activation did not reduce early pro-inflammatory cytokine gene expression in the setting of *in vitro* LPS-stimulated astrocyte cultures, was surprising. Other groups already showed that LXR activation was able to reduce cytokine mRNA and protein levels in primary astrocyte (Lee *et al.* 2009) and microglial (Kim *et al.* 2006; Zhang-Gandhi & Drew 2007) cultures. Nevertheless, these effects were observed using doses of T09 varying from 2.5 μ M to 20 μ M, which are substantially higher than the ones used in our study. We showed that 2 μ M T09 already caused LDH release into the medium of astrocyte cultures, indicative of cell toxicity. For that reason, doses no higher than 200 nM

were used in the present investigations. Higher doses of T09 may be effective in reducing cytokine expression, but increasing cell toxicity may obscure possible anti-inflammatory effects. In the setting of SCI, systemically administered T09 was found to induce transcription of the LXR-driven gene ABCA1 at 6 h after injury, indicative of T09 crossing the blood spinal cord barrier and inducing LXR signaling at the transcriptional level. Nevertheless, transcripts of pro-inflammatory cytokines were found unaffected at 6 h after SCI. Therefore, it may be reasoned that either the timing or treatment paradigm (single administration rather than repetitive administration) was not optimal or that LXR agonism triggers other, non-transcriptional-related mechanisms. In cell culture studies, we have recently observed that the RAR/RXR agonist retinoic acid also had stronger effects on the protein level than on gene transcription of IL-1 β (van Neerven, 2010 in press).

Post-transcriptional effects, like effects on RNA stability and translation of gene transcripts to proteins are poorly described mechanisms of NHR action. Retinoid receptors RAR/RXR, as members of the NHR family, and their natural ligand all-*trans* retinoic acid (RA) have been found to induce spine formation in hippocampal neurons via a membrane-bound receptor and a mitogen-activated-protein-kinase (MAPK)-dependent pathway. This MAPK-mediated RA effect was maintained in the presence of the transcription inhibitor actinomycin, but not in the presence of the translation inhibitor anisomycin (Chen & Napoli 2008). Hence, NHR such as RAR/RXR may have post-transcriptional mechanisms-of-action. Furthermore, RA has been reported to affect mRNA stability. Degradation of LPS-induced TNF α transcripts from macrophages was found to be significantly enhanced following RA treatment (Motomura *et al.* 2001). Although such post-transcriptional effects are reported for RA-members of NHR, it remains unclear whether such effects are also triggered by LXR.

In conclusion, we here show that LXR agonism using the synthetic molecule T09 does not affect pro-inflammatory cytokine gene expression at 6 h following rat spinal cord injury. Further *in vitro* data indicate that T09 does not reduce pro-inflammatory cytokine transcripts early after LPS-stimulation of astrocyte cultures. Hence, beneficial effects of LXR on early inflammatory processes related to the CNS are unlikely to involve alterations in gene transcription. Future experiments, focusing on different treatment time points after SCI or on non-transcriptional mechanisms of action, such as post-transcriptional effects, may shed more light on the nature of anti-inflammatory effects of LXR activation.

Acknowledgements

The authors are grateful to the Dutch Society for Anesthesiology (NVA) for their financial support via the Young Investigator Grant (to R.D.). This study was financially supported by a FP-6 Marie Curie Early Stage Training Site; grant number MEST-CT-2005-020013 (Euron Consortium; grant to J.M.).

References

- Adcock, I. M. (2001) Glucocorticoid-regulated transcription factors. *Pulm Pharmacol Ther*, **14**, 211–219
- Alexander, J. K. and Popovich, P. G. (2009) Neuroinflammation in spinal cord injury: therapeutic targets for neuroprotection and regeneration. *Prog Brain Res*, **175**, 125–137
- Baptiste, D. C. and Fehlings, M. G. (2006) Pharmacological approaches to repair the injured spinal cord. *J Neurotrauma*, **23**, 318–334
- Barnes, P. J. (1998) Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)*, **94**, 557–572
- Bethea, J. R. and Dietrich, W. D. (2002) Targeting the host inflammatory response in traumatic spinal cord injury. *Curr Opin Neurol*, **15**, 355–360
- Bethea, J. R., Nagashima, h., Acosta, M. C., Briceno, C., Gomez, F., Marcillo, A. E., Loor, K., Green, J. and Dietrich, W. D. (1999) Systemically administered interleukin-10 reduces tumor necrosis factor- α production and significantly improves functional recovery following traumatic spinal cord injury in rats. *J Neurotrauma*, **16**, 851–863
- Bracken, M. B., Shepard, M. J., Collins, W. F. *et al.* (1990) A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med*, **322**, 1405–1411
- Chen, N. and Napoli, J. L. (2008) All-*trans*-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RAR α . *FASEB J*, **22**, 236–245
- Cheng, O., Ostrowski, R. P., Liu, W. and Zhang, J. h. (2010) Activation of liver X receptor reduces global ischemic brain injury by reduction of nuclear factor- κ B. *Neuroscience*, **166**, 1101–1109
- Demjen, D., Klusmann, S., Kleber, S. *et al.* (2004) Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. *Nat Med*, **10**, 389–395
- Deumens, R., Joosten, E. A., Waxman, S. G. and Hains, B. C. (2008) Locomotor dysfunction and pain: the scylla and charybdis of fiber sprouting after spinal cord injury. *Mol Neurobiol*, **37**, 52–63
- Donnelly, D. J. and Popovich, P. G. (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol*, **209**, 378–388
- Fleming, J. C., Norenberg, M. D., Ramsay, D. A., Dekaban, G. A., Marcillo, A. E., Saenz, A. D., Pasquale-Styles, M., Dietrich, W. D. and Weaver, L. C. (2006) The cellular inflammatory response in human spinal cords after injury. *Brain*, **129**, 3249–3269
- Fu, E. S. and Saporita, S. (2005) Methylprednisolone inhibits production of interleukin-1 β and interleukin-6 in the spinal cord following compression injury in rats. *J Neurosurg Anesthesiol*, **17**, 82–85
- Gruner, J. A. (1992) A monitored contusion model of spinal cord injury in the rat. *J Neurotrauma*, **9**, 123–126; discussion 126–128
- Jee, Y. K., Gilmour, J., Kelly, A. *et al.* (2005) Repression of interleukin-5 transcription by the glucocorticoid receptor targets GATA3 signaling and involves histone deacetylase recruitment. *J Biol Chem*, **280**, 23243–23250

- Kim, O. S., Lee, C. S., Joe, E. h. and Jou, I. (2006) Oxidized low density lipoprotein suppresses lipopolysaccharide-induced inflammatory responses in microglia: oxidative stress acts through control of inflammation. *Biochem Biophys Res Commun*, **342**, 9–18
- Koopmans, G. C., Deumens, R., Buss, A. *et al.* (2009) Acute rolipram/thalidomide treatment improves tissue sparing and locomotion after experimental spinal cord injury. *Exp Neurol*, **216**, 490–498
- Lee, J. h., Park, S. M., Kim, O. S., Lee, C. S., Woo, J. h., Park, S. J., Joe, E. h. and Jou, I. (2009) Differential SUMOylation of LXRalpha and LXRbeta mediates transrepression of STAT1 inflammatory signaling in IFN-gamma-stimulated brain astrocytes. *Mol Cell*, **35**, 806–817
- Liberman, A. C., Druker, J., Perone, M. J. and Arzt, E. (2007a) Glucocorticoids in the regulation of transcription factors that control cytokine synthesis. *Cytokine Growth Factor Rev*, **18**, 45–56
- Liberman, A. C., Refojo, D., Druker, J., Toscano, M., Rein, T., Holsboer, F. and Arzt, E. (2007b) The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein-protein interaction. *FASEB J*, **21**, 1177–1188
- Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A. K., Bradbury, E. J. and McMahon, S. B. (2009) Effects of Etanercept and minocycline in a rat model of spinal cord injury. *Eur J Pain*, **13**, 673–681
- Morales, J. R., Ballesteros, I., Deniz, J. M., Hurtado, O., Vivancos, J., Nombela, F., Lizasoain, I., Castrillo, A. and Moro, M. A. (2008) Activation of liver X receptors promotes neuroprotection and reduces brain inflammation in experimental stroke. *Circulation*, **118**, 1450–1459
- Motomura, K., Ohata, M., Satre, M. and Tsukamoto, h. (2001) Destabilization of TNF-alpha mRNA by retinoic acid in hepatic macrophages: implications for alcoholic liver disease. *Am J Physiol Endocrinol Metab*, **281**, E420–429
- Park, S. W., Yi, J. h., Miranpuri, G., Satriotomo, I., Bowen, K., Resnick, D. K. and Vemuganti, R. (2007) Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther*, **320**, 1002–1012
- Paterniti, I., Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Galuppo, M., Bramanti, P. and Cuzzocrea, S. (2010) Liver X receptor agonist treatment regulates inflammatory response after spinal cord trauma. *J Neurochem*, **112**, 611–624
- Popovich, P. G. and Jones, T. B. (2003) Manipulating neuroinflammatory reactions in the injured spinal cord: back to basics. *Trends Pharmacol Sci*, **24**, 13–17
- Sironi, L., Mitro, N., Cimino, M., Gelosa, P., Guerrini, U., Tremoli, E. and Saez, E. (2008) Treatment with LXR agonists after focal cerebral ischemia prevents brain damage. *FEBS Lett*, **582**, 3396–3400
- Streit, W. J., Semple-Rowland, S. L., Hurley, S. D., Miller, R. C., Popovich, P. G. and Stokes, B. T. (1998) Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. *Exp Neurol*, **152**, 74–87
- Tsai, M. C., Wei, C. P., Lee, D. Y., Tseng, Y. T., Tsai, M. D., Shih, Y. L., Lee, Y. h., Chang, S. F. and Leu, S. J. (2008) Inflammatory mediators of cerebrospinal fluid from patients with spinal cord injury. *Surg Neurol*, **70 Suppl 1**, S1:19–24; discussion S11:24
- van Neerven, S., Kampmann, E. and Mey, J. (2008) RAR/RXR and PPAR/RXR signaling in neurological and psychiatric diseases. *Prog Neurobiol*, **85**, 433–451

- van Neerven, S. and Mey, J. (2007) RAR/RXR and PPAR/RXR Signaling in Spinal Cord Injury. *PPAR Res*, **2007**, 29275
- van Neerven, S., Regen, T., Wolf, D., Nemes, A., Johann, S., Beyer, C., Hanisch, U. K. and Mey, J. (2010) Inflammatory chemokine release of astrocytes *in vitro* is reduced by all-*trans* retinoic acid. *J Neurochem*, **114**, 1511-26
- Zhang-Gandhi, C. X. and Drew, P. D. (2007) Liver X receptor and retinoid X receptor agonists inhibit inflammatory responses of microglia and astrocytes. *J Neuroimmunol*, **183**, 50–59
- van Neerven, S., Nemes, A., Imholz, P., Regen, T., Denecke, B., Johan, S., Beyer C., Hanisch, U-K., and Mey, J. (2010) Inflammatory cytokine release of astrocytes in vitro is reduced by all-trans retinoic acid. *J Neuroimmunol*, **in press**

Repetitive intrathecal VEGF165 treatment has limited therapeutic effects after spinal cord injury in the rat

8

**S.G.A. van Neerven^{1,2,4,6}, E.A. Joosten^{1,6}, G.A. Brook^{3,6},
C.A. Lambert⁵, J. Mey^{4,6}, J. Weis^{3,6}, M.A.E. Marcus¹,
H.W.M. Steinbusch^{2,6}, M. van Kleef¹,
J. Patijn¹, R. Deumens^{1,3,6}**

¹Department of Anesthesiology, School for Mental Health
and Neuroscience, Maastricht, University Medical Center,
Maastricht, The Netherlands

²Department of Neuroscience, School for Mental Health and Neuroscience,
Maastricht University, Maastricht, The Netherlands

³Institute of Neuropathology, Medical Faculty, RWTH Aachen University,
Aachen, Germany

⁴Institute of Biology II, RWTH Aachen University, Aachen, Germany

⁵Laboratory of Connective Tissues Biology, GIGA-Cancer,
University of Liège, Liège, Belgium

⁶EURON European Graduate School of Neuroscience

Abstract

Neuropathic pain and motor deficits are detrimental consequences of injury to the spinal cord. In experimental settings, numerous neuroprotective agents are being explored for their therapeutic benefits. Vascular endothelial growth factor (VEGF) is an interesting candidate molecule in this respect since it is not only associated with angiogenesis, but also with neuroprotection and neurite growth. Other investigators have reported improved motor outcomes following intraparenchymal VEGF treatment. Here we demonstrate the therapeutic effects of daily intrathecal treatment of the contused thoracic rat spinal cord with the 165-isoform of VEGF during the first week after injury. We show that VEGF treatment resulted in a statistically significant attenuation of mechanical, but not thermal, hypersensitivity of the hindpaws, while motor deficits remained unaffected. Tissue sparing was also unchanged by VEGF treatment. Microglial responses at the lumbar spinal cord, which have been linked with spinal cord injury-induced hypersensitivity, were found to be unaffected by VEGF treatment. We conclude that repetitive intrathecal VEGF delivery has limited therapeutic effects on spinal cord injury outcome.

Keywords: central pain, glia, neuroplasticity, neuroprotection

Introduction

Tissue damage resulting from spinal cord injury (SCI) triggers inflammatory responses mediated primarily by resident glia and by blood-borne immune cells invading the spinal tissue via a compromised blood-spinal cord barrier. Tissue degeneration and inflammatory responses persist for weeks to months and are strongly related to functional outcome, including motor deficits and central neuropathic pain (Deumens *et al.*, 2008; Donnelly and Popovich, 2008). Indeed, SCI-induced hypersensitivity to mechanical stimuli has been reported to depend on the extent of tissue damage (Kloos *et al.*, 2005). Moreover, SCI triggers microglial responses described as “microgliosis” over several segments of the spinal cord (Detloff *et al.*, 2008), and these microglial responses mediate dorsal horn neuron hyperexcitability and symptoms of neuropathic pain (Carlton *et al.*, 2009; Gwak and Hulsebosch 2009; Hains and Waxman, 2006; Hulsebosch, 2008; Hulsebosch *et al.*, 2009; Zhao *et al.*, 2007a). Nevertheless, SCI-induced inflammatory responses are not only associated with detrimental effects on spinal tissue and functional outcome (Jones *et al.*, 2005; Schwartz *et al.*, 1999). It is now recognized that early inflammatory reactions also trigger processes such as scarring, which limit further tissue loss and preserve function (Faulkner *et al.*, 2004; Sofroniew 2005). Given the limited success of conventional immune suppressants in the therapeutic management of SCI, it is suggested that medications that prevent ongoing tissue loss, either by inhibition of apoptosis or by improving tissue vascularization (Koopmans *et al.*, 2009), are more promising.

Vascular endothelial growth factor (VEGF) is a key factor in the process of angiogenesis, but has also been associated with neuroprotection and neurite outgrowth (Rosenstein and Krum 2004; Sondell *et al.*, 1999). Increased levels of VEGF are detected around spinal injury sites (Bartholdi *et al.*, 1997; Herrera *et al.*, 2009; Skold *et al.*, 2000; Vaquero *et al.*, 1999). Treatment of the spinal cord parenchyma with anti-VEGF antibody decreased the number of surviving neurons around the epicenter of a contusion injury, indicating a neuroprotective role of endogenous VEGF (Herrera *et al.*, 2009). Delivery of the 165-isoform of VEGF (VEGF165) via a single intraparenchymal bolus injection at the time of spinal cord contusion was found to promote tissue preservation (Widenfalk *et al.*, 2003). In a more recent investigation, elevated levels of VEGF were maintained for at least 6 weeks following clip compression of the rat spinal cord by intraparenchymal injection of a viral vector containing a transcription factor that induces VEGF expression (Liu *et al.*, 2010). This treatment resulted in enhanced tissue sparing within a range of a millimeter distance from the lesion epicenter, and a mild improvement of motor function (Liu *et al.*, 2010), as measured by the Basso-Beattie-Bresnahan locomotor scale.

In the present study, we have investigated the effect of repetitive VEGF treatment on SCI-induced hypersensitivity. In addition, motor outcome was measured using an objective and sensitive gait analysis system. We show that daily intrathecal injections of VEGF165 for 1 week following SCI resulted in a slight attenuation of mechanical but not

thermal hypersensitivity, while motor deficits were unchanged. The extent of morphological preservation of spinal cord tissue was not affected by the treatment. Microglial responses associated with SCI-induced hypersensitivity were observed to a similar degree in both VEGF165-treated and vehicle-treated animals.

Material and Methods

All experimental procedures were performed according to the recommendations of the European Commission (European Communities Council Directive of 24 November 1986; 86/609/EEC), and protocols were approved by the committee on animal research of Maastricht University (DEC 2009–030). Twenty-one female Sprague-Dawley rats (Charles River, Wilmington, MA) were used in this study, weighing about 200 g at the start of the experiment. Throughout the study the animals were individually housed in a temperature- and humidity- conditioned room (19–24° C and 55±5%), with a normal 12-h:12-h light-dark cycle and background music to reduce stress. In the 2-week CatWalk-training period (see below) preceding surgery, and on each post-surgery day before recording of CatWalk runs, the animals were kept on a food-restriction protocol (12–15 g chow per day). Otherwise, the animals received standard rat chow ad libitum. The animals had continuous free access to water for the entire period of the study.

Surgery and experimental groups

The animals received a subcutaneous injection of buprenorphine (0.1 mg/kg body weight) at about 30–60 min before surgery. An Inventor 400® injection system vaporizer (Zevenaar, The Netherlands) with an open mask system was used for anesthesia. For induction and maintenance of anesthesia 5% and 1–2% isoflurane (in air) was used, respectively. First, a polyethylene catheter was implanted via an incision in the atlanto-occipital membrane. The tip of the catheter was gently maneuvered to the eighth thoracic (T8) spinal cord level. Then, the T9–T10 spinal cord was exposed by a laminectomy of the T8 and T9 vertebrae. A moderate spinal cord contusion was inflicted using the MASCIS impactor with a 10-g rod dropped from a distance of 12.5 mm onto the exposed dorsal surface of the spinal cord. Then the animals were randomly assigned to two treatment groups: animals either received an intrathecal bolus injection of 0.5 µg VEGF165 in 13 µL (n=9), or 13 µL vehicle solution (n=9), followed by a 17-µL saline flush over a period of about 20 sec. Human recombinant VEGF165 was produced by forced expression in HEK293 cells (Mineur *et al.*, 2007), and was purified from the conditioned medium by immuno-affinity using anti-VEGF antibodies (Avastin; Genentech, South San Francisco, CA), coupled to Affi-Gel Hydrazide gel (Bio-Rad, Hercules, CA). VEGF concentration was measured using a DuoSet ELISA (R&D Systems, Minneapolis, MN). Immediately after surgery, the bladders were manually voided

and the animals received 5 mL subcutaneous saline solution for rehydration. Subcutaneous antibiotic treatment with enrofloxacin (5 mg/kg body weight) was given once daily until normal bladder emptying returned (typically within the first week after SCI) to prevent urinary tract infection. At 1–7 days following surgery, the animals received the same daily treatment (VEGF165 or vehicle) as was given directly after surgery. As such, VEGF-treated animals received a total of 4 μ g VEGF165 over the first week following surgery. This amount of VEGF has previously been shown to limit spinal tissue degeneration in a rat model of severe spinal cord contusion injury (Widenfalk *et al.*, 2003). After the last intrathecal bolus injection, the catheter was carefully removed while the animal was anesthetized as described above. A total of three naïve animals served as controls in this experiment.

Behavioral testing

Motor function was assessed using CatWalk gait analysis (Hamers *et al.*, 2001), and the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso *et al.*, 1995). For gait analysis, the animals were trained on the CatWalk runway during the first 2 weeks preceding surgery according to previously documented protocols (Deumens *et al.*, 2007a). During this training period the rats were positioned at one end of the runway and sweet food pellet rewards (Noyes Precision pellets PJPPP-0045; Sandown Chemical Ltd., Hempton, U.K.) were located at the other end of the runway. The rats were allowed to explore the runway three times per day. Motivation of the animal was reinforced by use of a daily 12–15 g chow diet. After 2 weeks of training, the animals consistently displayed uninterrupted runs and such runs were recorded before surgery. Then CatWalk runs were recorded weekly starting at the fourth week after surgery. Only runs with runway-crossing times of 1–2 sec were used for analysis. This criterion was used to warrant a stable velocity of gait, which is of crucial importance because velocity of gait can largely influence gait parameters (Koopmans *et al.*, 2007). For each time point at least three runs per animal were analyzed using CatWalk XT v8.1 software (Noldus Information Technology, Wageningen). Here we focused on four CatWalk parameters that have previously been reported to be affected in rat models of SCI. First, stride length (i.e., the distance between two subsequent positioning of the same hindpaw; the values of both hindpaws were averaged); second, base-of-support (BOS) (i.e., the average distance between the center of the right and left hindpaws during the gait); third, print area (i.e., the total area that makes up the print of the hindpaws during gait, with the values of both hindpaws averaged); and fourth, mean intensity (i.e., the mean grey value of the pixels corresponding to the hindpaw print at maximum contact during a step cycle, with values of both hindpaws averaged). BBB scores were obtained before surgery and at 1, 3, 5, and 7 days after surgery, and thereafter once a week until the end of the experiment (8 weeks after surgery). Because reliable assessment of coordination using the BBB scale is limited, CatWalk-based coordination data were integrated into BBB scores at 4, 6, and 8 weeks following SCI, as previously reported (Koopmans *et al.*, 2005).

A dynamic plantar aesthesiometer (UgoBasile, Collegeville, PA) was used to assess sensitivity to mechanical stimuli. The animals were habituated to the testing environment for about 30 min until exploration and major grooming ceased. This testing environment consisted of transparent plastic boxes positioned on top of a plastic mesh floor. A metal probe was applied to the mid-plantar surface of the rat hindpaw, and this probe exerted a gradually increasing force (50 g in 10 sec) until a withdrawal reflex was elicited. The threshold evoking a withdrawal reflex was determined for each hindpaw (mean of three measurements, referred to as paw withdrawal threshold [PWT]). PWTs were determined before surgery and then weekly starting at the second week after surgery. To assess mechanical sensitivity, the area under the curve (AUC) of the PWT/time plot was calculated for each animal over the 8 weeks following SCI. Thermal sensitivity was determined using a thermal plantar algesia instrument (UgoBasile) as previously described (Hargreaves *et al.*, 1988). Following a habituation period of about 30 min, an infrared laser beam was positioned at the mid-plantar surface of the rat hindpaw. Before surgery, the intensity of the laser was fixed at a degree that elicited a withdrawal reflex of the hindpaws with a latency of 10–15 sec; this intensity was maintained throughout the experiment. Three withdrawal latencies were determined per hindpaw and averaged for each animal to render the paw withdrawal latency (PWL). PWLs were determined before surgery and then weekly starting at the second week after surgery. In addition, AUCs were determined as described above.

Histology

After the final behavioral tests at 8 weeks after surgery, the animals were terminally anesthetized using an intraperitoneal bolus injection of pentobarbital (180 mg/kg body weight). Subsequently they were transcardially perfused with ice-cold saline solution followed by ice-cold fixative (4% paraformaldehyde in 0.1M phosphate buffer [PB] with 15% picric acid, pH 7.4). The spinal cord was then isolated and immersion fixed at 4° C overnight. Cryoprotection was commenced by an overnight incubation in 10% sucrose (in PB), followed by an incubation in 25% sucrose (in PB) for at least 72 h (all incubations were at 4° C). Then each spinal cord was dissected into two tissue samples: a sample with a length of about 1.5 cm centered at the lesion epicenter (lesion samples), and a sample with a length of about 1.5 cm containing the lumbar region L3–L6 (lumbar samples). Each sample was frozen using dry ice powder and stored at -80° C until further use for histological processing. Cryosectioning was performed on a Leica CM3050S cryostat (Leica Microsystems, Bannockburn, IL). The samples were mounted in Tissue-Tek Optimal Cutting Temperature solution (O.C.T.; Sakura FineTek Europe, Zoeterwoude, The Netherlands). Transverse sections 30 µm thick were obtained over the range of the spinal lesion site and from the L4 and L5 spinal cord; all cryosectioning was performed at a temperature of -30° C. All samples were serially cut by taking every 12th section on the same (gelatin-coated) glass slide.

Every 12th section of the lesion site samples was stained with Luxol fast blue (LFB) to identify the injury epicenter. A Confocal Spinning Disk (SI-SD) system (MBF Bioscience, Williston, VT), consisting of a BX51 microscope (Olympus, San Jose, CA), a customized spinning disk unit (DSU; Olympus), and controlling software (Stereoinvestigator; MBF Bioscience) was used for the analysis. For each lesion sample, the total area of spinal tissue in an uninjured transverse section was measured. This value was then taken as a reference value for that particular lesion sample. Subsequently, the percentage of tissue sparing was determined as the area of preserved spinal tissue in a transverse section compared to the reference value of that lesion sample. The section with the lowest percentage of spared tissue was assigned as the lesion epicenter. Transverse sections with an interval of 360 μm rostral and caudal to this lesion epicenter were analyzed for percentage tissue sparing up to a distance of 2520 μm away from the lesion epicenter. Every 12th section of the lesion sample was stained for glial fibrillary acidic protein (GFAP) to study the scar tissue surrounding the central cavity at the spinal lesion site. Samples of each experimental group were investigated for astroglial scar tissue in a blinded manner by two independent observers.

A stain for ionized calcium-binding adapter molecule-1 (Iba-1) was used to study microglia in the L4–L5 spinal cord. Spinal sections were first incubated with blocking solution (Tris-buffered saline with 0.3% Triton-X100 [TBS-T] containing 5% normal donkey serum) for 1 h at 4° C; then overnight incubation was performed with primary antibody at 4° C (rabbit α -Iba-1; Wako, Osaka, Japan) 1:1000 in TBS-T containing 1% normal donkey serum. Staining with rabbit α -GFAP-antibody (1:1000 in TBS-T; Dako, Carpinteria, CA) was used to study astroglia in the L4–L5 spinal cord. A donkey α -rabbit-Alexa-488 antibody (1:100 in TBS-T; Invitrogen, Carlsbad, CA) was used as a secondary antibody. The stained sections were embedded in 80% glycerol/TBS and coverslipped. Photomicrographs were taken with identical exposure times using a microscope for detection of fluorescence, and an Olympus AX-70 grayscale F-view cooled CCD camera (Paes, Zoeterwoude, The Netherlands). For quantitative analysis, cell profiler software (CellP© Olympus) was used, and the dorsal horns were delineated as regions of interest (ROI) by drawing a line perpendicular to the central canal and then following the shape of the dorsal horn. After background signal subtraction, the average grey value (intensity) of the staining was measured in the ROI as described elsewhere (Deumens *et al.*, 2009). A total of 10 right and 10 left dorsal horns at L4–L5 were analyzed per animal and averaged.

Statistical analysis

All data were analyzed using SPSS 15.0 statistical software. Repeated-measures analysis of variance (ANOVA) followed by Bonferroni post hoc corrections was used to test for behavioural differences over time. Subsequently, VEGF treatment effects were tested using the independent Student's t-test. Effects of treatment on signal intensities of immunohistochemical stainings were tested using a one-way ANOVA with Bonferroni post-hoc corrections.

Results

Two out of the 18 vehicle-treated SCI animals were excluded from the study. One animal developed ascites during the first week after surgery, and the other animal developed a urinary tract infection despite enrofloxacin treatment; it lost more than 20% of its pre-surgery body weight and was then euthanized for ethical reasons.

VEGF treatment did not affect chronic motor function

SCI induced a large decrease in BBB score, which gradually recovered over the first week (Fig. 1A and B). CatWalk gait analysis was begun when weight-supported stepping of hindpaws returned (typically during the second week after injury). Between 4 and 8 weeks after injury, CatWalk-based coordination (regularity index) measures were obtained and integrated into the BBB score. This resulted in scores around 18 (Fig. 1B), reflecting nearly perfect coordination between fore- and hindpaws. In comparison with the control group, VEGF-treated animals did not show statistically significantly different scores on the BBB test at any time point of the investigation. CatWalk gait analysis showed only limited deficits in motor skills of the rats while crossing the glass runway. No statistically significant decrease in stride length (Fig. 1C), or the coordination measure “regularity index” (data not shown), could be observed from 4 weeks post-surgery onwards. The BOS (i.e., the distance between the centers of both hindpaws) was found to be significantly increased in vehicle-treated animals after injury compared to baseline ($F_{3,18} = 9.8$; $p < 0.01$), which was not the case for VEGF-treated animals (Fig. 1D). A reduction in print area of the hindpaws after injury was observed for both vehicle-treated ($F_{3,18} = 10.7$; $p < 0.01$) and VEGF-treated animals ($F_{3,24} = 8.5$; $p < 0.01$; Fig. 1E). Moreover, a reduction in mean intensity of the hindpaws after injury was observed for both vehicle treated ($F_{3,18} = 8.9$; $p < 0.01$) and VEGF-treated animals ($F_{3,24} = 6.4$; $p < 0.01$; Fig. 1F). Although reductions of print area and mean intensity were significantly lower in vehicle-treated animals than in VEGF-treated animals at 6 weeks after injury ($p < 0.05$), there was no group difference at 8 weeks after injury.

VEGF treatment slightly attenuated mechanical but not thermal hypersensitivity following SCI

Spinal cord contusion injury resulted in a significant drop of PWT to mechanical stimulation of the hindpaws in both vehicle-treated ($F_{7,42} = 7.2$; $p < 0.01$) and VEGF-treated animals ($F_{7,56} = 11.6$; $p < 0.01$; Fig. 2A). At baseline, PWTs were elicited at about 33 and 36 g in vehicle-treated and VEGF-treated animals, while at 8 weeks after SCI PWTs were reduced to 22 and 26 g, respectively. VEGF had a minor therapeutic effect on mechanical hypersensitivity. During 7 weeks following contusion injury, the PWT dropped continuously in control animals, whereas the VEGF-treated animals reached a plateau at a lower level of

Fig. 1. Unaffected chronic motor outcome following vascular endothelial growth factor (VEGF) treatment of the contused spinal cord. Basso-Beattie-Bresnahan (BBB) testing showed strong decreases in scores within the first weeks (A), which gradually recovered over the post-operative weeks (B), but no differences were observed between VEGF-treated and vehicle-treated animals. Stride length of the hind-paws remained unaffected by spinal cord injury (SCI) (C), while base-of-support (BOS) was only increased at 4 weeks after injury (D). No group differences were found for these two measures. Print area and mean intensity of the hindpaws were both decreased following SCI (E and F), and both showed significantly lower levels in vehicle-treated animals than in VEGF-treated animals only at 6 weeks after SCI (a.u., arbitrary units; BL, baseline; VEGF-treated animals, $n = 9$; vehicle-treated animals, $n = 7$; * $p < 0.05$).

hypersensitivity (Fig. 2A). Using a repeated-measures ANOVA for time, an overall difference between VEGF-treated and vehicle-treated animals was detected ($F_{1,14} = 5.8$; $p < 0.05$). Student's t-tests showed an approximate 15% reduction in the PWT of VEGF-treated animals compared to vehicle-treated animals at 7 and 8 weeks post-injury ($p < 0.05$). This quantitatively resulted in an 8% reduction of the AUC for VEGF-treated versus vehicle-treated animals (Fig. 2B, $p < 0.05$). The attenuation of mechanical hypersensitivity by VEGF was significant at 7 and 8 weeks after SCI (Fig. 2A). The PWL to thermal stimulation of the hindpaws was also strongly reduced by SCI ($F_{7,42} = 4.5$; $p < 0.01$ for vehicle-treated animals, and $F_{7,56} = 8.0$; $p < 0.01$ for VEGF-treated animals; Fig. 2C). While PWLs of about 13 sec were observed at baseline, group average values of 8 sec were observed at 8 weeks after SCI. There was no significant difference between the two groups of animals (Fig. 2C and D).

Fig. 2. Mechanical but not thermal hypersensitivity were slightly attenuated by vascular endothelial growth factor (VEGF) treatment. (A) Spinal cord injury (SCI) induced reductions in the paw withdrawal threshold (PWT) to mechanical stimulation of the hindpaws. VEGF treatment resulted in significantly lower reductions of PWT at 7 and 8 weeks after injury, and in a statistically significantly higher area under the curve (AUC; B). (C and D) Paw withdrawal latency (PWL) to thermal stimulation of the hindpaws was reduced by SCI to a similar degree in vehicle-treated and VEGF-treated animals (BL, baseline; VEGF-treated animals, $n=9$; vehicle-treated animals, $n=7$; * $p < 0.05$).

Astroglial scarring occurred around the spinal cord contusion site

GFAP staining of the injured spinal cord showed clear and dense astroglial scar tissue around the cystic cavities at 8 weeks following injury (Fig. 3A and B). The GFAP staining was too dense to clearly discriminate individual astroglial cells, although occasional hypertrophied cell profiles could be detected (arrows in Fig. 3C and D). GFAP staining of vehicle treated and VEGF treated animals did not show any apparent differences.

Spinal tissue sparing was unaffected by VEGF treatment

In order to assess the neuroprotective effects of VEGF treatment, morphometric analysis was performed on serial transverse spinal cord sections stained with Luxol fast blue. Only residual tissue without inflammatory cell infiltrates such as phagocytic macrophages was

Fig. 3. Astroglial scarring at the lesion site. (A and B) Low-power microphotographs of the glial fibrillary acidic protein (GFAP)-immunoreactive scar tissue surrounding the spinal lesion cavity (asterisk) in vehicle-treated and VEGF-treated animals, respectively. (C and D) High-power microphotographs in which hypertrophied cell profiles can be observed (arrows); vascular endothelial growth factor [VEGF]-treated animals, n=5; vehicle-treated animals, n=5; SCI, spinal cord injury).

regarded as spared spinal tissue, and sections containing the lowest amount of spared spinal tissue were defined as the lesion epicenter. At the epicentre only a rim of white matter remained intact and devoid of inflammatory cell infiltrates at 8 weeks after SCI (Fig. 4A–D). The amount of spared tissue was no different between VEGF-treated and vehicle-treated animals (Fig. 4E).

Fig. 4. Tissue sparing was not affected by vascular endothelial growth factor (VEGF) treatment. (A and C) Luxol fast blue (LFB)-stained sections at the lesion epicenter of a vehicle-treated and a VEGF-treated animal, respectively. (B and D) These show the traced spared tissue between the dotted and the solid lines. (E) This shows the results of the morphometric analysis of tissue sparing at intervals of 360 µm from the lesion epicenter. No differences were observed between VEGF-treated and vehicle-treated animals (VEGF-treated animals, n=5; vehicle-treated animals, n=5; SCI, spinal cord injury).

The microglial response to SCI remained unaffected by VEGF

Moderate spinal cord contusion of the thoracic spinal cord resulted in a strong upregulation of microglial Iba-1 staining (Fig. 5A and B). VEGF treatment did not show any overall change in staining compared to vehicle treatment (Fig. 5C). At higher magnification, intact naïve animals showed microglial cells with highly ramified morphologies (Fig. 5D), while SCI induced hypertrophy of the cell bodies and an increase in the density of cells in both vehicle-treated and VEGF-treated animals (Fig. 5E). Quantitative evaluation showed a statistically significant upregulation in both vehicle-treated and VEGF-treated animals compared to naïve control animals (Fig. 5G). GFAP staining was performed to assess astroglial responses at 8 weeks after SCI, but it was unchanged in SCI animals (both vehicle-treated and VEGF-treated) compared to naïve control animals (Fig. 5H–N).

Discussion

In the present study the effect of repetitive intrathecal delivery of VEGF165 during the first week after thoracic spinal cord contusion injury was assessed. We found that VEGF treatment had only limited therapeutic effects, which involved a slight but significantly attenuated mechanical hypersensitivity of the hindpaws. The extent of tissue loss and astroglial scar tissue were unaffected by the treatment, and no lasting improvement in functional recovery could be obtained. Microglial responses in the lumbar spinal cord were present in VEGF165-treated and vehicle-treated animals, while astroglial responses (i.e., GFAP upregulation) were absent at 2 months following injury.

Neuropathic pain is one of the most debilitating consequences of injury to the spinal cord (Felix *et al.*, 2007; Nepomuceno *et al.*, 1979; Siddall *et al.*, 2003). In this study, VEGF treatment resulted in a slight but statistically significant attenuation of mechanical, but not thermal, hypersensitivity of the hindpaws. Microglial responses have previously been shown to play a vital role in mechanical and thermal hypersensitivity at about 1 month after SCI (Hains and Waxman, 2006). Such microglial responses, classically characterized by morphological transformations such as hypertrophy and retraction of microglial processes, but also by proliferation, do not only occur at the level of the spinal injury, but can extend over several spinal segments (Detloff *et al.*, 2008), and can even be detected at supraspinal locations (Zhao *et al.*, 2007b). In the present study, upregulation of the microglial protein Iba-1 was observed in the dorsal horn of the lumbar spinal cord in both vehicle-treated and VEGF-treated animals. On the basis of these data, it is rather unlikely that microglial changes are involved in the minor reduction in mechanical hypersensitivity seen following VEGF treatment. It has to be stated that morphological changes are an oversimplified way to look at microglia, as such changes do not provide any information about alterations in cellular function. Indeed, microglia are thought to

Fig. 5. Lumbar glial responses to spinal cord injury (SCI) in vehicle-treated and VEGF-treated animals. Iba-1 (A–G) and GFAP (H–N) staining was performed on the L4–L5 spinal cord of naïve animals (A and D, and h and K, respectively), vehicle-treated SCI animals (B and E, and I and L, respectively), and VEGF-treated SCI animals (C and F, and J and M, respectively). Higher magnification shows highly ramified microglia in the lumbar dorsal horn of a naïve control animal (D), while SCI with vehicle or VEGF treatment shows hypertrophied microglia and an increased density of microglia in the lumbar dorsal horn (E and F, respectively). (G) Quantitative analysis demonstrates significant increases in the intensity of Iba-1 staining in the dorsal horn of SCI animals (vehicle-treated and VEGF-treated), compared to naïve controls. (H–J) Lowpower photomicrographs of GFAP-stained lumbar spinal cord sections of a naïve control animal (H), a vehicle-treated (I), and a VEGF-treated animal with SCI (J). (K–M) High-power photomicrographs of this staining did not show any apparent differences in astroglial morphologies or densities, which is in agreement with the quantitative analyses of GFAP staining in the lumbar dorsal horn of the three animal groups (N; a.u., arbitrary units; VEGF, vascular endothelial growth factor; Iba-1, ionized calcium-binding adapter molecule-1; GFAP, glial fibrillary acid protein; VEGF treated animals, n = 5; vehicle-treated animals, n = 5); **p < 0.01; *p < 0.05).

have many different functional states, many if not all of which are characterized by morphological changes (Ransohoff and Perry, 2009). With respect to functional microglial states, in particular the expression of the eicosanoid prostaglandin E2 (PGE2) has been linked to microglial-mediated hypersensitivity following SCI (Hains *et al.*, 2001; Zhao *et al.*, 2007a). However, since VEGF treatment did not change thermal hypersensitivity of the hindpaws in the present study, and such hypersensitivity depends on PGE2 (Zhao *et al.*, 2007a), it is unlikely that the VEGF-induced reduction of mechanical hypersensitivity was

due to modified PGE2 signaling. Surprisingly, we did not find any indications of astroglial responses in the lumbar spinal cord at 8 weeks after SCI, while astroglial responses are often suggested to be involved in symptoms of neuropathic pain (Gwak and Hulsebosch, 2009; Ji *et al.*, 2006; Nestic *et al.*, 2005; Tanga *et al.*, 2006). Nevertheless, the link between such astroglial responses and neuropathic pain symptoms is typically seen within the first 4–5 weeks, while later time points are not frequently investigated. Interestingly, astroglial responses in the lumbar dorsal horn at 3 months after sciatic nerve injury have been found to be inversely related to mechanical hypersensitivity (Deumens *et al.*, 2009).

The VEGF-induced attenuation of mechanical hypersensitivity of the hindpaws was only minor, and therefore its physiological relevance remains uncertain. A subset of investigations has nevertheless claimed therapeutic effects of VEGF treatment in animal models of SCI (Facchiano *et al.*, 2002; Liu *et al.*, 2010; Widenfalk *et al.*, 2003), while others have found no effects or even negative effects, including an increase in the permeability of the blood–spinal cord barrier (Benton and Whittemore, 2003; Vaquero *et al.*, 1999). Therapeutic effects have been related to enhanced plasticity in neuronal structures, which has also been frequently associated with mechanical hypersensitivity (Christensen and Hulsebosch, 1997; Deumens *et al.*, 2007b, 2008; Hofstetter *et al.*, 2005). Injured corticospinal axons that showed particularly weak spontaneous regenerative responses following SCI (Deumens *et al.*, 2005) were found to re-grow following VEGF165 treatment (Facchiano *et al.*, 2002). In addition, VEGF has been associated with neuroprotective effects. A single 4- μ g bolus injection of VEGF165 into the damaged spinal parenchyma at the time of spinal contusion injury was found to result in enhanced spared spinal tissue (Widenfalk *et al.*, 2003). In the present study, no such neuroprotective effects were observed. The amount of tissue loss at the lesion epicenter reached up to 90% in both vehicle-treated and VEGF-treated animals.

The lack of neuroprotective effects of VEGF treatment found in the present study may be attributable to our treatment design. It was previously shown that intraparenchymal injection of 4 μ g, but not 1 or 20 μ g of VEGF165 resulted in motor improvements according to the BBB scale (Widenfalk *et al.*, 2003). In the present study, a total of 4 μ g of VEGF165 was given intrathecally, equally distributed over a total of eight daily bolus injections within the first week following injury. Tissue penetration of VEGF165 may be less extensive following intrathecal administration compared to intraparenchymal administration. Although astroglia also express VEGF receptors (Krum and Rosenstein, 1998; Krum *et al.*, 2002), astroglial scar tissue around the spinal lesion site did not appear to be affected by VEGF treatment. An alternative explanation for the lack of neuroprotective effects of VEGF seen in the present study may involve the duration of treatment. In this study treatment was restricted to the first week, because tissue sparing after VEGF165 treatment has previously been reported during the first week following SCI (Widenfalk *et al.*, 2003). Recent findings show that experimentally-induced upregulation of VEGF at the site of spinal cord compression injury for a total of at least 6 weeks substantially reduced tissue

loss around the lesion epicenter (Liu *et al.*, 2010). Moreover, BBB scores were significantly improved following prolonged elevations in VEGF levels (Liu *et al.*, 2010); however, in our study BBB scores were unaffected by VEGF treatment. Of note, CatWalk-based BBB scores were found to be particularly high (around 18) from the fourth week onwards, indicating the general well-being of the animals in this study. Gait analysis using the CatWalk system further showed that the motor impairments seen following moderate spinal cord contusion were relatively mild. Gait analysis can be reliably used to assess motor outcome after SCI in the rat (Deumens *et al.*, 2006b, 2006c; Hamers *et al.*, 2006; Koopmans *et al.*, 2007, 2009). Previously, the base-of-support of the hindpaws was found to be increased by spinal cord contusion in female Wistar rats, and this was thought to reflect compensatory behavior for an injury-induced reduction in the stability of gait (Koopmans *et al.*, 2006). In the present study, the base-of-support was only increased during the first 4 weeks after injury, after which pre-operative levels were reached. Stride length of the hindpaw (i.e., the distance between two subsequent placements of the same hindpaw), which was previously found to be affected following dorsal hemisection injury of the spinal cord (Deumens *et al.*, 2006a), remained unaffected after spinal cord contusion. Chronic deficits in motor function were unaffected following VEGF treatment in the present study, a finding in agreement with recent results that showed no lasting motor improvement induced by VEGF treatment (Patel *et al.*, 2009).

On the basis of the present study it may be concluded that VEGF has only limited therapeutic benefits for the treatment of SCI. It is clear that suboptimal conditions related to the treatment design (including dosing, timing, and drug delivery route) may partly explain the relatively poor therapeutic outcome of VEGF treatment seen in the present study, with only mild reductions in mechanical hypersensitivity and transient motor improvements. Nevertheless, VEGF has a rather narrow therapeutic range of action with respect to neuroprotection following SCI (Widenfalk *et al.*, 2003). Thus alternative drug candidates need to be explored in the search for neuroprotective therapies to improve SCI outcomes. Anti-inflammatory therapies may be of interest in this respect, as such therapies may prevent inflammation-induced exacerbation of tissue damage, and some have been reported to attenuate SCI-induced hypersensitivity and motor deficits (Gris *et al.*, 2004; Koopmans *et al.*, 2009; Park *et al.*, 2007; Plunkett *et al.*, 2001). Nevertheless, anti-inflammatory therapies may also interfere with beneficial effects associated with the inflammatory processes seen following SCI (Faulkner *et al.*, 2004; Moalem *et al.*, 1999; Schwartz *et al.*, 1999; Sofroniew, 2005). Other neuroprotective approaches that selectively interfere with apoptosis may therefore be more suitable for the treatment of SCI (Citron *et al.*, 2008; Li *et al.*, 2000).

In conclusion, repetitive intrathecal delivery of VEGF165 over the first week after rat spinal cord contusion injury showed only minor therapeutic effects, which included slightly attenuated mechanical but not thermal hypersensitivity and unaffected motor outcome. Microglial responses, normally linked to SCI-induced hypersensitivity, were present in the

lumbar dorsal horn after spinal cord contusion following both vehicle treatment and VEGF treatment. Tissue sparing at the lesion site and astroglial scarring were unaffected by VEGF treatment. As such, VEGF has only limited beneficial effects on SCI outcomes.

Acknowledgments

The authors thank Dr. Pierre Mineur for his efforts regarding the production of VEGF165. In addition, the authors wish to thank Ms. Marijke Lemmens for her assistance in the use of the confocal SI-SD system. The authors are grateful to the Royal Dutch Academy of Arts and Sciences (KNAW) for financial support of this work via a KNAW-Hendrik Casimir Karl Ziegler Research Stipend (to R.D.), and a KNAW-van Leersum Fonds grant (to R.D.). Special thanks also go to the Dutch Society for Anesthesiology (NVA) for their financial support of this study via the Young Investigator Grant (to R.D.). The SI-SD system was funded by grant no. 911-06-003 from the Medical Section of the Dutch Scientific Organization (NWO). This work was supported by an FP6-Marie Curie Early Stage Training Site, grant number MEST-CT-2005-020013 (Euron consortium) to J.M. Author Disclosure Statement No competing financial interests exist.

References

- Bartholdi, D., Rubin, B. P. and Schwab, M. E. (1997) VEGF mRNA induction correlates with changes in the vascular architecture upon spinal cord damage in the rat. *Eur J Neurosci*, **9**, 2549–2560
- Basso, D. M., Beattie, M. S. and Bresnahan, J. C. (1995) A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma*, **12**, 1–21
- Benton, R. L. and Whittemore, S. R. (2003) VEGF165 therapy exacerbates secondary damage following spinal cord injury. *Neurochem Res*, **28**, 1693–1703
- Carlton, S. M., Du, J., Tan, h. Y. *et al.* (2009) Peripheral and central sensitization in remote spinal cord regions contribute to central neuropathic pain after spinal cord injury. *Pain*, **147**, 265–276
- Christensen, M. D. and Hulsebosch, C. E. (1997) Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn in the rat. *Exp Neurol*, **147**, 463–475
- Citron, B. A., Arnold, P. M., Haynes, N. G., Ameenuddin, S., Farooque, M., Santacruz, K. and Festoff, B. W. (2008) Neuroprotective effects of caspase-3 inhibition on functional recovery and tissue sparing after acute spinal cord injury. *Spine (Phila Pa 1976)*, **33**, 2269–2277
- Detloff, M. R., Fisher, L. C., McGaughy, V., Longbrake, E. E., Popovich, P. G. and Basso, D. M. (2008) Remote activation of microglia and pro-inflammatory cytokines predict the onset and severity of below-level neuropathic pain after spinal cord injury in rats. *Exp Neurol*, **212**, 337–347
- Deumens, R., Jaken, R. J., Knaepen, L., van der Meulen, I. and Joosten, E. A. (2009) Inverse relation between intensity of GFAP expression in the substantia gelatinosa and degree of chronic mechanical allodynia. *Neurosci Lett*, **452**, 101–105
- Deumens, R., Jaken, R. J., Marcus, M. A. and Joosten, E. A. (2007a) The CatWalk gait analysis in assessment of both dynamic and static gait changes after adult rat sciatic nerve resection. *J Neurosci Methods*, **164**, 120–130
- Deumens, R., Joosten, E. A., Waxman, S. G. and Hains, B. C. (2008) Locomotor dysfunction and pain: the scylla and charybdis of fiber sprouting after spinal cord injury. *Mol Neurobiol*, **37**, 52–63
- Deumens, R., Koopmans, G. C., Honig, W. M., Hamers, F. P., Maquet, V., Jerome, R., Steinbusch, h. W. and Joosten, E. A. (2006a) Olfactory ensheathing cells, olfactory nerve fibroblasts and biomatrices to promote long-distance axon regrowth and functional recovery in the dorsally hemisected adult rat spinal cord. *Exp Neurol*, **200**, 89–103
- Deumens, R., Koopmans, G. C., Honig, W. M., Maquet, V., Jerome, R., Steinbusch, h. W. and Joosten, E. A. (2006b) Chronically injured corticospinal axons do not cross large spinal lesion gaps after a multifactorial transplantation strategy using olfactory ensheathing cell/olfactory nerve fibroblast-biomatrix bridges. *J Neurosci Res*, **83**, 811–820
- Deumens, R., Koopmans, G. C., Honig, W. M., Maquet, V., Jerome, R., Steinbusch, h. W. and Joosten, E. A. (2006c) Limitations in transplantation of astroglia-biomatrix bridges to stimulate corticospinal axon regrowth across large spinal lesion gaps. *Neurosci Lett*, **400**, 208–212
- Deumens, R., Koopmans, G. C. and Joosten, E. A. (2005) Regeneration of descending axon tracts after spinal cord injury. *Prog Neurobiol*, **77**, 57–89

- Deumens, R., Lubbers, M., Jaken, R. J., Meijs, M. F., Thurlings, R. M., Honig, W. M., Schachner, M., Brook, G. A. and Joosten, E. A. (2007b) Mice lacking L1 have reduced CGRP fibre in-growth into spinal transection lesions. *Neurosci Lett*, **420**, 277–281
- Donnelly, D. J. and Popovich, P. G. (2007) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol*
- Facchiano, F., Fernandez, E., Mancarella, S. *et al.* (2002) Promotion of regeneration of corticospinal tract axons in rats with recombinant vascular endothelial growth factor alone and combined with adenovirus coding for this factor. *J Neurosurg*, **97**, 161–168
- Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B. and Sofroniew, M. V. (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*, **24**, 2143–2155
- Felix, E. R., Cruz-Almeida, Y. and Widerstrom-Noga, E. G. (2007) Chronic pain after spinal cord injury: What characteristics make some pains more disturbing than others? *J Rehabil Res Dev*, **44**, 703–716
- Gris, D., Marsh, D. R., Oatway, M. A., Chen, Y., Hamilton, E. F., Dekaban, G. A. and Weaver, L. C. (2004) Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. *J Neurosci*, **24**, 4043–4051
- Gwak, Y. S. and Hulsebosch, C. E. (2009) Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. *Neuroscience*, **161**, 895–903
- Hains, B. C. and Waxman, S. G. (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci*, **26**, 4308–4317
- Hains, B. C., Yucra, J. A. and Hulsebosch, C. E. (2001) Reduction of pathological and behavioral deficits following spinal cord contusion injury with the selective cyclooxygenase-2 inhibitor NS-398. *J Neurotrauma*, **18**, 409–423
- Hamers, F. P., Koopmans, G. C. and Joosten, E. A. (2006) CatWalk-assisted gait analysis in the assessment of spinal cord injury. *J Neurotrauma*, **23**, 537–548
- Hamers, F. P., Lankhorst, A. J., van Laar, T. J., Veldhuis, W. B. and Gispen, W. h. (2001) Automated quantitative gait analysis during overground locomotion in the rat: its application to spinal cord contusion and transection injuries. *J Neurotrauma*, **18**, 187–201
- Hargreaves, K., Dubner, R., Brown, F., Flores, C. and Joris, J. (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain*, **32**, 77–88
- Herrera, J. J., Nestic-Taylor, D. O. and Narayana, P. A. (2009) Reduced Vascular Endothelial Growth Factor Expression In Contusive Spinal Cord Injury. *J Neurotrauma*
- Hofstetter, C. P., Holmstrom, N. A., Lilja, J. A. *et al.* (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. *Nat Neurosci*, **8**, 346–353
- Hulsebosch, C. E. (2008) Gliopathy ensures persistent inflammation and chronic pain after spinal cord injury. *Exp Neurol*, **214**, 6–9
- Hulsebosch, C. E., Hains, B. C., Crown, E. D. and Carlton, S. M. (2009) Mechanisms of chronic central neuropathic pain after spinal cord injury. *Brain Res Rev*, **60**, 202–213

- Ji, R. R., Kawasaki, Y., Zhuang, Z. Y., Wen, Y. R. and Decosterd, I. (2006) Possible role of spinal astrocytes in maintaining chronic pain sensitization: review of current evidence with focus on bFGF/JNK pathway. *Neuron Glia Biol*, **2**, 259–269
- Jones, T. B., McDaniel, E. E. and Popovich, P. G. (2005) Inflammatory-mediated injury and repair in the traumatically injured spinal cord. *Curr Pharm Des*, **11**, 1223–1236
- Kloos, A. D., Fisher, L. C., Detloff, M. R., Hassenzahl, D. L. and Basso, D. M. (2005) Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. *Exp Neurol*, **191**, 251–265
- Koopmans, G. C., Brans, M., Gomez-Pinilla, F., Duis, S., Gispen, W. h., Torres-Aleman, I., Joosten, E. A. and Hamers, F. P. (2006) Circulating insulin-like growth factor I and functional recovery from spinal cord injury under enriched housing conditions. *Eur J Neurosci*, **23**, 1035–1046
- Koopmans, G. C., Deumens, R., Brook, G., Gerver, J., Honig, W. M., Hamers, F. P. and Joosten, E. A. (2007) Strain and locomotor speed affect over-ground locomotion in intact rats. *Physiol Behav*, **92**, 993–1001
- Koopmans, G. C., Deumens, R., Buss, A. *et al.* (2009) Acute rolipram/thalidomide treatment improves tissue sparing and locomotion after experimental spinal cord injury. *Exp Neurol*, **216**, 490–498
- Koopmans, G. C., Deumens, R., Honig, W. M., Hamers, F. P., Steinbusch, h. W. and Joosten, E. A. (2005) The assessment of locomotor function in spinal cord injured rats: the importance of objective analysis of coordination. *J Neurotrauma*, **22**, 214–225
- Krum, J. M., Mani, N. and Rosenstein, J. M. (2002) Angiogenic and astroglial responses to vascular endothelial growth factor administration in adult rat brain. *Neuroscience*, **110**, 589–604
- Krum, J. M. and Rosenstein, J. M. (1998) VEGF mRNA and its receptor flt-1 are expressed in reactive astrocytes following neural grafting and tumor cell implantation in the adult CNS. *Exp Neurol*, **154**, 57–65
- Li, M., Ona, V. O., Chen, M., Kaul, M., Tenneti, L., Zhang, X., Stieg, P. E., Lipton, S. A. and Friedlander, R. M. (2000) Functional role and therapeutic implications of neuronal caspase-1 and -3 in a mouse model of traumatic spinal cord injury. *Neuroscience*, **99**, 333–342
- Liu, Y., Figley, S., Spratt, S. K., Lee, G., Ando, D., Surosky, R. and Fehlings, M. G. (2010) An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. *Neurobiol Dis*, **37**, 384–393
- Mineur, P., Colige, A. C., Deroanne, C. F. *et al.* (2007) Newly identified biologically active and proteolysis-resistant VEGF-A isoform VEGF111 is induced by genotoxic agents. *J Cell Biol*, **179**, 1261–1273
- Moalem, G., Leibowitz-Amit, R., Yoles, E., Mor, F., Cohen, I. R. and Schwartz, M. (1999) Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med*, **5**, 49–55
- Nepomuceno, C., Fine, P. R., Richards, J. S., Gowens, h., Stover, S. L., Rantanuaboli, U. and Houston, R. (1979) Pain in patients with spinal cord injury. *Arch Phys Med Rehabil*, **60**, 605–609
- Nesic, O., Lee, J., Johnson, K. M. *et al.* (2005) Transcriptional profiling of spinal cord injury-induced central neuropathic pain. *J Neurochem*, **95**, 998–1014
- Park, S. W., Yi, J. h., Miranpuri, G., Satriotomo, I., Bowen, K., Resnick, D. K. and Vemuganti, R. (2007) Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. *J Pharmacol Exp Ther*, **320**, 1002–1012

- Patel, C. B., Cohen, D. M., Ahobila-Vajjala, P., Sundberg, L. M., Chacko, T. and Narayana, P. A. (2009) Effect of VEGF treatment on blood-spinal cord barrier permeability in experimental spinal cord injury: dynamic contrast-enhanced magnetic resonance imaging. *J Neurotrauma*, **26**, 1005–1016
- Plunkett, J. A., Yu, C. G., Easton, J. M., Bethea, J. R. and Yezierski, R. P. (2001) Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat. *Exp Neurol*, **168**, 144–154
- Ransohoff, R. M. and Perry, V. h. (2009) Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol*, **27**, 119–145
- Rosenstein, J. M. and Krum, J. M. (2004) New roles for VEGF in nervous tissue—beyond blood vessels. *Exp Neurol*, **187**, 246–253
- Schwartz, M., Moalem, G., Leibowitz-Amit, R. and Cohen, I. R. (1999) Innate and adaptive immune responses can be beneficial for CNS repair. *Trends Neurosci*, **22**, 295–299
- Siddall, P. J., McClelland, J. M., Rutkowski, S. B. and Cousins, M. J. (2003) A longitudinal study of the prevalence and characteristics of pain in the first 5 years following spinal cord injury. *Pain*, **103**, 249–257
- Skold, M., Cullheim, S., Hammarberg, h., Piehl, F., Suneson, A., Lake, S., Sjogren, A., Walum, E. and Risling, M. (2000) Induction of VEGF and VEGF receptors in the spinal cord after mechanical spinal injury and prostaglandin administration. *Eur J Neurosci*, **12**, 3675–3686
- Sofroniew, M. V. (2005) Reactive astrocytes in neural repair and protection. *Neuroscientist*, **11**, 400–407
- Sondell, M., Lundborg, G. and Kanje, M. (1999) Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. *J Neurosci*, **19**, 5731–5740
- Tanga, F. Y., Raghavendra, V., Natile-McMenemy, N., Marks, A. and Deleo, J. A. (2006) Role of astrocytic S100beta in behavioral hypersensitivity in rodent models of neuropathic pain. *Neuroscience*, **140**, 1003–1010
- Vaquero, J., Zurita, M., de Oya, S. and Coca, S. (1999) Vascular endothelial growth/permeability factor in spinal cord injury. *J Neurosurg*, **90**, 220–223
- Widenfalk, J., Lipson, A., Jubran, M., Hofstetter, C., Ebendal, T., Cao, Y. and Olson, L. (2003) Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience*, **120**, 951–960
- Zhao, P., Waxman, S. G. and Hains, B. C. (2007a) Extracellular signal-regulated kinase-regulated microglia-neuron signaling by prostaglandin E2 contributes to pain after spinal cord injury. *J Neurosci*, **27**, 2357–2368
- Zhao, P., Waxman, S. G. and Hains, B. C. (2007b) Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J Neurosci*, **27**, 8893–8902

Discussion

9

General discussion

9.1 Modulation of neuroinflammation to treat the injured spinal cord

Detailed knowledge of the processes that occur during the course of inflammation is crucial to enable accurate SCI treatment in place and time. The first minutes to hours after injury, are mainly dominated by an innate immune reaction providing a fast, a-specific inflammatory response. In the first place this response is driven by glial activation and after 24 h after SCI also by neutrophil and macrophage infiltration from the blood (Donnelly & Popovich 2008, Popovich & Hickey 2001, Jones *et al.* 2005, Jones *et al.* 2002). The a-specificity of this early immune response causes so called 'bystander damage' due to the release of toxic substances (normally to fight pathogens), such as free radicals and cytokines by these cells. These processes lead to further tissue destruction after SCI. Additionally at approximately one week after SCI also the adaptive immune system is activated, providing a specific immune response that is characterized by anti-body production mainly against myelin debris followed by activation of cytotoxic T-cells (Popovich *et al.* 1997). Nevertheless, the inflammatory reaction following SCI is like a two edged blade; on the one hand events occur that are detrimental to the spinal nervous tissue, whereas on the other hand events take place that are beneficial and lead to repair of damaged tissue. One of these beneficial mechanisms may be the formation of a glial scar which occurs during the first week after SCI. The scar creates a barrier which contains the inflammation. As such, inhibition of its formation has been associated with worsened functional outcome (Faulkner *et al.* 2004, Bush *et al.* 1999, Okada *et al.* 2006). The fact that also remyelination and axonal sprouting take place during the inflammatory reaction is indicative of the induction of repair mechanisms after SCI (Hill *et al.* 2001). Moreover growth factor production has been shown to start immediately after SCI and stays elevated up to weeks after SCI. The neurotrophic actions of growth factors may be involved in neuroprotection and may reduce the detrimental effects of inflammation (Brown *et al.* 2004, Ikeda *et al.* 2001, Donnelly & Popovich 2008). Despite the dual character of the inflammatory reaction, most therapies to modulate inflammation after SCI have been directed towards inhibition of the pro-inflammatory events.

9.1.1 Inhibition of pro-inflammatory events by activation of nuclear hormone receptors

The glucocorticoid and nuclear hormone receptor (NHR) agonist methylprednisolone (MP) is the only recognized pharmacological treatment to modulate the early inflammatory reaction after SCI in humans. In the last decade, other NHR family members have been investigated for their anti-inflammatory properties in different animal models of CNS inflammation. This increasing amount of data on the effects of PPAR and RAR activa-

tion in CNS pathologies, including SCI, are described in **chapters 2 and 3** of this thesis. These reviews provided solid ground to further investigate the possible anti-inflammatory effect of NHR activation in cell cultures and models of SCI, described in chapters 4, 5, 6 and 7. **Chapters 4 and 5** describe the anti-inflammatory effect of RAR activation by the natural ligand RA, which was able to reduce the expression as well as the release of inflammatory chemokines and cytokines in LPS-challenged primary astrocytes cultures.

Anti-inflammatory effects of NHR are likely to take place via non-genomic actions such as direct interference with pro-inflammatory transcription factor activation, such as NF- κ B activation. The two *in vitro* studies on RA demonstrate that when RA was given as a pre-treatment followed by LPS and a second dose of RA, the anti-inflammatory effects were stronger than when RA was only administered simultaneous to LPS and no pre-treatment was done. This may indicate that the mechanisms underlying the anti-inflammatory effects of RA not only involve direct interference with pro-inflammatory transcription factors such as NF- κ B, but that possibly also indirect mechanisms such as RA-target gene transcription and *de novo* synthesis of anti-inflammatory proteins may be involved in the anti-inflammatory effects observed.

Moreover effects of RA were stronger on protein level than on mRNA level, suggesting that the anti-inflammatory effect of RA may also include post-transcriptional effects on cytokine release. Investigation of pro-inflammatory transcription factor activation did not show any interference of RA with signalling of the transcription factors NF- κ B, STAT3, p38 or pJNK. It should be noted however that nuclear levels of NF- κ B were measured, and that this only provides information about translocation and not about NF- κ B DNA-binding activities (Na *et al.* 1999). **Chapter 6** describes the *in vivo* application of RA, demonstrating reduced pro-inflammatory cytokine transcript levels already at 6 h after SCI. NF- κ B activation was however not measured in this study. Hence molecular mechanisms that underlie the anti-inflammatory effects of RA remain largely unclear to date.

Chapter 7 describes the potential anti-inflammatory effect of NHR member LXR. Activation of LXR by the synthetic ligand T09 after experimental SCI or in primary astrocytes challenged with LPS, did not attenuate transcript levels of pro-inflammatory cytokines. Nevertheless, others demonstrated cytokine reducing effects on protein level after SCI accompanied by attenuated translocation of NF- κ B into the cell nucleus, suggesting a direct interference of LXR with NF- κ B activation (Paterniti *et al. in press*). This study was performed in mice however, and treatment with T09 was performed twice, at 1 h and 6 h after SCI. In the contrary, we investigated rats that were injected once, immediately after SCI. The effect of multiple injections was not tested in our study, and may underlie the differences in outcome observed. Alternatively, the lack of effect of LXR treatment on pro-inflammatory cytokine transcripts may be explained by different working mechanisms of NHR. LXR may inhibit cytokines only at the protein level, suggesting post-transcriptional effects rather than interference with pro-inflammatory transcription factor

signalling may be involved. However to verify whether such mechanisms are indeed in question, additional (*in vitro*) experiments might study whether translation into protein can be blocked using an translational inhibitor such as anisomycin.

9.1.2 Stimulation of anti-inflammatory events by application of growth factors

The therapies described in chapters 6 and 7 were aiming on inhibition of pro-inflammatory cytokine transcription. Most strategies to limit secondary injury after SCI have been directed towards inhibition of the pro-inflammatory events that occur. As previously described, the inflammatory reaction following SCI is also characterized by beneficial events such as growth factor production that occur simultaneously to the detrimental events. So what about aiming at the beneficial effects of inflammation? Are we able to achieve neuroprotection and subsequent improved long term outcome when growth factor production is boosted? This question was addressed in **chapter 8**. The growth factor VEGF was already shown to exhibit neuroprotective properties after CNS injury, including SCI (Widenfalk *et al.* 2003, Liu *et al.* 2010 *in press*, Krum *et al.* 2008). Effects of VEGF treatment on central neuropathic pain (CNP) after SCI were not yet investigated however. VEGF was administered intrathecally during the first week after SCI via daily bolus injections. This treatment did not improve motor function and had only limited beneficial effects on CNP. The limited effect on behavioural outcome was also reflected by unaltered lesion size following VEGF treatment, as well as glial activation at lumbar levels of the spinal cord. Even when internal VEGF expression levels were elevated via a vector construct for 6 weeks after SCI, motor scores were moderately improved which was accompanied by improved tissue sparing. Effects on CNP were not investigated in this study (Liu *et al.* 2010 *in press*). Recently a study appeared demonstrating pain-inducing effects of VEGF treatment after SCI which was accompanied by growth of myelinated axons into the dorsal columns of injured animals (Nesic *et al.* 2010 *in press*). These data demonstrate that VEGF treatment, if beneficial at all, has only limited neuro-protective effects after SCI.

9.2. Immunomodulation in place

Drug treatment at the right place is vital for optimal treatment. The route of administration of immunomodulating drugs determines the efficacy of a drug as well determines the risk of adverse side effects, which are typically associated with systemic administration of drugs. Methylprednisolone (MP) has not been included in standard care of SCI-patients worldwide, since its use has been shown to have adverse side effects including metabolic complications and increased risk of wound infection. In order to reduce adverse side effects, drugs are often applied locally. To determine whether the route of application of a drug differentially affects the effectiveness of the drug we administered

RA locally via intrathecal catheters, or we applied RA systemically (**Chapter 6**). Intrathecally administered RA up to doses of 100 ng was unable to reduce transcript levels of pro-inflammatory cytokines, whereas a high systemic dose was able to do so.

Although systemic, but not local administration, of RA was able to suppress pro-inflammatory cytokine expression effectively after SCI, clinical use of systemic RA may be limited by adverse side effects. High systemic doses of RA can cause hypervitaminosis, a condition where liver capacity is exceeded leading to toxicity and multiple organ failure (Hathcock *et al.* 1990). It may be argued however that such side effects are not likely to occur, since only one single high dose was administered.

9.3. Current concepts of immunomodulation in time

In the search for potential therapeutic targets, increasing attention has been paid understanding the molecular events that occur immediately after SCI. The direct mechanical damage to neurons, axons, glia cells and blood vessels induces the release of molecules that induce glial activation and initiation of secondary inflammation characterized by the infiltration of cells from the circulation. Different strategies to interfere with these events have been tested experimentally and some are pursued in human clinical trial. The following paragraphs summarize the different molecular targets investigated.

9.3.1. Attenuation of oxidative stress

The impairment of blood flow results in oxidative stress and energy depletion, inducing production of ROS and free radicals that lead to damaged membranes, DNA and proteins. As a strategy against this oxidative damage, free radical scavengers, such as edaravone (MC-186) and resveratrol (polyphenol in red wine) have experimentally been investigated and proven to be effective in experimental SCI (Aoyama *et al.* 2008, Hashizume *et al.* 2005, Takahashi *et al.* 2004, Ates *et al.* 2006, Kaplan *et al.* 2005). Erythropoietin (EPO) increases the oxygen capacity of the blood and has for that reason been used as a drug in patients that suffer from dysfunctional kidneys or anemia. EPO also functions as a pro-inflammatory cytokine inhibitor (Savino *et al.* 2006). In experimental SCI, acute EPO treatment increased spared tissue, decreased cavitation, apoptosis and cytokine levels, reduced lipid peroxidation and improved motor performance (Kaptanoglu *et al.* 2004, Arishima *et al.* 2006, Gorio *et al.* 2002, Gorio *et al.* 2005). On the contrary, other studies reported no beneficial effects of EPO (Mann *et al.* 2008, Pinzon *et al.* 2008a). In humans, free radical scavengers have not been tested yet, however currently clinical trials for the safety and efficacy of EPO (in comparison to MP) in acute SCI were started (<http://clinicaltrials.gov/ct2/home>).

9.3.2 Restoring of energy balance

Energy disbalance is a second major sub-acute problem after SCI. The sudden drop in energy levels leads to dysfunction of ion-pumps, and disbalance of electrolytes. Release of ATP triggers glia cells to become reactive and induces the excessive firing and apoptosis of neurons. Blockers of ATP receptors have therefore been proven to be effective in reducing inflammation after experimental SCI (Peng *et al.* 2009, Wang *et al.* 2004). Inosine, a nucleoside that is formed by the deamination of adenosine has been shown to have neuroprotective properties after SCI in animals (Conta & Stelzner 2008, Liu *et al.* 2006). Though pre-clinical work demonstrated beneficial effects, none of these substances have been tested in clinical trials yet.

9.3.3 Glutamate scavenging

An additional major sub-acute problem after SCI is the excessive release of glutamate, which creates a micro-environment that is toxic to neurons and glial cells. This extracellular glutamate is not only derived from damaged neurons, axons and glial cells, but also supposed to be due to impaired glutamate handling of glial cells during inflammatory conditions (Tilleux & Hermans 2007). This may induce activation of glutamate receptors on neurons and glial cells leading to excessive neuronal firing, glial cell toxicity and eventually cell death (Choi 1988, Park *et al.* 2004, Liu *et al.* 1999). Inhibition of glutamate receptors has been postulated as a possible mechanism to dam the excessive release of glutamate after injury. Nevertheless glutamate is the main excitatory neurotransmitter in the CNS and responsible for the majority of neurotransmission in the CNS. As such, antagonizing glutamate receptors resulted in unwanted side effects. For that reason, glutamate receptor antagonists have not been tested for neuroprotective effects in human clinical trials after CNS injury.

Antagonizing α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate/ kainate (AMPA/KA) receptors by 2,3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) has been shown to attenuate neurotoxicity after experimental SCI (Wrathall *et al.* 1997), suggesting that targeting non-NMDA glutamate receptors may be beneficial after SCI. Although these antagonists have less side effects, clinical trials in humans have not been started yet due to sub-optimal pharmacokinetic properties of the drug. Additionally other non-NMDA antagonists, like gacyclidine, a potent and specific phencyclidine (PCP) analog, has been shown to be neuroprotective in experimental SCI (Feldblum *et al.* 2000, Gaviria *et al.* 2000). Moreover in clinical trials of acute SCI, gacyclidine treatment resulted in inconclusive results on improvements in motor performance at 1 year follow up. Therefore the drug is not longer pursued for the treatment of SCI.

Activation of voltage-gated Na⁺ channels has been associated with excessive neuronal firing, cell damage and glutamate release. As such the Na⁺ channel blocker and indirect NMDA receptor blocker riluzole was shown to have protective properties in experimental SCI (Ates *et al.* 2007, Schwartz & Fehlings 2001), although glutamate levels were not changed (McAdoo *et al.* 2005). Clinically riluzole has already been approved for the treatment of amyotrophic lateral sclerosis and currently clinical trials for the safety of this drug in SCI patients have been started (<http://clinicaltrials.gov/ct2/home>).

9.3.4 Inhibition of gliosis

Glia cells are the first to respond to damaged cells, which makes them an additional sub-acute therapeutic target after SCI. Damaged neurons, axons, glial cells and blood vessels release molecules that trigger glial activation (Zhao *et al.* 2007, Schachtrup *et al.* *in press*, Davalos *et al.* 2005). Glial activation can be detected immediately after the injury, persists up to weeks (Popovich & Hickey 2001, Donnelly & Popovich 2008) and spreads into rostral and caudal direction in time (Popovich *et al.* 1997). The cross-talk between neurons, astrocytes, microglia and the vascular system plays a crucial role in this activation pattern. Crosstalk between microglia and astrocytes was already elegantly shown by Davalos and co-workers where microglia did not respond to laser induced injury when ATP release from astrocytes was blocked by a pharmacological agent (Davalos *et al.* 2005). Astrocytes and microglia produce cytokines and chemokines that activate and attract immune cells from the blood (Pineau *et al.* 2010 *in press*). This property makes glia cells important initiators of the secondary inflammatory reaction that follows SCI. Inhibition of gliosis can therefore be a strategy to prevent initiation of secondary inflammation.

The broad-spectrum, tetracyclic anti-biotic minocyclin, which is meanwhile known for its inhibitory properties on microglial activation (Hains & Waxman 2006, Lai & Todd 2006, Ledeboer *et al.* 2005) has been investigated in models of SCI and in most studies proven to have beneficial effects on tissue sparing after SCI (Kwon *et al.* *in press*). Treatment with minocycline starting within the first hours after SCI was found to be neuroprotective as it attenuated SCI-induced microgliosis, neuronal apoptosis and improved tissue preservation and motor outcome (Wells *et al.* 2003, Festoff *et al.* 2006, Stirling *et al.* 2004, Teng *et al.* 2004, Yune *et al.* 2007), whereas also only moderate neuroprotective effects of minocyclin were found (Saganova *et al.* 2008, Pinzon *et al.* 2008b). Besides increased tissue sparing and motor performance also neuropathic pain behaviour was affected by minocycline treatment. SCI-induced decreases in withdrawal thresholds for thermal and mechanical stimuli were restored after minocycline treatment starting at day 31 after SCI and lasting for 2 days (Hains & Waxman 2006). When minocycline was administered immediately after SCI it also prevented the development of mechanical and thermal hy-

persensitivity (Marchand *et al.* 2009). Moreover attenuation of activated glia by intrathecal propentofylline treatment during the first week after SCI, reduced symptoms of below-level hypersensitivity (Gwak & Hulsebosch 2009). These data indicate that inhibition of gliosis after SCI can be a promising strategy to reduce secondary injury and improve long term outcome. Following these promising preclinical data, clinical trials with minocycline to assess clinical safety and tolerance of the drug were started in SCI patients (<http://clinicaltrials.gov>) and are currently ongoing.

9.3.5 Specific inhibition of cytokines

In experimental models early inhibition of TNF α with infliximab (Genovese *et al.* 2008, Kurt *et al.* 2009), etanercept (Genovese *et al.* 2006) or neutralizing anti-TNF α antibodies (Lee *et al.* 2000) reduced downstream inflammatory mediators such as iNOS and COX2, reduced tissue loss and apoptosis, improved motor outcome and attenuated hypersensitivity (Marchand *et al.* 2009) after SCI. These TNF α inhibitors were already tested for other inflammatory conditions in humans, such as reumatoid arthritis, Crohn's disease and psoriasis (<http://clinicaltrials.gov>) and may therefore be also promising candidates to modulate inflammation following human SCI. Additionally the protein family of interleukins, including IL-1 β and IL-6, have been investigated as potential therapeutic targets in experimental SCI as well. Exogenous application of the interleukin 1 receptor antagonist (IL-1RA) (an endogenous receptor antagonist that blocks activities of both IL-1 α and IL-1 β) was able to reduce IL-1 β mRNA and protein levels, which was accompanied with reduced apoptosis after SCI (Nesic *et al.* 2001). Anakinra, a recombinant form of the human IL-1RA, may also hold particular promise in treatment of SCI as it has already been used for the treatment of joint pain and structural damage in reumatoid arthritis patients.

Also inhibition of early IL-6 signalling has been proposed as a neuroprotective strategy: inhibition of early IL-6 signalling reduced secondary injury after SCI (Tuna *et al.* 2001). Moreover blocking of the IL-6 receptor resulted in attenuated astrogliosis, reduced infiltration of blood-borne immune cells and improved motor performance (Okada *et al.* 2004).

Antibodies against the human IL-6 receptor (MRA: Tocilizumab) are already used in the clinic to treat patients with Castleman's disease and reumatoid arthritis since 2008 and 2009, respectively. All together these studies demonstrate that targeting cytokines can be a powerful tool to control the inflammatory reaction following SCI. Since cytokines play also an important role in the sensitization of spinal nociceptive networks underlying chronic pain conditions, the previously mentioned drugs could also be promising candidates to reduce pain after SCI. Experimentally this has already been tested with etanercept after SCI and with the IL-1RA in other chronic pain conditions, but not yet with the IL-6 receptor antagonists (Marchand *et al.* 2009, Sweitzer *et al.* 2001).

9.3.6 Interference with prostaglandin synthesis

Prostaglandins play an important role in the inflammatory response that follows injury to the CNS and are produced by activated glia, injured neurons, but also endothelial cells (Resnick *et al.* 1998, Phillis *et al.* 2006). Non-steroidal anti-inflammatory drugs (NSAID), including naproxen, ibuprofen and aspirin have been tested in a wide variety of experimental inflammatory conditions and their main mechanism of action is thought to be mediated by blocking COX2 activity, which is responsible for the production of prostaglandins (Phillis *et al.* 2006, Farooqui *et al.* 2007). Since prostaglandins are also involved in the sensitization of nociceptive networks, these drugs not only have anti-inflammatory properties, but also function as analgesics (Zeilhofer 2007). After experimental SCI, ibuprofen decreased secondary injury accompanied with improved functional outcome in rats (Wang *et al.* 2009). On the contrary, naproxen did not improve motor outcome (Wang *et al.* 2009). Likewise, data on the use of indomethacin were not conclusive since reduced (Harada *et al.* 2006), improved (Pantovic *et al.* 2005, Resnick *et al.* 1998, Simpson *et al.* 1991) and unaffected (Guth *et al.* 1994, Sharma 2005, Winkler *et al.* 1993) motor function have been reported. Aspirin treatment was shown to have beneficial effects on motor outcome (Fujita *et al.* 1985).

NSAIDs have been shown to reduce pain in experimental settings as well as in non-SCI patients and have therefore been tested as analgesics. Whether NSAID also reduce secondary injury or pain after SCI has not been tested in clinical trials yet.

9.3.7 Interference with extravasation

Extravasation of blood-born immune cells is a key event in the inflammatory reaction after traumatic SCI, and plays an important role in the expansion of tissue damage. In the extravasation process, integrins expressed on leukocytes bind to adhesion molecules on endothelial cells and in that way extravasation of macrophages, neutrophils and lymphocytes into the spinal cord parenchyma is facilitated. Anti-CD11d/CD18 integrin antibodies reduced infiltration of macrophages and neutrophils, which was accompanied with reduced tissue damage, decreased ROS production, lipid peroxidation, apoptosis and pro-inflammatory gene expression after experimental SCI (Bao *et al.* 2005, Gris *et al.* 2005, Gris *et al.* 2009, Weaver *et al.* 2005). Behaviorally, improvement of motor function and reduction in mechanical hypersensitivity could be observed (Ditor *et al.* 2006, Gris *et al.* 2004, Oatway *et al.* 2005). Anti-integrin antibodies have not been tested yet in clinical studies. The infiltration of blood-borne immune cells has however also been associated with repair mechanisms (Hofstetter *et al.* 2003, Shechter *et al.* 2009). This dual function of blood born cells makes it extremely difficult to limit only the harmful effects of these cells.

9.4. Immunomodulation following SCI, a lost cause?

As shown in this thesis, many therapeutics have been explored to reduce inflammation after SCI, demonstrating limited recovery and improved tissue sparing after experimental SCI. Nevertheless, this tremendous set of pre-clinical data resulted in only a few clinical trials. Why are many substances not tested in human clinical trials? Obviously data do not provide sufficient evidence to start, expensive, time consuming and demanding studies in humans with SCI. As such the answer to the question why we find only limited neuroprotective effects, by inhibiting detrimental or boosting beneficial events after SCI, remains unanswered.

During the first minutes and hours after SCI, the inflammatory reaction is mainly dominated by the innate immune response, which is a-specific and causes bystander damage, whereas after one week the more specific adaptive immune system is activated. Simultaneously, also beneficial events occur and repair mechanisms are activated. These two faces of the inflammatory reaction may underlie the limited effects observed, since inhibition of the early detrimental effects may also impair neuroprotective events and repair mechanisms. So the beneficial and detrimental effects that occur after SCI take place at the same time and may be inevitably interdependent. This makes it extremely difficult, or even impossible to modulate the detrimental or beneficial events separately.

Up to now most strategies have been directed either towards inhibition of damaging events, or towards stimulation of repair mechanisms that occur.

9.5. So which way should we go then?

Changing the direction of the inflammatory reaction following SCI, may be a promising new strategy to provide therapeutic benefit after SCI. Mukaino and co-workers already demonstrated that blocking of the IL-6 receptor using an antibody (MR16-1) administered immediately after SCI, changed the character of the inflammatory response.

The inflammatory reaction that normally is characterized by the activation of hematogenous macrophages was now microglia-dominated. This change of character was also accompanied by reduced expression of the macrophage-recruiting chemokines CCL2, CCL5 and CXCL10 and increased expression of microglia mitogen GM-CSF (Mukaino *et al. in press*). Macrophages are important for the clearance of cell debris; as such the phagocytic capacity of the microglia was assessed. Interestingly, microglia showed an increased expression of the lysosomal marker LAMP2, phagocytosis marker Mac2 and increased engulfment of Oil red O labelled lipids when compared to macrophages (Mukaino *et al. in press*). These data suggest that microglia-dominated inflammation leads to increased tissue clearance after SCI, which is known to be beneficial to axonal re-growth

and cell survival (Silver & Miller 2004). The beneficial effects of changing the nature of inflammation were also reflected by the analysis of myelin and fibre sparing, which was significantly improved in MR16-1 treated animals (Mukaino *et al. in press*).

This study points out the importance of studying the different characters of inflammatory cells that determine the beneficial or detrimental effects of inflammation. The molecular cues glial cells encounter early after SCI, may play a crucial role in these different activation patterns (Ransohoff & Perry 2009, White & Jakeman 2008). Different activation patterns of cells in response to environmental cues could already be observed in macrophages; incubation of macrophages with peripheral nerves and skin resulted in activated cells that induced repair (Bomstein *et al.* 2003, Schwartz *et al.* 1999), whereas macrophages activated by the yeast product, zymosan resulted in activated cells with a detrimental phenotype (Popovich *et al.* 2002). This may also apply to the activation of glial cells early after SCI. Whether different activation patterns of glia are able to change the nature of the inflammatory reaction needs further investigations in the future however.

As such, it can be suggested that effective treatment of SCI thus may reside in modulation of SCI-induced inflammation in order to change its character rather than to tackling detrimental or boosting beneficial effects *per se*.

References

- Aoyama, T., Hida, K., Kuroda, S., Seki, T., Yano, S., Shichinohe, h. and Iwasaki, Y. (2008) Edaravone (MCI-186) scavenges reactive oxygen species and ameliorates tissue damage in the murine spinal cord injury model. *Neurol Med Chir (Tokyo)*, **48**, 539–545; discussion 545
- Arishima, Y., Setoguchi, T., Yamaura, I., Yone, K. and Komiya, S. (2006) Preventive effect of erythropoietin on spinal cord cell apoptosis following acute traumatic injury in rats. *Spine (Phila Pa 1976)*, **31**, 2432–2438
- Ates, O., Cayli, S., Altinoz, E., Gurses, I., Yucel, N., Kocak, A., Yologlu, S. and Turkoz, Y. (2006) Effects of resveratrol and methylprednisolone on biochemical, neurobehavioral and histopathological recovery after experimental spinal cord injury. *Acta Pharmacol Sin*, **27**, 1317–1325
- Ates, O., Cayli, S. R., Gurses, I., Turkoz, Y., Tarim, O., Cakir, C. O. and Kocak, A. (2007) Comparative neuroprotective effect of sodium channel blockers after experimental spinal cord injury. *J Clin Neurosci*, **14**, 658–665
- Bao, F., Dekaban, G. A. and Weaver, L. C. (2005) Anti-CD11d antibody treatment reduces free radical formation and cell death in the injured spinal cord of rats. *J Neurochem*, **94**, 1361–1373
- Bomstein, Y., Marder, J. B., Vitner, K., Smirnov, I., Lisaey, G., Butovsky, O., Fulga, V. and Yoles, E. (2003) Features of skin-coincubated macrophages that promote recovery from spinal cord injury. *J Neuroimmunol*, **142**, 10–16
- Brown, A., Ricci, M. J. and Weaver, L. C. (2004) NGF message and protein distribution in the injured rat spinal cord. *Exp Neurol*, **188**, 115–127
- Bush, T. G., Puvanachandra, N., Horner, C. h., Polito, A., Ostenfeld, T., Svendsen, C. N., Mucke, L., Johnson, M. h. and Sofroniew, M. V. (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. *Neuron*, **23**, 297–308
- Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron*, **1**, 623–634
- Conta, A. C. and Stelzner, D. J. (2008) Immunomodulatory effect of the purine nucleoside inosine following spinal cord contusion injury in rat. *Spinal Cord*, **46**, 39–44
- Davalos, D., Grutzendler, J., Yang, G., Kim, J. V., Zuo, Y., Jung, S., Littman, D. R., Dustin, M. L. and Gan, W. B. (2005) ATP mediates rapid microglial response to local brain injury *in vivo*. *Nat Neurosci*, **8**, 752–758
- Ditor, D. S., Bao, F., Chen, Y., Dekaban, G. A. and Weaver, L. C. (2006) A therapeutic time window for anti-CD 11d monoclonal antibody treatment yielding reduced secondary tissue damage and enhanced behavioral recovery following severe spinal cord injury. *J Neurosurg Spine*, **5**, 343–352
- Donnelly, D. J. and Popovich, P. G. (2008) Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Exp Neurol*, **209**, 378–388
- Farooqui, A. A., Horrocks, L. A. and Farooqui, T. (2007) Modulation of inflammation in brain: a matter of fat. *J Neurochem*, **101**, 577–599
- Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B. and Sofroniew, M. V. (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J Neurosci*, **24**, 2143–2155
- Feldblum, S., Arnaud, S., Simon, M., Rabin, O. and D'Arbigny, P. (2000) Efficacy of a new neuroprotective agent, gacyclidine, in a model of rat spinal cord injury. *J Neurotrauma*, **17**, 1079–1093

- Festoff, B. W., Ameenuddin, S., Arnold, P. M., Wong, A., Santacruz, K. S. and Citron, B. A. (2006) minocycline neuroprotects, reduces microgliosis, and inhibits caspase protease expression early after spinal cord injury. *J Neurochem*, **97**, 1314–1326
- Fujita, Y., Shingu, T., Kurihara, M., Miyake, h., Kono, T., Tsujimura, M. and Mori, K. (1985) Evaluation of a low dose administration of aspirin, dipyridamol and steroid. Therapeutic effects on motor function and protective effects on Na⁺-K⁺-activated ATPase activity against lipid peroxidation in an experimental model of spinal cord injury. *Paraplegia*, **23**, 56–57
- Gaviria, M., Privat, A., d'Arbigny, P., Kamenka, J., Haton, h. and Ohanna, F. (2000) Neuroprotective effects of a novel NMDA antagonist, Gacyclidine, after experimental contusive spinal cord injury in adult rats. *Brain Res*, **874**, 200–209
- Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Muia, C., Bramanti, P. and Cuzzocrea, S. (2006) Immunomodulatory effects of etanercept in an experimental model of spinal cord injury. *J Pharmacol Exp Ther*, **316**, 1006–1016
- Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Muia, C., Esposito, E., Bramanti, P. and Cuzzocrea, S. (2008) TNF-alpha blockage in a mouse model of SCI: evidence for improved outcome. *Shock*, **29**, 32–41
- Gorio, A., Gokmen, N., Erbayraktar, S. *et al.* (2002) Recombinant human erythropoietin counteracts secondary injury and markedly enhances neurological recovery from experimental spinal cord trauma. *Proc Natl Acad Sci U s A*, **99**, 9450–9455
- Gorio, A., Madaschi, L., Di Stefano, B., Carelli, S., Di Giulio, A. M., De Biasi, S., Coleman, T., Cerami, A. and Brines, M. (2005) Methylprednisolone neutralizes the beneficial effects of erythropoietin in experimental spinal cord injury. *Proc Natl Acad Sci U s A*, **102**, 16379–16384
- Gris, D., Marsh, D. R., Dekaban, G. A. and Weaver, L. C. (2005) Comparison of effects of methylprednisolone and anti-CD11d antibody treatments on autonomic dysreflexia after spinal cord injury. *Exp Neurol*, **194**, 541–549
- Gris, D., Marsh, D. R., Oatway, M. A., Chen, Y., Hamilton, E. F., Dekaban, G. A. and Weaver, L. C. (2004) Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. *J Neurosci*, **24**, 4043–4051
- Gris, P., Tighe, A., Thawer, S., Hemphill, A., Oatway, M., Weaver, L., Dekaban, G. A. and Brown, A. (2009) Gene expression profiling in anti-CD11d mAb-treated spinal cord-injured rats. *J Neuroimmunol*, **209**, 104–113
- Guth, L., Zhang, Z., DiProspero, N. A., Joubin, K. and Fitch, M. T. (1994) Spinal cord injury in the rat: treatment with bacterial lipopolysaccharide and indomethacin enhances cellular repair and locomotor function. *Exp Neurol*, **126**, 76–87
- Gwak, Y. S. and Hulsebosch, C. E. (2009) Remote astrocytic and microglial activation modulates neuronal hyperexcitability and below-level neuropathic pain after spinal injury in rat. *Neuroscience*, **161**, 895–903
- Hains, B. C. and Waxman, S. G. (2006) Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. *J Neurosci*, **26**, 4308–4317
- Harada, N., Taoka, Y. and Okajima, K. (2006) Role of prostacyclin in the development of compression trauma-induced spinal cord injury in rats. *J Neurotrauma*, **23**, 1739–1749

- Hashizume, K., Ueda, T., Shimizu, h., Mori, A. and Yozu, R. (2005) Effect of the free radical scavenger MCI-186 on spinal cord reperfusion after transient ischemia in the rabbit. *Jpn J Thorac Cardiovasc Surg*, **53**, 426–433
- Hathcock, J. N., Hattan, D. G., Jenkins, M. Y., McDonald, J. T., Sundaresan, P. R. and Wilkening, V. L. (1990) Evaluation of vitamin A toxicity. *Am J Clin Nutr*, **52**, 183–202
- Hill, C. E., Beattie, M. S. and Bresnahan, J. C. (2001) Degeneration and sprouting of identified descending supraspinal axons after contusive spinal cord injury in the rat. *Exp Neurol*, **171**, 153–169
- Hofstetter, h. h., Sewell, D. L., Liu, F., Sandor, M., Forsthuber, T., Lehmann, P. V. and Fabry, Z. (2003) Autoreactive T cells promote post-traumatic healing in the central nervous system. *J Neuroimmunol*, **134**, 25–34
- Ikeda, O., Murakami, M., Ino, h., Yamazaki, M., Nemoto, T., Koda, M., Nakayama, C. and Moriya, h. (2001) Acute up-regulation of brain-derived neurotrophic factor expression resulting from experimentally induced injury in the rat spinal cord. *Acta Neuropathol*, **102**, 239–245
- Jones, T. B., Basso, D. M., Sodhi, A., Pan, J. Z., Hart, R. P., MacCallum, R. C., Lee, S., Whitacre, C. C. and Popovich, P. G. (2002) Pathological CNS autoimmune disease triggered by traumatic spinal cord injury: implications for autoimmune vaccine therapy. *J Neurosci*, **22**, 2690–2700
- Jones, T. B., Hart, R. P. and Popovich, P. G. (2005) Molecular control of physiological and pathological T-cell recruitment after mouse spinal cord injury. *J Neurosci*, **25**, 6576–6583
- Kaplan, S., Bisleri, G., Morgan, J. A., Cheema, F. h. and Oz, M. C. (2005) Resveratrol, a natural red wine polyphenol, reduces ischemia-reperfusion-induced spinal cord injury. *Ann Thorac Surg*, **80**, 2242–2249
- Kaptanoglu, E., Solaroglu, I., Okutan, O., Surucu, h. S., Akbiyik, F. and Beskonakli, E. (2004) Erythropoietin exerts neuroprotection after acute spinal cord injury in rats: effect on lipid peroxidation and early ultrastructural findings. *Neurosurg Rev*, **27**, 113–120
- Krum, J. M., Mani, N. and Rosenstein, J. M. (2008) Roles of the endogenous VEGF receptors flt-1 and flk-1 in astroglial and vascular remodeling after brain injury. *Exp Neurol*, **212**, 108–117
- Kurt, G., Ergun, E., Cemil, B., Borcek, A. O., Borcek, P., Gulbahar, O. and Ceviker, N. (2009) Neuroprotective effects of infliximab in experimental spinal cord injury. *Surg Neurol*, **71**, 332–336, discussion 336
- Kwon, B. K., Okon, E., Hillyer, J., Mann, C., Baptiste, D., Weaver, L. C., Fehlings, M. G. and Tetzlaff, W. (in press) A Systematic Review of Non-Invasive Pharmacologic Neuroprotective Treatments for Acute Spinal Cord Injury. *J Neurotrauma*
- Lai, A. Y. and Todd, K. G. (2006) Hypoxia-activated microglial mediators of neuronal survival are differentially regulated by tetracyclines. *Glia*, **53**, 809–816
- Ledeboer, A., Sloane, E. M., Milligan, E. D., Frank, M. G., Mahony, J. h., Maier, S. F. and Watkins, L. R. (2005) minocycline attenuates mechanical allodynia and proinflammatory cytokine expression in rat models of pain facilitation. *Pain*, **115**, 71–83
- Lee, Y. B., Yune, T. Y., Baik, S. Y. et al. (2000) Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury. *Exp Neurol*, **166**, 190–195
- Liu, D., Xu, G. Y., Pan, E. and McAdoo, D. J. (1999) Neurotoxicity of glutamate at the concentration released upon spinal cord injury. *Neuroscience*, **93**, 1383–1389
- Liu, F., You, S. W., Yao, L. P., Liu, h. L., Jiao, X. Y., Shi, M., Zhao, Q. B. and Ju, G. (2006) Secondary degeneration reduced by inosine after spinal cord injury in rats. *Spinal Cord*, **44**, 421–426

- Liu, Y., Figley, S., Spratt, S. K., Lee, G., Ando, D., Surosky, R. and Fehlings, M. G. (2010 in press) An engineered transcription factor which activates VEGF-A enhances recovery after spinal cord injury. *Neurobiol Dis*, **37**, 384–393
- Mann, C., Lee, J. H., Liu, J., Stammers, A. M., Sohn, h. M., Tetzlaff, W. and Kwon, B. K. (2008) Delayed treatment of spinal cord injury with erythropoietin or darbepoetin—a lack of neuroprotective efficacy in a contusion model of cord injury. *Exp Neurol*, **211**, 34–40
- Marchand, F., Tsantoulas, C., Singh, D., Grist, J., Clark, A. K., Bradbury, E. J. and McMahon, S. B. (2009) Effects of Etanercept and minocycline in a rat model of spinal cord injury. *Eur J Pain*, **13**, 673–681
- McAdoo, D. J., Hughes, M. G., Nie, L., Shah, B., Clifton, C., Fullwood, S. and Hulsebosch, C. E. (2005) The effect of glutamate receptor blockers on glutamate release following spinal cord injury. Lack of evidence for an ongoing feedback cascade of damage --> glutamate release --> damage --> glutamate release --> etc. *Brain Res*, **1038**, 92–99
- Mukaino, M., Nakamura, M., Yamada, O. *et al.* (in press) Anti-IL-6-receptor antibody promotes repair of spinal cord injury by inducing microglia-dominant inflammation. *Exp Neurol*, **224**, 403–414
- Na, S. Y., Kang, B. Y., Chung, S. W., Han, S. J., Ma, X., Trinchieri, G., Im, S. Y., Lee, J. W. and Kim, T. S. (1999) Retinoids inhibit interleukin-12 production in macrophages through physical associations of retinoid X receptor and NFkappaB. *J Biol Chem*, **274**, 7674–7680
- Nesic, O., Sundberg, L. M., Herrera, J. J., Mokkalapati, V. U., Lee, J. and Narayana, P. A. (2010 in press) Vascular endothelial growth factor and spinal cord injury pain. *J Neurotrauma*, **27**, 1793–1803
- Nesic, O., Xu, G. Y., McAdoo, D., High, K. W., Hulsebosch, C. and Perez-Pol, R. (2001) IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. *J Neurotrauma*, **18**, 947–956
- Oatway, M. A., Chen, Y., Bruce, J. C., Dekaban, G. A. and Weaver, L. C. (2005) Anti-CD11d integrin antibody treatment restores normal serotonergic projections to the dorsal, intermediate, and ventral horns of the injured spinal cord. *J Neurosci*, **25**, 637–647
- Okada, S., Nakamura, M., Katoh, h. *et al.* (2006) Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med*, **12**, 829–834
- Okada, S., Nakamura, M., Mikami, Y. *et al.* (2004) Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. *J Neurosci Res*, **76**, 265–276
- Pantovic, R., Draganic, P., Erakovic, V., Blagovic, B., Milin, C. and Simonovic, A. (2005) Effect of indomethacin on motor activity and spinal cord free fatty acid content after experimental spinal cord injury in rabbits. *Spinal Cord*, **43**, 519–526
- Park, E., Velumian, A. A. and Fehlings, M. G. (2004) The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma*, **21**, 754–774
- Paterniti, I., Genovese, T., Mazzon, E., Crisafulli, C., Di Paola, R., Galuppo, M., Bramanti, P. and Cuzzocrea, S. (in press) Liver X receptor agonist treatment regulates inflammatory response after spinal cord trauma. *J Neurochem*, **112**, 611–624
- Peng, W., Cotrina, M. L., Han, X. *et al.* (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. *Proc Natl Acad Sci U S A*, **106**, 12489–12493

- Phillis, J. W., Horrocks, L. A. and Farooqui, A. A. (2006) Cyclooxygenases, lipoxigenases, and epoxygenases in CNS: their role and involvement in neurological disorders. *Brain Res Rev*, **52**, 201–243
- Pineau, I., Sun, L., Bastien, D. and Lacroix, S. (2010 in press) Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion. *Brain Behav Immun*, **24**, 540–553
- Pinzon, A., Marcillo, A., Pabon, D., Bramlett, h. M., Bunge, M. B. and Dietrich, W. D. (2008a) A re-assessment of erythropoietin as a neuroprotective agent following rat spinal cord compression or contusion injury. *Exp Neurol*, **213**, 129–136
- Pinzon, A., Marcillo, A., Quintana, A., Stamler, S., Bunge, M. B., Bramlett, h. M. and Dietrich, W. D. (2008b) A re-assessment of minocycline as a neuroprotective agent in a rat spinal cord contusion model. *Brain Res*, **1243**, 146–151
- Popovich, P. G., Guan, Z., McGaughy, V., Fisher, L., Hickey, W. F. and Basso, D. M. (2002) The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. *J Neuropathol Exp Neurol*, **61**, 623–633
- Popovich, P. G. and Hickey, W. F. (2001) Bone marrow chimeric rats reveal the unique distribution of resident and recruited macrophages in the contused rat spinal cord. *J Neuropathol Exp Neurol*, **60**, 676–685
- Popovich, P. G., Wei, P. and Stokes, B. T. (1997) Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats. *J Comp Neurol*, **377**, 443–464
- Ransohoff, R. M. and Perry, V. h. (2009) Microglial physiology: unique stimuli, specialized responses. *Annu Rev Immunol*, **27**, 119–145
- Resnick, D. K., Graham, S. h., Dixon, C. E. and Marion, D. W. (1998) Role of cyclooxygenase 2 in acute spinal cord injury. *J Neurotrauma*, **15**, 1005–1013
- Saganova, K., Orendacova, J., Cizkova, D. and Vanicky, I. (2008) Limited minocycline neuroprotection after balloon-compression spinal cord injury in the rat. *Neurosci Lett*, **433**, 246–249
- Savino, C., Pedotti, R., Baggi, F. *et al.* (2006) Delayed administration of erythropoietin and its non-erythropoietic derivatives ameliorates chronic murine autoimmune encephalomyelitis. *J Neuroimmunol*, **172**, 27–37
- Schachtrup, C., Ryu, J. K., Helmrick, M. J., Vagena, E., Galanakis, D. K., Degen, J. L., Margolis, R. U. and Akassoglou, K. (*in press*) Fibrinogen triggers astrocyte scar formation by promoting the availability of active TGF-beta after vascular damage. *J Neurosci*, **30**, 5843–5854
- Schwartz, G. and Fehlings, M. G. (2001) Evaluation of the neuroprotective effects of sodium channel blockers after spinal cord injury: improved behavioral and neuroanatomical recovery with riluzole. *J Neurosurg*, **94**, 245–256
- Schwartz, M., Cohen, I., Lazarov-Spiegler, O., Moalem, G. and Yoles, E. (1999) The remedy may lie in ourselves: prospects for immune cell therapy in central nervous system protection and repair. *J Mol Med*, **77**, 713–717
- Sharma, h. S. (2005) Pathophysiology of blood-spinal cord barrier in traumatic injury and repair. *Curr Pharm Des*, **11**, 1353–1389
- Shechter, R., London, A., Varol, C. *et al.* (2009) Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice. *PLoS Med*, **6**, e1000113

- Silver, J. and Miller, J. h. (2004) Regeneration beyond the glial scar. *Nat Rev Neurosci*, **5**, 146–156
- Simpson, R. K., Jr., Baskin, D. S., Dudley, A. W., Bogue, L. and Rothenberg, F. (1991) The influence of long-term nifedipine or indomethacin therapy on neurologic recovery from experimental spinal cord injury. *J Spinal Disord*, **4**, 420–427
- Stirling, D. P., Khodarahmi, K., Liu, J., McPhail, L. T., McBride, C. B., Steeves, J. D., Ramer, M. S. and Tetzlaff, W. (2004) minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury. *J Neurosci*, **24**, 2182–2190
- Sweitzer, S., Martin, D. and DeLeo, J. A. (2001) Intrathecal interleukin-1 receptor antagonist in combination with soluble tumor necrosis factor receptor exhibits an anti-allodynic action in a rat model of neuropathic pain. *Neuroscience*, **103**, 529–539
- Takahashi, G., Sakurai, M., Abe, K., Itoyama, Y. and Tabayashi, K. (2004) MCI-186 reduces oxidative cellular damage and increases DNA repair function in the rabbit spinal cord after transient ischemia. *Ann Thorac Surg*, **78**, 602–607
- Teng, Y. D., Choi, h., Onario, R. C. *et al.* (2004) minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury. *Proc Natl Acad Sci U S A*, **101**, 3071–3076
- Tilleux, S. and Hermans, E. (2007) Neuroinflammation and regulation of glial glutamate uptake in neurological disorders. *J Neurosci Res*, **85**, 2059–2070
- Tuna, M., Polat, S., Erman, T., Ildan, F., Gocer, A. I., Tuna, N., Tamer, L., Kaya, M. and Cetinalp, E. (2001) Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5'-triphosphatase and superoxide dismutase activation, and ultrastructural changes. *J Neurosurg*, **95**, 64–73
- Wang, X., Arcuino, G., Takano, T. *et al.* (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med*, **10**, 821–827
- Wang, X., Budel, S., Baughman, K., Gould, G., Song, K. h. and Strittmatter, S. M. (2009) Ibuprofen enhances recovery from spinal cord injury by limiting tissue loss and stimulating axonal growth. *J Neurotrauma*, **26**, 81–95
- Weaver, L. C., Gris, D., Saville, L. R., Oatway, M. A., Chen, Y., Marsh, D. R., Hamilton, E. F. and Dekaban, G. A. (2005) Methylprednisolone causes minimal improvement after spinal cord injury in rats, contrasting with benefits of an anti-integrin treatment. *J Neurotrauma*, **22**, 1375–1387
- Wells, J. E., Hurlbert, R. J., Fehlings, M. G. and Yong, V. W. (2003) Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice. *Brain*, **126**, 1628–1637
- White, R. E. and Jakeman, L. B. (2008) Don't fence me in: harnessing the beneficial roles of astrocytes for spinal cord repair. *Restor Neurol Neurosci*, **26**, 197–214
- Widenfalk, J., Lipson, A., Jubran, M., Hofstetter, C., Ebendal, T., Cao, Y. and Olson, L. (2003) Vascular endothelial growth factor improves functional outcome and decreases secondary degeneration in experimental spinal cord contusion injury. *Neuroscience*, **120**, 951–960
- Winkler, T., Sharma, h. S., Stalberg, E. and Olsson, Y. (1993) Indomethacin, an inhibitor of prostaglandin synthesis attenuates alteration in spinal cord evoked potentials and edema formation after trauma to the spinal cord: an experimental study in the rat. *Neuroscience*, **52**, 1057–1067

- Wrathall, J. R., Teng, Y. D. and Marriott, R. (1997) Delayed antagonism of AMPA/kainate receptors reduces long-term functional deficits resulting from spinal cord trauma. *Exp Neurol*, **145**, 565–573
- Yune, T. Y., Lee, J. Y., Jung, G. Y., Kim, S. J., Jiang, M. h., Kim, Y. C., Oh, Y. J., Markelonis, G. J. and Oh, T. h. (2007) minocycline alleviates death of oligodendrocytes by inhibiting pro-nerve growth factor production in microglia after spinal cord injury. *J Neurosci*, **27**, 7751–7761
- Zeilhofer, h. U. (2007) Prostanoids in nociception and pain. *Biochem Pharmacol*, **73**, 165–174
- Zhao, P., Waxman, S. G. and Hains, B. C. (2007) Modulation of thalamic nociceptive processing after spinal cord injury through remote activation of thalamic microglia by cysteine cysteine chemokine ligand 21. *J Neurosci*, **27**, 8893–8902

Abbreviations

A

AA: arachidonic acid
 A β : beta amyloid peptide
 ABCA: ATP-binding cassette transporter
 AD: Alzheimer's disease
 ADH: alcohol dehydrogenase
 ALS: amyotrophic lateral sclerosis
 AMPA: α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
 ANOVA: analysis of variance
 AP: activator protein
 APP: amyloid precursor protein
 ATP: adenosine tri-phosphate
 AUC: area under the curve

B

BBB: Basso, Beattie, and Bresnahan locomotor scale
 BDA: biotinylated dextran amine
 BDNF: brain-derived neurotrophic factor
 BOS: base of support
 BSCB: blood spinal cord barrier

C

CD: cluster of differentiation
 cDNA: copy deoxyribonucleic acid
 CSPG: chondroitin sulphate proteoglycan
 CCL: CC chemokine ligand
 CXCL: CXC chemokine ligand
 CNP: central neuropathic pain
 CNS: central nervous system
 CNTF: ciliary neurotrophic factor
 COX: cyclooxygenase
 CRABP: cellular retinoic acid binding protein
 CRBP: cellular retinol binding protein
 CREB: cAMP responsive element binding protein

D

DHA: docosahexaenoic acid
 DMSO: dimethylsulfoxide
 DAPI: 4',6-diamidino-2-phenylindole dihydrochloride
 dpo: dies post operationem

E

EAE: experimental allergic encephalomyelitis
 EDTA: ethylenediaminetetraacetic
 EGF: epidermal growth factor
 Egr: EGF receptor
 ELISA: enzyme-linked immunosorbent assay
 EPA: eicosapentaenoic acid
 EPO: erythropoietin
 EPSP: excitatory postsynaptic potential
 ErbB: Tyrosine kinase related to the EGF receptor
 ERK: extracellular signal-regulated kinase

F

FABP: fatty acid binding protein

G

GABA: gamma-aminobutyric acid
 GAP43: growth associated protein 43
 GAPDH: glyceraldehyde 3-phosphate dehydrogenase
 GDNF: glial-cell derived neurotrophic factor
 GFAP: glial fibrillary acidic protein
 GR: glucocorticoid receptor

H

HD, Huntington's disease

HPRT: hypoxanthine-guanine phosphoribosyltransferase

HSP: heat shock protein

I

IBA: ionized calcium binding adaptor molecule 1

ICAM: intercellular cell adhesion molecule

IFN: interferon

IκB: inhibitor of NF-κB

IL: interleukin

IL1-RA: interleukin 1 receptor antagonist

iNOS: inducible NO synthase

IPSP: inhibitory postsynaptic potential

IRF: Interferon regulatory factor

J

Jak: Janus kinase

JNK: Jun amino-terminal kinase

K

KA: Kainate

L

LA: linolenic acid

LDH: lactate dehydrogenase

LFB: luxol fast blue

LPS: lipopolysaccharide

LXR: liver X receptor

LTB₄: leukotriene B₄

M

MAPK: mitogen-activated protein kinase

MBP: myelin basic protein

MC-186: edaravone

MCP: macrophage chemoattractant protein

MHC: major histocompatibility complex

MIP: macrophage inflammatory protein

MMP: matrix metalloproteinase

MP: methylprednisolone

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRI: magnetic resonance imaging; MS, multiple sclerosis

N

NASCIS: National Acute Spinal Cord Injury Study

NBM: neurobasal medium

NGF: nerve growth factor

NHR: nuclear hormone receptor

NF-κB: nuclear factor κB

NMDA: N-methyl-D-aspartic acid

NO: nitric oxide

NOS: nitric oxide synthase

NR: nuclear receptor

NSAID: non-steroidal anti-inflammatory drug

O

OD: optical density

P

PB: phosphate buffer

PCP: phencyclidine

PCR: polymerase chain reaction

PD: Parkinson's disease

PEA: palmitoyl ethanolamide

PG: prostaglandin

PGE: prostaglandin E

15d-PGJ₂: 15-deoxy-delta PG J₂

PI3-K: Phosphoinositide 3-kinase

PNS: peripheral nervous system

PPAR: peroxisome proliferator activated receptor

PPRE: PPAR response element

PUFA: polyunsaturated fatty acid

PWL: paw withdrawal latency

PWT: paw withdrawal threshold

R

RA: retinoic acid
 RALDH: retinaldehyde dehydrogenase
 RANTES: regulated upon activation normal T-cell expressed and secreted
 RAR: RA receptor
 RARE: retinoic acid response element
 RBP: retinol binding protein
 RNA: ribonucleic acid
 ROI: region of interest
 ROS: reactive oxygen species
 RT: reverse transcription
 RXR: retinoid X receptor

S

SCI: spinal cord injury
 SDR: short-chain dehydrogenase/reductase
 SEM: standard error of the mean
 SOCS: suppressor of cytokine signaling
 SOD: superoxide dismutase
 STAT: signal transducer and activator of transcription

T

T0901317: N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide
 T9: thoracic level 9 of the spinal cord
 TBST: tris-buffered saline with 0.4% Triton X-100
 TGF: transforming growth factor
 Tgm-2: transglutaminase-2
 THR: thyroid hormone receptor
 Th: T-helper cell
 TLR: toll-like receptor
 TNF: tumor necrosis factor
 TR: thyroid hormone receptor
 TTNPB: 4-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid
 2,4-TZD: 2,4-thiazolidinedione

V

VCAM: vascular cell adhesion molecule
 VEGF: vascular endothelial growth factor

Summary

The aim of the present thesis was to study novel therapeutic approaches to modulate the inflammatory reaction that follows experimental SCI to induce improved motor function and reduced pain. As early pro-inflammatory cytokine production after SCI is essential for the initiation and the maintenance of the inflammatory reaction, as a first strategy the cytokine transcript reducing effect of the nuclear hormone receptor ligands retinoic acid (RA) and T0901317 (T09) was investigated.

As a second strategy, boosting of beneficial effects of the inflammatory reaction was studied via exogenous application of the growth factor VEGF after SCI.

The first chapter of this thesis briefly gives an introduction into the clinical problems that arise after damage to the spinal cord. Patients with spinal cord injury not only suffer from the devastating consequences of sensory and motor loss, a substantial percentage develops chronic pain after injury. Sufficient pain relief cannot be achieved with the current analgesics available and therefore this pain often leads to decreased quality of life, depression and even suicide in SCI patients. This first chapter gives also an impression how we study these aspects of long term outcome in animal models of SCI. A crucial event that determines long term outcome after SCI is the inflammatory reaction that follows the initial damage. As such, this first chapter describes the cellular and molecular events that occur during this inflammatory reaction.

The second chapter of this thesis provides information about two members of the nuclear hormone receptor family; peroxisome proliferator activated receptors (PPAR/RXR) and retinoic acid receptors (RAR/RXR). This chapter sheds light on how the activation of these receptors may be involved in different neurological and psychiatric diseases, and how their activation may provide neuroprotection.

The third chapter of this thesis describes the relevance of PPAR and RAR/RXR activation in spinal cord injury. The data demonstrate that RAR/RXR and PPAR/RXR are activated after SCI and that targeting PPAR/RXR and RAR/RXR activation by natural or synthetic ligands may be able to reduce inflammatory processes after SCI.

Chapter four describes the *in vitro* testing of RAR/RXR natural ligand all-*trans* RA as a potential anti-inflammatory agent. To mimic an inflammatory reaction *in vitro*, primary astrocyte cultures were stimulated with bacterial products and RA was administered as an intervention method to reduce this inflammatory reaction. The data described

in this chapter not only demonstrate the anti-inflammatory effect of RA on chemokine production by astrocytes, it also sheds light on the molecular mechanisms that may underlie this RA effect.

Chapter five of this thesis demonstrates that the anti-inflammatory effect of RA is not only restricted to chemokine production by primary astrocytes. RA also reduced the expression and production of pro-inflammatory cytokines *in vitro*.

Chapter six describes the *in vivo* application of RA as a strategy to reduce pro-inflammatory cytokine expression after experimental SCI. Two different routes of application were tested in this study, local application via intrathecal catheters and systemic application via an intraperitoneal injection. Only after systemic application, but not after local administration, RA was able to reduce transcript levels of pro-inflammatory cytokines. This study points out that the route of administration of a drug differentially affects the efficacy of a drug.

Chapter seven describes the application of T09, a ligand for nuclear hormone receptor LXR, as a strategy to reduce inflammation after SCI. T09 was also tested *in vitro* for its potential pro-inflammatory cytokine gene transcription reducing properties. Unfortunately T09 was unable to reduce pro-inflammatory cytokine expression in primary astrocytes stimulated with bacterial products, nor was it able to reduce transcript levels of pro-inflammatory cytokines in an experimental model of SCI.

In chapter eight boosting of beneficial effects of the inflammatory reaction was studied via exogenous application of the growth factor VEGF. VEGF was administered during the first week after SCI via single intrathecal bolus injections every day. This treatment resulted in only limited therapeutic benefit, since glial scar formation, tissue sparing and motor performance appeared unaffected after SCI by VEGF treatment. Pain behavior after SCI was slightly affected by VEGF treatment; VEGF had a small reducing effect on mechanical hypersensitivity, whereas thermal hypersensitivity appeared unaffected.

Finally chapter nine summarizes the results of the studies described in this thesis. It provides also an overview about the chemical compounds that are used to modulate the inflammatory reaction after SCI. This overview shows us that there are many substances pre-clinically tested, whereas only a few are in clinical trials yet. So probably these pre-clinical data give insufficient confidence to start human clinical trials? But why do we find than only limited beneficial effects pre-clinically? This gives us some reason to consider the way we think about inflammatory reactions in general. We now focused mainly on either stimulating beneficial or inhibiting detrimental events after SCI, although both events

occur often simultaneously along the course of inflammation. Good and bad events may be inevitably interdependent on each other, which makes it very difficult or even impossible to achieve neuroprotection by just boosting good or reducing bad events.

Changing the character of inflammation may be a good alternative that may provide neuroprotection after SCI. A crucial role within this concept may be dedicated to different activation patterns of glial cells that result from the molecular cues these cells encounter. Modulation of glial activation patterns may be able to change the nature of the inflammatory reaction to achieve effective treatment for SCI.

Samenvatting

Het doel van het huidige proefschrift was om nieuwe therapeutische strategieën te testen die de ontstekingsreactie die volgt na een dwarslaesie kunnen moduleren, om uiteindelijk ervoor te zorgen dat motor uitkomst verbeterd en pijn verminderd wordt na dwarslaesie. Sinds vroege productie van pro-inflammatoire cytokinen een essentiële rol speelt in het ontstaan en onderhouden van deze ontstekingsreactie, werd als eerste strategie het transcriptie verminderende effect op pro-inflammatoire cytokinen onderzocht door toediening van de liganden voor nucleaire hormoon receptoren retinolzuur (RA) en T0901317 (T09).

Als tweede strategie werd er gekeken wat het effect was van het stimuleren van de goede effecten van een ontstekingsreactie, zoals door het additioneel toedienen van de groeifactor VEGF na dwarslaesie.

Het eerste hoofdstuk geeft kort een inleiding in het klinische probleem dat ontstaat in patiënten met een dwarslaesie. Patiënten met een dwarslaesie lijden niet alleen onder de ernstige consequenties van het niet kunnen lopen en gevoelloosheid, een aanzienlijk gedeelte ontwikkelt ook chronische neuropathische pijn. Deze pijn kan niet behandeld worden met de huidige voorhanden medicatie en leidt daardoor vaak tot een sterk verminderde kwaliteit van leven in deze patiënten. Depressie en zelfmoord komen dan ook niet zelden voor. Dit eerste hoofdstuk geeft ook een idee over hoe we deze aspecten van lange termijn uitkomsten na dwarslaesie kunnen nabootsen en bestuderen in diermodellen. Een cruciale gebeurtenis, die ook de lange termijn uitkomsten na dwarslaesie bepaald, is de ontstekingsreactie die volgt op de initiële schade aan het ruggemerg. Daarom beschrijft het eerste hoofdstuk van dit proefschrift de cellulaire en moleculaire gebeurtenissen die plaatsvinden gedurende deze ontstekingsreactie.

Het tweede hoofdstuk van dit proefschrift geeft informatie over twee receptoren die behoren tot de familie van nucleaire hormoon receptoren, en wel peroxisome proliferator activated receptor (PPAR/RXR) en de retinolzuur receptoren (RAR/RXR). Dit hoofdstuk licht toe hoe activatie van deze receptoren betrokken kan zijn in verschillende neurologische en psychiatrische ziekten en hoe activatie van deze receptoren mogelijk tot neuroprotectie kan leiden.

Het derde hoofdstuk van dit proefschrift beschrijft de relevantie van PPAR/RXR en RAR/RXR activatie na dwarslaesie. De data beschreven in dit hoofdstuk laten zien dat RAR/RXR worden geactiveerd na dwarslaesie en dat het activeren van PPAR/RXR en RAR/RXR door natuurlijke dan wel synthetische liganden mogelijk zou kunnen leiden tot een verminderde ontstekingsreactie na dwarslaesie.

Het vierde hoofdstuk van dit proefschrift beschrijft het bestuderen van het mogelijke anti-inflammatoire effect van retinolzuur, het natuurlijk ligand voor RAR/RXR. Om een ontstekingsreactie *in vitro* na te bootsen werden primaire astrocyten bloot gesteld aan producten van bacteriën. Dit veroorzaakt een ontstekingsreactie in astrocyten welke gepaard gaat met de productie en expressie van ontstekingsfactoren. Retinolzuur werd als interventie methode gegeven om deze ontstekingsreactie tegen te gaan, wat resulteerde in verminderde chemokine expressie en productie door deze cellen. Dit hoofdstuk licht ook toe welke moleculaire mechanismen ten grondslag liggen aan dit chemokine verminderende effect.

Hoofdstuk vijf van dit proefschrift laat zien dat het ontstekingsremmende effect van retinolzuur zich niet alleen beperkt tot verminderde chemokine afgifte door astrocyten. Productie van ontsteking stimulerende cytokinen door astrocyten bleek namelijk ook verminderd door behandeling met retinolzuur.

Het zesde hoofdstuk van dit proefschrift beschrijft de toepassing van retinolzuur als een strategie om cytokine expressie te verminderen in een model voor dwarslaesie. In dit experiment werden twee routes van toepassing van retinolzuur onderzocht, enerzijds werd het retinolzuur lokaal gegeven via een intrathecale catheter, anderzijds werd het retinolzuur systemisch gegeven via een intraperitoneale injectie in de buikholte. Retinolzuur was alleen na systemische toediening in staat om transcriptie niveaus van pro-inflammatoire cytokinen te remmen, niet na locale toediening. Deze studie benadrukt dat de route van toepassing van een medicament de effectiviteit van een medicijn verschillend bepaald.

Hoofdstuk zeven laat de toepassing van T09, welke een ligand is voor de nucleaire hormoon receptor LXR, als een strategie om genexpressie van ontsteking stimulerende cytokinen te remmen in een model voor dwarslaesie. T09 werd ook in getest in primaire astrocyt culturen gestimuleerd met bacteriële producten als mogelijk cytokine expressie verminderend medicament. Helaas was T09 niet in staat expressie van cytokinen te remmen in primaire astrocyten. Eveneens in het model voor dwarslaesie was T09 niet in staat de expressie van cytokinen te verminderen.

In hoofdstuk 8 werd onderzocht of het stimuleren van de goede effecten van een ontstekingsreactie door het exogeen toedienen van de groeifactor VEGF kan leiden tot neuroprotectie en functionele verbeteringen na experimentele dwarslaesie. VEGF werd gedurende de eerste week na dwarslaesie gegeven via een intrathecale catheter bestaande uit een enkele bolus injectie per dag. Deze behandeling leidde echter maar tot beperkte therapeutische verbetering na dwarslaesie. Litteken weefsel vorming door glia cellen, weefsel sparing en motorische verbeteringen bleken niet veranderd door de behandeling met VEGF. Ook werd er in deze studie naar pijn-gedrag gekeken van geletselde dieren; VEGF bleek een klein verbeterend effect te hebben op mechanische hypersensitiviteit, thermische hypersensitiviteit bleek echter niet veranderd.

Het laatste hoofdstuk vat de resultaten van dit proefschrift samen. Het geeft ook een overzicht over de literatuur die al bekend is over verschillende pre-klinisch onderzochte therapieën met het oog op het verkrijgen van neuroprotectie na dwarslaesie. Dit overzicht laat ons zien dat al vele medicamenten pre-klinisch getest zijn voor hun neuroprotectieve eigenschappen, hoewel er momenteel maar weinig medicamenten daadwerkelijk in klinische trials getest worden. Blijkbaar bieden deze pre-klinische studies dus onvoldoende vertrouwen, om klinische trials in mensen met dwarslaesie te starten. Maar hoe komt dit dan, dat we eigenlijk maar beperkte neuroprotectieve effecten vinden van de medicamenten die we pre-klinisch testen? Dit geeft stof tot nadenken over hoe wij momenteel tegen een ontstekingsreactie aankijken. We hebben onze strategieën nu vooral gericht op het enerzijds remmen van slechte effecten, dan wel het anderzijds stimuleren van de goede effecten van een ontstekingsreactie, terwijl vele van deze goede en slechte effecten juist gelijktijdig plaatsvinden. Goede en slechte gebeurtenissen gaan misschien hand in hand en zijn daardoor onlosmakelijk met elkaar verbonden gedurende de ontstekingsreactie. Dit maakt het wel heel moeilijk of misschien wel onmogelijk om neuroprotectie te bereiken door enkel en alleen goede dan wel slechte effecten te stimuleren dan wel te remmen.

Een goed alternatief om neuroprotectie te bereiken, kan het veranderen van het karakter van een ontstekingsreactie na dwarslaesie zijn. Een cruciale rol binnen dit concept kan weggelegd zijn voor de verschillende activatie patronen die glia cellen kennen als reactie op de moleculen die deze cellen tegenkomen. Modulatie van deze activatie patronen kan er misschien toe leiden dat een ontstekingsreactie anders verloopt en dat dit gepaard gaat met een meer behoudende aard en neuroprotectie.

Acknowledgments/Dankwoord

Zo mijn boekje is af! Dit eindresultaat zou echter nooit mogelijk zijn geweest zonder de hulp en steun van collegas, vrienden en familie. Daarom wil ik bij deze een speciaal woordje richten aan degenen die mij tijdens mijn promotie begeleid en ondersteund hebben.

Prof. Dr. Steinbusch, mijn promotor. Ik wil u hartelijk bedanken voor de steun en begeleiding tijdens mijn promotieonderzoek. Niet alleen tijdens de eerste twee jaar van mijn promotie die ik in de groep van Dr. Mey doorbracht was u altijd op de achtergrond aanwezig via de EURON Marie Curie dagen en EURON dagen, toen ik naar Maastricht kwam in mijn derde jaar werd uw rol binnen mijn promotieonderzoek steeds groter. U zorgde ervoor dat ik nog een jaartje langer in Maastricht aan mijn promotieproject kon werken zodat er voldoende artikelen gepubliceerd konden worden voor een promotie in Nederland. Hartelijk dank hiervoor.

Prof. Dr. Van Kleef, mijn promotor. Ik dank u hartelijk voor steun en begeleiding tijdens mijn promotie. Hartelijk dank ook dat ik direct het gevoel had dat ik deel uitmaakte van de afdeling Anaesthesiologie toen ik in mijn derde jaar naar Maastricht kwam. U zorgde er ook voor dat ik nog een jaar langer in Maastricht kon blijven, hartelijk dank hiervoor.

Dank ook aan de beoordelingscommissie onder leiding van Prof. Dr. De Baets voor het lezen en beoordelen van mijn proefschrift.

Dr. Mey, lieber Jörg, mein co-promotor. Danke für deine Hilfe und Unterstützung während meine Doktorarbeit. Danke auch für die viele Diskussionen über Data, Paper und Experimenten. Von dir habe ich nicht nur viel gelernt auf der Arbeitsebene, ich habe auch viel gelernt über Geschichte und Deutschland, vor allem über Deutsche Politik ☺ Ich wünsche dir ganz viel Erfolg mit deiner wissenschaftlichen Karriere in der Zukunft!

Dr. Deumens, Ronnos, mijn co-promotor. Super bedankt voor alle hulp, advies en inzet die je voor mij getoond hebt, ik waardeer dit echt enorm. Ik heb tijdens die twee jaar in Maastricht veel van jou geleerd, van het werken met de ratten tot en met het schrijven van artikelen en het proefschrift toe. Bedankt voor alle nuttige discussies over data en artikelen en bedankt ook voor al jouw enthousiasme in het onderzoek. Het is fijn samen te werken met mensen die zo gemotiveerd zijn. Bedankt ook voor alle grappige momenten; ik vind het wel een geruststelling dat er ook mensen zijn die nog erger zijn dan ik zelf met het kwijtraken van portemonnees, sleutels en pasjes ☺. Ik wens je heel veel succes toe in de toekomst, maar dat zal zeker wel goed komen!

Dan wil ik vervolgens de experimentele pijn groep van de Afdeling Anaesthesiologie in Maastricht bedanken:

Prof. Dr. Marcus, Dr. Patijn, Dr. Joosten, Bert hartelijk dank voor jullie steun en hulp tijdens mijn promotieproject. Bedankt ook voor de nuttige discussies over data en artikelen.

Robby, Rupke, Super bedankt voor al je hulp, ik heb veel van jou geleerd (o.a. de dwarslaesie operaties). Ik ben bovendien nog steeds onder de indruk van jouw gestructureerdheid ☺. Ik vond het ook ontzettend leuk om bij jouw promotie paranimf te zijn (wat hebben we gelachen bij het maken van het filmpje!) Bedankt ook voor alle gezelligheid en belgische woorden die ik nu ook regelmatig gebruik (spaveuh!)

Liesbeth, ontzettend bedankt voor alles! Ik heb erg veel steun aan jou gehad, niet alleen in het lab leerde ik veel van jou, ook konden wij altijd goed over experimenten discussiëren en wat nog meer allemaal interessant zou kunnen zijn om te onderzoeken in de toekomst. Niet alleen op werk-gebied klikte het goed, we hadden ook veel dezelfde interesses, het snowboarden, festivals, kleren, schoenen (uiteeraard ☺). Ik vond het dan ook super om samen met jou Robbies paranimfen te zijn en dit te organiseren. Ik wens je heel veel succes verder met je promotie, maar dat zit wel goed met jou, jij komt er wel!

Bart, Bartjûh! Ik denk wel dat jij nu de koning van het PCR lab bent ☺, zoveel platen als jij er doorheen gejaat hebt, ongelooflijk! Ik vond het leuk je wegwijs te maken in de wonder wereld van de mRNAs en DNAs. Jammer dat je eigenlijk al wel redelijk snel naar San Diego vertrokken bent, goed voor jou wel ☺, maar we missen "The Rat wisperer" wel hier in Maastricht. Ik wens je heel veel succes met je promotie verder, dat komt zeker goed!

Michiel, hahaha wat heb ik altijd om jou moeten lachen, ik vind nog steeds het "evidence beest" je beste creatie! Al hadden onze onderwerpen niet direct met elkaar te maken, ik wil je toch bedanken voor alle nuttige discussies over data en experimenten. Mooi ook dat we uiteindelijk tot de conclusie gekomen zijn dat retinolzuur gewoon stinkt... Intussen zijn we beiden klaar met ons promotieproject, toch denk ik dat we die uitgebouwde hangmat op de markt moeten brengen, dan lopen we binnen als je het mij vraagt...

Wouter, werkelijk! Fijn was het altijd om iemand te hebben waarmee je over data en vooral over (vreselijke) statistiek kunt discussieren! Ook super dat je al mijn klinische vragen kon beantwoorden, bedankt hiervoor. Wat goed ook dat het gewoon gelukt is om een wielengroepje op te richten samen met Bart! Als ik jouw verhalen zo hoor, dan denk ik dat het echt tijd wordt dat ik weer op mijn renfiets spring, anders krijg ik jullie helemaal niet meer bijgehouden ☺.

Sofie, Sofieke! Onze kleine Belg met Nederlandse uitspraken! Bedankt Sofieke, voor al je vrolijkheid en gezelligheid, maar ook voor al je nuttige input tijdens discussies over data en experimenten. Ik kan nu niet meer naar "Das Rote Pferd" of naar "Ich bin ein Döner" luisteren zonder aan jou te denken! Ik wens je in ieder geval heel veel succes bij je verdere carrière!

Wiel, jij was mijn roomie gedurende de eerste tijd dat ik in Maastricht werkte. Ik wil je hartelijk bedanken voor alle hulp met mijn computer, ik waardeer dit echt heel erg omdat ik zelf een ongelofelijke digibeet ben ("plaats de muis in de linker bovenhoek"). En jep, ik heb een backup van mijn proefschrift gemaakt ☺.

Helwin, we hebben niet zo heel lang samen gewerkt, maar toch wil ik je bedanken voor de gezelligheid, vooral mooie verhalen ("Mission Impossible"), en vooral ook voor het feit dat we eindeloos over muziek kunnen praten. Ik wens je veel succes bij het afronden van je promotie!

Marjolein, hartelijk dank voor het analyseren van de Catwalk data voor het VEGF stuk, je hebt een berg werk verzet! Succes ook met het afronden van je masterstage en het vinden van een AIO baan!

Een groot gedeelte van de experimenten beschreven in dit proefschrift zijn uitgevoerd gedurende de eerste twee jaar van mijn promotie in Aken, Duitsland. Daarom wil ik ook graag mijn collegas bedanken aan de afdeling Zoologie, Bio2 van de RWTH-Aachen voor hun hulp en steun tijdens mijn promotieonderzoek.

Prof. Dr. Wagner, Lieber Hermann, danke das ich Teil der Bio2 Gruppe sein dürfte. Ich habe mich immer sehr zu Hause gefühlt bei der Abteilung. Danke auch für die Unterstützung und das Vertrauen während meine Doktorarbeit.

Liebe Julia, es war schön jemand zu haben womit man gut reden kann, nicht nur über Experimenten ☺. Obwohl wir nicht am gleichen Projekt gearbeitet haben, hat es mir immer Spaß gemacht mit dir zusammen zu arbeiten. Danke auch für alle schöne Chococroissant- und Kaffeemomenten beim Moss! Ich hoffe wir werden uns noch regelmäßig sehen und ich wünsche dir alles bestens bei deiner Karriere als Ärztin.

Lieve Ilona, hartelijk dank voor al uw hulp en steun! Zeker in de eerste maanden dat ik in Aken was heb ik veel aan u gehad. Heel fijn om iemand te hebben waar je gewoon even lekker Nederlands mee kunt praten ☺ Ook heel erg bedankt voor alle uitleg en hulp in het lab, de westernblots, kleuringen en cell kweken die u voor mij gedaan hebt, ik waardeer dit echt enorm. Ik hoop dat we elkaar nog regelmatig blijven zien, wens u al het beste en ik hoop dat u lekker van uw pensioen mag genieten!

Marie, Danke für die Zusammenarbeit, es hat richtig Spaß gemacht mir dir zu arbeiten! Ich hoffe es geht dir gut da an der Nordsee ☺! Steffi, wir haben nicht so lange zusammen gearbeitet aber ich danke dir herzlich für deine Hilfe bei den letzten PCR Experimenten!

Liebe Studenten der Mey-Gruppe; Nadine, Philipp Imholz, Jeremie, Andrei, Jeya, Dhana, Isabell, Cordula, Philipp Apperdanier, Milena, René, Ruman, obwohl ich nicht immer am gleichen Projekt gearbeitet habe mit euch, möchte ich euch ganz herzlich Danken fuer die schöne Zusammenarbeit, es hat richtig Spaß gemacht mit euch in der Bio2 zu arbeiten!

Gerne möchte ich auch alle andere mitarbeiter von der Bio2 danken die nicht direkt bei meinem Projekt involviert waren, die aber trotzdem dafür gesorgt haben dass die Arbeitsatmosphäre super war.

Liebe Ulrike, Es war super mit dir den Büro zu teilen. Danke auch für dein Verständnis, alle gute Gesprächen und Unterstützung, das hat mir sehr geholfen. Ich hoffe wir werden uns noch regelmäßig sehen, wünsche dir alles bestens und viel Erfolg bei Novartis!

Katrin, es war echt super in die USA, ich muss noch immer lachen wenn ich an unseren Shit-Trip zum Freiheitsstatue denke hahaha. Ich wünsche dir ganz viel Erfolg als Post-doc!

Laura, danke für alle nette Gesprächen, Diskussionen, Cocktails und Kaffee ☺. Es war super dich als Kollegin zu haben! Ich wünsche dir ganz viel Erfolg bei deinem neuen Job.

Martin, danke für alles, du warst einen super Kollege. Ich glaube, ich habe dich noch immer nicht überzeugen können dass nicht alle Holländer Graß rauchen, oder ☺?

Suzanne, Marcus, Sandra, Wolf und Petra und alle andere Mitarbeiter der Bio2 herzlichen Dank für ihr Interesse immer!

Aangezien een groot gedeelte van de experimenten binnen de afdeling Neuroscience in Maastricht is uitgevoerd wil ik natuurlijk ook graag mijn collegas van de afdeling Neuroscience bedanken voor hun hulp en steun gedurende de laatste twee jaar van mijn promotieproject.

Kimberly, dank je voor alle relaxte DE-momentjes "even afschakelen", opbeurende kaartjes en ook voor de leuke discussies over onze projecten. Ik wens je heel veel succes met alles, alles kump good! ☺

Evi and Maria, thank you for working together, it was really fun explaining things to you about the microglia and astrocyte cultures! I still favour the pancake-shaped astros ☺!

Sven, Jochen and Nathalia, thank you for all the nice soccer games, great way to lose some stress!

Dank ook aan alle andere collegas van de afdeling neuroscience die ervoor zorgden dat de werksfeer goed was door samen in het lab te staan, dan wel door een babbeltje te maken op de gang!

Het analistenteam; bedankt dat ik altijd bij jullie terecht kon met vragen. De mensen van het CPV; bedankt voor alle hulp met de dieren!

Nicole, bedankt voor alle administratieve hulp en interesse tijdens mijn Marie-Curie Fellowship!

Marie-Therese en Lisa, zeker tijdens het laatste gedeelte van mijn promotie heb ik veel aan jullie hulp en goede raad gehad. Super dat ik altijd even snel het secretariaat binnen kon wandelen om wat te vragen!

Dit proefschrift zou nooit mogelijk geweest zijn zonder de samenwerking met andere onderzoeksgroepen. Daarom wil ik bij deze ook graag mijn samenwerkingspartners bedanken.

Afdeling Neuroscience, Universiteit Maastricht, The Netherlands

Prof. Dr. De Baets, Mario Losen and Piluca Martinez thank you very much for the nice cooperation on the LXR project. Thank you for all the help in the lab and the discussions about the LXR data and article.

Afdeling interne geneeskunde, Erasmus Medisch Centrum Rotterdam, The Netherlands

Dr. Mulder, Monique hartelijk bedankt voor de fijne samenwerking bij het LXR project.

Institut für Neuroanatomie, Universitätsklinikum Aachen, Germany

Prof. Dr. Beyer, danke für die gute Zusammenarbeit, die nützliche Diskussionen über Daten und Paper, was letztendlich resultierte in zwei schöne Publikationen. Danke auch dafür das ich mich immer sehr willkommen gefühlt habe in Ihrem Labor.

Liebe Sonja, Dr. J., Es hat richtig Spaß gemacht mit dir über Daten zu diskutieren und Experimenten aus zu denken (Unser Schlagplan ☺). Danke auch für all deine Hilfe mit den Zellkulturen, Färbungen, PCRs und was nicht alles noch mehr! Echt klasse! Du warst nicht nur eine super Kollegin, auch hat es richtig Spaß gemacht mit dir ein wenig ab zu schalten von der Arbeit beim Film gucken, Konzert besuchen oder Sushi essen (wann ist unserem nächsten Sushi-Termin ☺?) Ich hoffe dann auch das wir noch lange befreundet

bleiben, vielleicht auch wieder zusammenarbeiten werden und ich wünsche dir auf jeden Fall ganz viel Erfolg bei deine wissenschaftlichen Karriere. Vielleicht sollen wir doch jetzt das Pocket-Lab auf den Markt bringen ☺!

Tim, Jon, Markus, Magda, Shilpee, Suzanne, Akvile, Uta, Petra, Helga ganz vielen Dank das ihr mich so lieb aufgenommen habt in eurer Gruppe. Es hat richtig Spaß gemacht mit euch zusammen zu arbeiten. Danke auch das ich immer mit fragen bei euch vorbei kommen konnte.

Institut für Neuropathologie, Universitätsklinikum Göttingen, Germany

Prof. Dr. Hanisch ganz herzlichen Dank für die gute Zusammenarbeit. Es freut mich sehr dass wir zusammen zwei Paper publiziert haben!

Tommy Regen, ganz herzlichen Dank für die viele viele ELISAs die du für mich gemacht hast! Die Zusammenarbeit war super und ich danke dir auch für alle Infos über die microglia Färbungen und Clodronat Experimenten, und natürlich auch für die Tafel Choci hahaha, mmm! ☺

Institut für Neuropathologie, Universitätsklinikum Aachen, Germany

Prof. Dr. Weis Herzlichen Dank für die gute Zusammenarbeit und nützliche Diskussionen über das VEGF paper.

Dr. Brook, Gary thank you for all the helpful discussions about the VEGF paper and data! Thank you also for all the nice soccer games (I promise, I will spare your shin-bones next time ☺) .

Tobias, Danke dass ich dir immer Sachen fragen konnte mit Bezug auf Immunfärbungen! Ich wünsche dir ganz viel Erfolg bei deiner wissenschaftlichen Karriere und ich hoffe es geht dir gut Down Under!

Interdisziplinäres Zentrum für klinische Forschung (IZKF Biomat), Universitätsklinikum Aachen, Germany

Dr. Denecke, Danke für die Zusammenarbeit bei den Gene-chip arrays.

Laboratory of Connective Tissues Biology, GIGA-Cancer, University of Liège, Belgium

Dr. Lambert, thank you for your collaboration in the VEGF study. I would also like to thank to Dr. Mineur for the production of the VEGF165.

Many thanks go to Michal Slawiński for the typesetting, layout and printing of this thesis. Thank you very much for the accurate responses, great competence and large amount of work you did. This cooperation worked very well!

Graag wil ik ook mijn familie en vrienden bedanken voor hun steun, interesse dan wel voor het zorgen voor afleiding tijdens mijn promotieonderzoek!

Bedankt lieve voetbalteamvriendinnetjes voor alle gezellige en ontspannende uurtjes op het veld en sorry voor alle ballen richting de ballenvanger (Nit mit Gewalt!!! ☺) Lieve fietsvriendinnetjes jullie ook veel dank voor alle ontspannen uurtjes op de renfiets en alle slopende Keutenberg momenten! Sport biedt toch altijd weer een mooie uitlaatklep na een dag hard werken!

Claire, Clairos wat ben ik blij dat jij er altijd voor mij bent! Heerlijk even met jou afschakelen op monotone spinning muziek van Henk (zonder zang) ☺. Dank je voor alle lekkere avond-etentjes die je voor mij klaar gemaakt hebt als ik het heel druk had. Echt geweldig ☺!

Monique, Monnik, dank je voor de momenten waar ik even samen met jou, Claire en Simone kon ontspannen. Echt geweldig die twee kleine jongens van je, laat maar weer eens zien dat er ook nog andere dingen in het leven zijn dan alleen maar werken he ☺.

Simone, Simmie, dank je wel dat je altijd achter me staat, dat geeft echt een goed gevoel! Dank je ook voor alle hilarische momenten op 30 m onder de zeespiegel (de French Angelfish rules!) en voor alle aangebrande pizzas ☺. Geweldig ook dat ik bij jou altijd kon crashen (in de über relaxte Pullman ☺) als ik vroeg op moest voor experimenten, toppie!

Nadine, Nadiëntje! dank je voor al die ontspannen koffie-momentjes en dat je altijd naar mij luisterde als er weer eens een experiment niet gelukt was of als ik moe was van het schrijven. Ik wens je heel veel succes met je eigen promotie en ik hoop dat we nog heel lang over henna, zelfgemaakte zeep en muffins kunnen praten ☺.

Nicole, Heini, dank je wel voor alle momentjes waar ik even bij jou kon afschakelen van het werk. Even de "Dog wisperer" of "Survivalman/woman" kijken, lekker op jou megabank hangen met een kopje groene thee (voor de anti-oxidanten he ☺) . Ik hoop ook dat we dit nog lang samen mogen doen!

Veronique, Verootje, we zien elkaar niet zo heel vaak, maar als we elkaar zien is het altijd super leuk met je twee kindjes en Ruud. Bedankt daarvoor!

Dank je wel lieve schoonfamilie, Antoon, Jeanne, Martine, Roger, Femke, Nienke en Maaike dat ik bij jullie altijd even met andere dingen bezig was dan met mijn werk.

Tante Gerrie en oom Ton, een betere peettante en peetoom kan ik me niet wensen. Bedankt voor jullie support en interesse altijd!

Tom bedankt wa brother ☺. Bedankt voor alle hulp en support ten alle tijden. Met name ook tijdens de kritieke uren van het versturen van veel te grote posterfiles tot en met mijn proefschrift (drie dubbele zip files) en alle andere computer-issues! Ik ben toch wel blij dat de big brother altijd een beetje op mij past.

In this bright future, you can't forget your past... (Bob Marley, No woman no cry)

Lieve mamma en pappa bedankt dat jullie mij gesteund hebben in de dingen die ik deed, dat jullie altijd tegen me gezegd hebben "niet opgeven, laat ze maar eens wat zien!" Jullie zorgden ervoor dat ik kon gaan studeren in Utrecht, zodat ik daarna met mijn promotieproject kon beginnen en nu is het boekje en de promotie klaar! Dank voor al het interesse dat jullie steeds getoond hebben in mijn onderzoek. Mam, dank je dat je altijd voor me klaar staat en echt geweldig dat je steeds alle artikelen over neurowetenschap bewaard hebt voor me ☺! Pappa, bedankt ook dat jij altijd voor me klaar staat en voor alle geruststellende woorden op de momenten waar het eens een keertje niet zo soepel verliep met mijn promotie. Bedankt voor jullie onvoorwaardelijk steun en liefde en dat ik altijd op jullie kan terugvallen!

Lieve Jeroen, dank je wel voor alle begrip en ondersteuning. Het is denk ik niet altijd makkelijk geweest, maar ik ben heel blij dat je mij de ruimte gegeven hebt om de dingen te doen die belangrijk voor mij zijn, zoals het volbrengen van dit promotieproject. Ik hoop dat we nog vele mooie momenten tegemoet gaan samen.

Curriculum Vitae

Sabien Geraldine Antonia van Neerven werd geboren op 22 Februari 1983 te Heerlen en groeide op in Trintelen. In 2001 rondde zij haar VWO af aan het Sintermeertencollege in Heerlen in de profielrichting Natuur en Gezondheid. Aansluitend in datzelfde jaar begon zij aan de studie Biologie aan de Universiteit van Utrecht. Daar studeerde zij drie jaar in de richting Experimentele Zoologie waarna zij, na het behalen van haar Bachelor in de Biologie, startte met het Masterprogramma Neuroscience and Cognition and de faculteit Biomedische Wetenschappen van het Utrecht Universitair Medisch Centrum. Tijdens deze masteropleiding volgde zij haar twee wetenschappelijke stages, eerst 9 maanden bij de afdeling Psychoneuroimmunologie van het Utrecht Universitair Medisch Centrum binnen de groep van Prof. Dr. C. Heijnen en vervolgens 6 maanden bij de afdeling Neuroscience van de Universiteit Maastricht onder begeleiding van Dr. G. Kenis. Na in Oktober 2006 deze masteropleiding succesvol afgerond te hebben, startte zij in November 2006 als promovendus in Aken (Duitsland) bij de faculteit Biologie en vakgroep Zoologie 2 van de Reinisch-Westfälische Technische Hochschule Aachen onder supervisie van Prof. Dr. J. Mey en Prof. Dr. H. Wagner. Daar werkte zij aan haar promotieonderzoek binnen het EURON Marie-Curie Fellowship Training Programma. Tijdens de eerste twee jaar van haar promotieonderzoek was zij vooral werkzaam binnen de afdeling Zoologie 2, maar werkte zij ook samen met de afdeling Neuroanatomie binnen de groep van Prof. Dr. C. Beyer en met de afdeling Neuropathologie binnen de groep van Prof. Dr. Weis van het Universitätsklinikum Aachen. De daaropvolgende twee jaar was zij vooral werkzaam in Maastricht binnen de afdeling Neuroscience en het Pain Management and Research Center van de afdeling Anesthesiologie. Tijdens deze 4 jaar onderzocht zij hoe de neuroinflamatoire repons na een ruggemergletsel beïnvloed kan worden door farmacologische interventie zodat schade die veroorzaakt wordt door de ontsteking beperkt kan worden na het letsel. De resultaten van dit onderzoek zijn te lezen in dit proefschrift.

Sabien Geraldine Antonia van Neerven was born on February 22, 1983 in Heerlen (The Netherlands) and grew up in Trintelen. In 2001 she graduated at the Sintermeertencollege in Heerlen in the direction "Environment and Health". In that same year she started her study Biology at the University of Utrecht, with as main topic Experimental Zoology. After receiving her bachelor degree, she started with her master Neuroscience and Cognition at the University Medical Center Utrecht. During this master she worked as a trainee, first 9 months at the department of Psychoneuroimmunology at the University Medical Centrum Utrecht under supervision of Prof. Dr. C. Heijnen, and subsequently at the department of Neuroscience of the Maastricht University under supervision of Dr. G. Kenis for 6 months. After receiving her master degree in October 2006, she

started her PhD project in Aachen, Germany at the Biology Department, Zoology 2 of the Rheinisch-Westfälische Technische Hochschule Aachen under the supervision of Dr. Mey and Prof. Dr. Wagner in November 2006. She participated as PhD student in the EURON Marie Curie Training Program. During the first two years of her PhD project she worked mainly at the department of Zoology, but she also collaborated with the department of Neuroanatomy of the University Clinic Aachen under the supervision of Prof. Dr. C. Beyer and with the department of Neuropathology of the University Clinic Aachen under the supervision of Prof. Dr. Weis. The following two years she worked mainly in Maastricht at the department of Neuroscience and the Pain Management and Research Centre of the department of Anaesthesiology. During the 4 years of her PhD project she investigated different therapeutic approaches to modulate the inflammatory response that follows spinal cord injury, with the intention to reduce damage caused by this inflammation. The results of this work are included in the present thesis.

List of publications

Peer-reviewed publications

van Neerven, S, Nemes, A, Imholz, P, Regen, T, Denecke, B, Hansisch, UK, Mey, J (2010) Inflammatory cytokine release of astrocytes *in vitro* is reduced by *all-trans* retinoic acid. In press J Neuroimmunol

van Neerven S, Joosten EA, Brook GA, Lambert CA, Mey J, Weiss J, Marcus MA, Steinbusch HW, van Kleef M, Patijn J, Deumens R. (2010) J Neurotrauma 27(10): 1781-91 Repetitive intrathecal VEGF165 treatment has limited therapeutic effects after spinal cord injury in the rat.

van Neerven S, Mey J, Joosten EA, Steinbusch HW, van Kleef M, Marcus MA, Deumens R. (2010) Neurosci Lett.: 485(1):21-5. Systemic but not local administration of retinoic acid reduces early transcript levels of pro-inflammatory cytokines after experimental spinal cord injury.

van Neerven, S, Regen, T, Wolf, D, Nemes, A, Johann, S, Beyer, C, Hansisch, UK, Mey, J (2010) J Neurochem.: 114(5):1511-26 Inflammatory chemokine release of astrocytes *in vitro* is reduced by *all-trans* retinoic acid.

van Neerven, S, Kampmann, E, Mey, J (2008) Prog Neurobiol 85(4):433-51. RAR/RXR and PPAR/RXR signaling in neurological and psychiatric diseases.

van Neerven, S, Mey, J (2007) PPAR Res 2007: 29275. RAR/RXR and PPAR/RXR signaling in spinal cord injury.

Peer reviewed publications – Co-author

Kampmann, E, Johann, S, **van Neerven, S**, Beyer, C, Mey, J (2008). J Neurochem 106(1): 320-32. Anti-inflammatory effects of retinoic acid on prostaglandin synthesis in cultured cortical astrocytes.

Publications in progress

van Neerven, S, Jaken, RJP, Joosten, EA, Steinbusch, HWM, van Kleef, M, Mey, J, Mulder, M, Martinez-Martinez, P, Losen, M, De Baets, M, Marcus, MAE, Deumens, R. Liver X receptor activation does not attenuate early pro-inflammatory cytokine gene expression after experimental spinal cord injury.

Submitted to Experimental Neurology

Deumens, R, Bozkurt, A, van Gorp, S, **van Neerven, S**, Führmann, T, Marcus, MAE, Tolba, R, Weis, J, Joosten, EAJ, Brook, GA. Repair of the injured adult rat thoracic spinal cord: effect of collagen implants seeded with olfactory ensheathing cells

In preparation intended to submit to Experimental Neurology

Abstracts – poster presentations

van Neerven, S, Joosten, EA, Deumens, R. (2010) 3rd International Congress on Neuropathic Pain (NeuPSIG), Athens, Greece. Effects of early VEGF treatment on evoked pain behavior in a rat model of central neuropathic pain.

van Neerven, S, Deumens, R, Jaken, R, Joosten, EA, Mey, J. (2010) 3rd International Congress on Neuropathic Pain (NeuPSIG), Athens, Greece. Effects of early retinoic acid treatment on inflammation in a rat model of central neuropathic pain.

van Neerven, S, Regen, T, Hanisch, U-K, Johann, S, Beyer, C, Mey, J. (2008) The Society for Neuroscience 38th Annual Meeting, Washington-DC (US). Retinoic acid modulates the inflammatory response of astrocytes *in vitro*.

van Neerven S, Hanisch U-K Mey J. (2007) Deutsche Zoologische Gesellschaft Annual Meeting, Cologne (D) Retinoic acid modulates microglial inflammatory responses.

Abstracts – oral presentations

Retinoic acid modulates the inflammatory reaction of astrocytes (Plenary Lecture).

8th EndoNeuroPsycho Meeting, Doorwerth, The Netherlands.

Retinoic acid modulates the inflammatory reaction of astrocytes *in vitro* (Plenary Lecture).

12th EURON PhD student days, RWTH Aachen University, Aachen, Germany.

Retinoic acid modulates the inflammatory responses of astrocytes (Plenary Lecture).

11th EURON PhD student days, Université catholique de Louvain, Brussels, Belgium.