Little by little : perinatal asphyxia and the developing spinal cord

Citation for published version (APA):

de Louw, A. J. A. (2002). *Little by little : perinatal asphyxia and the developing spinal cord*. [Doctoral Thesis, Maastricht University]. Datawyse / Universitaire Pers Maastricht.
<https://doi.org/10.26481/dis.20020920al>

Document status and date:

Published: 01/01/2002

DOI:

[10.26481/dis.20020920al](https://doi.org/10.26481/dis.20020920al)

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.
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Little by Little

perinatal asphyxia and the developing spinal cord

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PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Universiteit Maastricht,
op gezag van de Rector Magnificus
Prof. dr. A.C. Nieuwenhuijzen Kruseman,
volgens het besluit van het College van Decanen
in het openbaar te verdedigen
op vrijdag 20 september 2002 om 16.00 uur

door

Antonius Jacobus Arduinus de Louw
geboren op 14 november 1968 te Schayk

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scent of funis before head, &c., without mechanical injury to head and neck—I am justified in referring the spastic rigidity which follows asphyxia at birth to lesion of spinal cord, and not to lesion of brain or medulla oblongata, it is obvious, from the greater frequency of this evidence of lesion

Layout and cover illustration

Annebeth Nies, o'bidesign

The studies in this thesis were financially supported by:

Dr. W.M. Phelps stichting voor spastici, profileringsfonds azM, and Ipsen BV.

The publication of this thesis was financially supported by:

Medtronic BV, Cotop BV and GlaxoWellcome

Little by Little: perinatal asphyxia and the developing spinal cord.

A.J.A. de Louw

Thesis University Maastricht

ISBN 90 5278 355 1

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and the duration and severity of the asphyxia [61]. Only between 8 and 20 percent of the cases of cerebral palsy are related to perinatal asphyxia [50, 56]. Other pathophysiological mechanisms involved in cerebral palsy are genetic/metabolic disorders, intrauterine infection and prematurity [29, 51].

The CNS damage due to perinatal asphyxia can lead to mental retardation, epilepsy and motor abnormalities such as spasticity [23]. While mental retardation and epilepsy are obvious clinical signs of brain dysfunction, the locomotor abnormalities of cerebral palsy may not be solely derived from cerebral dysfunction but could also partially be caused by dysfunction of the spinal cord neural network.

Studies on the effect of perinatal asphyxia in human neonates are rare. In a study by Sladky and Rorke, necropsies were performed on 21 asphyxiated human neonates. In this study, six of nine infants with pathological features of spinal cord infarction were premature and lesions were found to be more severe at lumbosacral levels [62]. In a later study it was found that ischemic injury to anterior horn cells within the spinal cord gray matter is relatively common among hypotonic-hyporeflexic neonates following severe perinatal asphyxia [7]. Furthermore, in this study, the motor abnormalities after perinatal asphyxia were partially attributed to spinal cord injury.

In the first detailed description of cerebral palsy by Little in 1861 the origin of the spastic features of cerebral palsy were attributed to lesions of the spinal cord. In later years this localization was viewed upon as inaccurate. The spastic rigidity accompanying cerebral palsy was thought to be brain derived, and has been so to date. Nevertheless, some authors attribute the features of spasticity to abnormalities of interneuronal activity in the spinal cord [4, 5, 20].

Some clinical findings in neonates after perinatal asphyxia and in children with cerebral palsy point to the spinal cord as part of the origin of the spastic features of cerebral palsy as suggested by Little: a) the observation of paroxysmal (epileptic) features in neonates after perinatal asphyxia without abnormal brain function on EEG monitoring; b) the findings of both extensor and flexor spasms in

the lower extremities of children with cerebral palsy. While extensor spasms are generally contributed to the loss of supraspinal control after brain damage, flexor spasms are mainly observed after spinal cord injury [67].

While abundant studies have been done concerning the effects of asphyxia on the developing brain, there are virtually no studies on the effects of perinatal asphyxia on the spinal cord. Little by little we started to investigate the effects of perinatal asphyxia on the developing spinal cord in order to better understand the pathophysiological mechanisms of spasticity in children with cerebral palsy. Understanding the mechanisms of spasticity will lead to more knowledgeable (and perhaps more sensible) treatment options for a devastating condition.

Normal locomotion

The coordination of normal locomotion requires an intact CNS. Three levels of control can be distinguished: the first level consisting of the cerebral cortex, limbic system and basal ganglia. A second level controlled by the cerebellum and the brainstem and a lower level in which the spinal cord has a central role [47]. The most basic pattern of alternating flexion and extension during mammalian locomotion is controlled by spinal central pattern generators (CPGs). These CPG's are under supraspinal control by the cerebral cortex, basal ganglia, cerebellum and brainstem through specific pathways known as the corticospinal (pyramidal) and parapyramidal tracts [2]. In the lumbar spinal cord afferent input from muscle spindles has been shown to regulate sensorimotor control of locomotion through an effect on CPGs by spinal interneurons [44].

The spinal segmental stretch reflex arc is one of the major pathways of afferent input on the spinal cord. Fast conducting sensory fibres (Ia afferents) relay excitatory impulses originated in muscle spindles after muscle stretch. After excitation of the primary afferent Ia fibres an excitatory action on the alpha-motoneuron of the agonistic muscle is established. The Ia afferents also connect with inhibitory interneurons that project directly to the antagonist motoneuron,

known as reciprocal inhibition. In the stretch reflex most excitatory activity is mediated via oligosynaptic and polysynaptic pathways [67].

Besides by corticospinal and afferent input from muscle spindles, spinal interneuron activity is regulated by other inhibitory and excitatory supraspinal descending systems known as the parapyramidal tracts. The key parapyramidal tracts are: the dorsal reticulospinal tract (DRT), the medial reticulospinal tract (MRT) and the vestibulospinal tract (VRT). The MRT and the VRT are the two main excitatory pathways while the DRT is a inhibitory pathway. Lesions in either one of the above mentioned systems will have an effect on normal locomotion.

Spasticity

Spasticity is defined as a sensorimotor disorder with a velocity dependent increase in tonic stretch reflexes and exaggerated tendon reflexes as a result of hyperexcitability of the stretch reflex [30]. Spasticity can be a very disabling feature: limited locomotor abilities, contractures (lasting stiffness of muscles, tendons, ligaments and joints), pain and limitations for self-care and care givers, amount to considerable healthcare costs.

Spasticity is looked upon as a positive component of the upper motor neuron (UMN) syndrome [59]. The UMN is defined by all long descending tracts that control or influence movement and muscle tone [2, 59]. The UMN syndrome is divided in positive and negative phenomena. The negative phenomena are characterised by a reduction in motor activity and the positive phenomena are characterized by excessive motor activity . In contrast to the general opinion, spasticity is not due to a pyramidal tract syndrome. Isolated lesions of the pyramidal tract are not associated with the combination of weakness, spasticity and hyperreflexia as seen in the upper motor neuron (UMN) syndrome [2].

The exact pathophysiological mechanisms of spasticity in cerebral palsy remain unclear. Nevertheless, it is generally accepted that the balance between excitation and inhibition is disturbed in favour of the excitatory forces. The Ia afferent stretch reflex arc is the most basic neural circuit within the

pathophysiology of spasticity [27]. In spasticity, this reflex circuit is impaired in favour of the excitatory action by an enhanced processing of afferent information within the spinal cord [43, 58].

Spinal interneurons are thought to play a role in the pathophysiological mechanisms of spasticity [24]. The role of interneurons in the stretch reflex arc is thought to be even larger than the direct connections between sensory neurons and motor neurons [67]. It is likely that interference with normal development of spinal (inter-) neurons will have a profound effect on the development of locomotor abnormalities such as spasticity.

Treatment options for spasticity

The management of spasticity is focussed on the improvement and/or prevention of complications like contractures. Furthermore, an emphasis on functional improvement and/or facilitating rehabilitation after treatment should be made. The management of spasticity with the aid of physical therapy in order to improve functionality and avoid painful contractures has been the only treatment option for years. Besides physical therapy, roughly three treatment options are available nowadays; oral pharmacotherapeutic, chemical denervation and surgery [21].

Drugs mostly used for oral administration are: Baclofen, Diazepam, Dantrolene and Tizanidine. All these drugs, besides Dantrolene, enhance inhibition by inhibiting excitatory neurotransmitters or augmenting inhibitory neurotransmitters at the spinal cord level, in order to reduce spasticity. An important side effect of oral drug is sedation due to an effect on brain neurotransmitters. These side effects are a limiting factor in the use of oral pharmacotherapy.

The second therapeutical option is chemical denervation. Before the introduction of Botulinum toxin type A (BTX-A), local injections with Phenol or alcohol were performed. Nowadays, intramuscular injections with BTX-A made the use of Phenol as a local denervation agent almost obsolete. The beneficial effect of

BTX-A on function and muscle tone has been extensively proven in randomized double blinded clinical trials [9, 22]. The major drawback of BTX is the recurrence of spasticity, the mean duration of the effect is approximately 3 months. Moreover, adverse events of repeated BTX therapy have been reported [46].

Besides orthopaedic interventions for the relief of contractures, the surgical management of spasticity is mainly focussed on selective dorsal rhizotomies (SDR) and intrathecal baclofen pumps. In all neurosurgical interventions, reduction of spasticity is established through interruption of the stretch reflex in order to decrease excitation [63]. After the first description of the use of dorsal rhizotomies in the treatment of spasticity by Foerster, several authors have modified the technique to the SDR used nowadays [12, 13]. SDR has several disadvantages. The invasive techniques used in the SDR are complicated and time consuming procedures only to be performed by highly specialized neurosurgeons. Moreover, considerable cooperation of the patient is required in order to successfully complete the post operative rehabilitation program during one year.

An alternative for the SDR is the percutaneous radiofrequency lesion adjacent to the dorsal root ganglion (RF-DRG). Accumulated clinical experience with RF-DRG has shown that it is possible to alleviate pain without clinical signs of nerve damage [65]. Furthermore, RF-DRG is a simple and safe treatment with little side effects [57]. In this thesis, the morphological effects of RF-DRG on the rat spinal cord and the clinical effect of RF-DRG on spasticity in children with cerebral palsy are investigated.

Morphology: spinal development and cell death

The occurrence of cell death in the CNS during normal development and under pathological circumstances has been well established during the past decades. Two types of cell death are known: programmed cell death, or apoptosis, and necrosis. At first, necrosis and apoptosis were viewed upon as totally different mechanisms of cell death. Nowadays, the two mechanisms are not thought to be so strictly divided [32].

Apoptosis will lead to cell death characterized by: fragmentation of DNA into nucleosomal fragments, shrinkage of the cell, the formation of apoptotic bodies and phagocytosis of cell debris without features of inflammation [41]. Fragmentation of the nucleolus is a hallmark of apoptosis which can be observed using a routine histochemical stain like the hematoxylin and eosin (HE) stain. However, apoptosis is more easily detected using the TUNEL (TdT-mediated dUTP-biotin nick end labelling) stain [19].

The concept of apoptosis was first thought of in 1972 by Kerr et al [28]. The exact mechanisms that trigger apoptosis are still poorly understood. However, the current opinion is that the major trigger for apoptosis are a series of events that activate caspases. Caspases are present in living cells in an inactivated isoform. For the activation of caspases two pathways have been described. Firstly, through so called "death receptors" like the Fas receptor and the tumor necrosis factor (TNF) receptor [3]. Occupation of these receptors leads to activation of intracellular caspase-8. This will lead to further activation of other caspases such as caspase-3, caspase-6 and caspase-7. After activation of caspase-3 a "point of no return" is reached, the cell will surely die. The second pathway leading to caspase activation and thus to apoptosis is through cytochrome-c from mitochondria. Cytochrome-c is exclusively located in the intermembrane space of mitochondria. During apoptosis, the outer membrane of mitochondria becomes permeable for cytochrome-c. The regulation of cytochrome-c is dependent on anti-apoptotic (Bcl-2 and Bcl-x_L) and pro-apoptotic (Bax, Bim, Bad and Bak) proteins [1, 52].

During normal development of the brain programmed cell death has been described for both neural and glial cells and an estimated half of the original cell population is eliminated in this way [54]. Developmental apoptosis of motor neurons in the spinal cord is grossly completed in the embryonal stage, hardly any motor neuron death is observed during postnatal development [53, 66]. Nevertheless, a wave of apoptotic cell death is described in the spinal cord during normal postnatal development [31, 39]. In these studies, apoptotic cells were

identified as interneurons based on their anatomical location within the spinal cord and their immunocytochemical profile.

Besides programmed cell death during development, apoptosis in the spinal cord has been demonstrated in several pathological conditions like ischemia, infection, radiation or trauma [25, 36, 40].

Apoptosis after (oxidative) cell injury has been related to the NO-cGMP pathway through activation of the NMDA receptor. Unregulated NO production can cause cell death (both apoptosis and necrosis) through, among others, oxidative stress [49]. NO-mediated apoptosis of cardiomyocytes has been found to be cGMP dependent [60]. The selective and delayed neuronal death in the spinal cord after ischemia has been related to NO [68]. Furthermore, an increase of cGMP has been described in the rat striatum after severe perinatal asphyxia [37].

Biochemistry: the NO-cGMP signal transduction system

The discovery of the biological function of NO in the cardiovascular system by Furchott and Zawadzki and the reported role of NO in the brain by Garthwaite et al., has raised considerable interest of neuroscientist for the molecule in the past decade [16, 18]. Several different mechanisms of action of NO within the CNS are known. Firstly, NO is a neurotransmitter with totally different properties than other known neurotransmitters. As a neurotransmitter, NO acts as an intercellular messenger molecule after activation of the NMDA receptor by glutamate (Figure 1). Activation of the NMDA receptor results in a Ca^{++} influx in the cell. Intracellularly, NO synthase (NOS) catalyses the conversion of L-arginine and oxygen to L-citrulline and NO (a Ca^{++} /Calmodulin and NADPH dependent process). After diffusion across the cell membrane and into surrounding cells, NO activates soluble guanylyl cyclase (sGC) which in turn synthesizes the second messenger cyclic guanosine 3',5' monophosphate (cGMP) [42]. Secondly, NO is known to be a highly reactive free radical with a strong cytotoxic action through the formation of peroxynitrite ($\text{O}_2^- + \text{.NO} \rightarrow \text{ONOO}^-$) [26].

From a functional point of view NO has been implicated in the regulation of excitation, long term potentiation, long term depression and in memory processes [55]. In the CNS, cGMP is synthesized by guanylyl cyclases. This class of enzymes can be differentiated into two main groups, i.e. conventionally called the membrane-bound or particulate guanylyl cyclase (pGC) and the cytosolic or soluble guanylyl cyclase (sGC). While activation of sGC is mainly NO-mediated, pGC is activated by natriuretic peptides [10]. Once cGMP is formed, it is rapidly hydrolyzed to the biologically inactive non cyclic form 5'-GMP by the family of cyclic nucleotide phosphodiesterases (PDE's) [11]. All members of the PDE family, 11 different isoforms have been described thus far, differ in their tissue distribution, affinity for cGMP or cAMP and regulatory mechanisms [15].

Although the exact function of cGMP in the CNS is largely unknown, cGMP is thought to have several effects in the (developing) CNS. In the spinal cord, cyclic nucleotides like cGMP contribute to spinal hyperalgesia [17]. Recent studies provide evidence for a contribution of the NO-cGMP pathway in the development and maintenance of central sensitization of spinothalamic tract neurons by reducing the effectiveness of spinal inhibition [33, 34]. Furthermore, cyclic nucleotides are thought to be involved in the guidance of neural growth cones during development [6].

Immunocytochemistry studies revealed that, in response to NMDA, cGMP accumulated in a population of small cells and neuropil in laminae II and III of the dorsal horn [48]. The localization and function of cGMP in the spinal cord in rats after perinatal asphyxia is presently unknown.

Figure 1. The NO-cGMP pathway

Schematic representation of the NO-cGMP pathway superimposed on a drawing of the synaptical cleft. The explanation of the figure is given in the text.

Perinatal asphyxia in the rat

Several models for cerebral asphyxia have been described in the literature. Most of these models are arterial occlusion models in combination with an hypoxic environment, most probably more representative for local ischemia and not for the asphyxia as we have used in our perinatal asphyxia model [14, 25]. We used a model for perinatal asphyxia described by Loidl et al. which is a more representative model for the clinical situation of perinatal asphyxia [38].

In short, time pregnant Wistar rats were used (Figure 2). The rats were decapitated and hysterectomized after their first pup was allowed to be delivered vaginally (control, CVD). Both the uterus horns were placed in a water bath at 37°C for 20 minutes (severe perinatal asphyxia, SPA). After 20 minutes the uterus horns

Since approximately 150 years doctors and scientists have studied the patient with impairments in locomotion which originated in childhood, initially known as Little's disease and later termed cerebral palsy. The first detailed description of cerebral palsy was done by Little in 1861 in a paper presented for the London Obstetrical Society [35]. The clinical features observed by Little were attributed to difficult birth. Nowadays the term cerebral palsy is used for a group of conditions mainly characterized by motor disabilities resulting from a permanent and non progressive injury of the developing central nervous system (CNS) [45]. The injury of the CNS can take place during several stages of development namely: pre- peri- or post- natal.

The care for neonates is constantly changing. Due to the improving care for increasingly younger and sicker neonates, an increasingly higher survival rate is achieved. Two of the long term consequences of this changing care are an increase in the incidence and prevalence of cerebral palsy [8, 64].

Perinatal asphyxia, a condition which may lead to the clinical picture of cerebral palsy, is a common cause of mortality and morbidity in childhood with a prevalence of 1.5 to 2.5 per 1000 live term births [29]. In perinatal asphyxia a combination of ischemia (no bloodflow), hypercapnia and hypoxia (low oxygenation of the blood) can cause tissue damage in all organ systems. The severity of CNS damage after asphyxia is dependent on the developmental stage of the newborn,

were opened, the pups removed, resuscitated and allowed to recover in an incubator at 37°C. The pups were placed with a surrogate mother. The advantages and disadvantages of this model are discussed in the general discussion.

Figure 2. Rat model for perinatal asphyxia

The used rat model for perinatal asphyxia. Adapted from Loid et al[37].

Thesis outline

For the outline of this thesis we choose to make a clinically oriented arrangement of the different chapters, starting with the studies describing normal development of the rat neonatal spinal cord, both biochemical and morphological, followed by the studies describing the pathological condition (perinatal asphyxia) and the studies on the (pharmaco-) therapeutical interventions. The initial emphasis on the animal model changes to a more clinical emphasis in the last chapters.

In chapter 2 the localization of NO and ANP mediated cGMP synthesis in the developing rat spinal cord are investigated. Furthermore, the developmental pattern of apoptosis in the spinal cord white matter is described in chapter 3.

The effects of perinatal asphyxia on developmental apoptosis in the spinal cord of neonatal rats is described in chapter 4.

In chapters 5 and 6 the effects of the spasmolytic drug Baclofen on the cGMP synthesis in the spinal cord are studied, both during normal development and after perinatal asphyxia. The effects of the radiofrequency lesion of the dorsal root ganglion on the rat spinal cord are studied in chapter 7.

In chapters 8 and 9 the clinical implications of RF-DRG as a treatment option for spasticity in children with cerebral palsy are reviewed and investigated. Finally, in the general discussion, the results of the different studies and the future aims are discussed

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Abstract

An immunocytochemical technique was used to study the localization and developmental aspects of GMP (cGMP)-synthesizing structures in the cervical spinal cord of 2 weeks and 3 months old Lewis rats in response to the nitric oxide (NO) donor sodium nitroprusside (SNP) and / or atrial natriuretic peptide (ANP). By using cell-specific markers, the cell structures involved were investigated. To visualize cGMP a combined technique of low- and high-power magnification, using a confocal laser scanning microscope was used. NOS-mediated cGMP synthesis was observed in the cervical spinal cord in laminae I, II and III in 14 days old rats, which activity was mainly absent at the age of 3 months. The involvement of NO in the NMDA-mediated increase in cGMP immunostaining (cGMP-IS) was demonstrated by the absence of cGMP-IS in slices incubated in the presence of NMDA together with the NOS inhibitor N^G -nitro-L-arginine methyl ester (L-NAME). This NO-mediated effect of NMDA on cGMP-IS was completely absent in the 3-month-old rats. ANP-mediated cGMP synthesis resulted in an increase in cGMP in laminae I and II which was generally similar at both ages. Astrocytes in both white and gray matter were found to be cGMP-IS in the basal, NO- and ANP-stimulated conditions. Using confocal laser microscopy, NO-mediated cGMP synthesis was observed in large cholinergic terminals nearby motor neurons in the ventral horn. An extensive colocalization between NO-stimulated cGMP synthesis and parvalbumin-positive (GABAergic) neurons and fibers was observed in all laminae. In the ANP-stimulated condition a colocalization with parvalbumin structures was found in laminae II and III. No NO- or ANP-mediated cGMP synthesis was found in fibers immunopositive for the presynaptic glutamate transporter, serotonin, or tyrosine hydroxylase.

Introduction

In the central nervous system (CNS) the second messenger cyclic GMP (cGMP) is synthesized by guanylyl cyclases. This class of enzymes can be differentiated into two main groups, i.e. conventionally called the membrane-bound or particulate guanylyl cyclase (pGNC) and the cytosolic or soluble guanylyl cyclase (sGNC) [36, 37, 43, 63]. pGNC's are large transmembrane molecules which have a receptor domain at the outside and the catalytic site at the inside of the cell. pGNC is activated through interaction of peptide hormones with the receptor domain. In the rat CNS, activation of pGNC has only been demonstrated for natriuretic peptides (ANP's). sGNC, in the form which can be activated by NO, is composed of two different subunits associated with a heme group [20, 24, 36, 37, 43]. In recent years, evidence has accumulated that NO is an important endogenous activator of sGNC in the CNS [5, 17, 18, 24, 44, 52]. The distributions of neuronal NOS (nNOS) and NO-mediated cGMP synthesis, as visualized using immunocytochemistry, were shown to be overlapping throughout the CNS [5, 52]. Although it has been demonstrated that cGMP synthesis may occur in a few NOS-containing structures such a colocalization proved to be an exception [5, 6].

The cGMP- signalling pathways in the rat brain changes significantly during postnatal development, and both, sources and targets of NO seem to be involved [8, 34, 55]. pGNC and sGNC activities are high after birth and decline until adult levels of enzyme activity are reached at post-natal age of 21 days. Not only the enzyme activities change, but also the cellular structures containing the active enzymes change with age [34].

The distribution of nNOS in the spinal cord of the rat has been described in detail [3, 35, 54, 56, 61, 66]. In contrast, little is known about the regulation and especially the localization of cGMP synthesis in the spinal cord. Morris et al. showed that the NO-cGMP pathway in the neonatal rat spinal cord is located primarily in the superficial layers of the dorsal horn (lamina I and II) [35]. There is evidence that cGMP in the dorsal horn facilitates hyperalgesia, although this has

been questioned recently [16, 26]. However, at present it is not known which cell-types are involved in the cGMP response. In addition, although ANP receptors have been described in the spinal cord, there are no data on ANP-mediated cGMP synthesis in this part of the nervous system [42, 58, 68].

The aim of this study was to describe the localization of cGMP synthesis in the spinal cord in response to the NO-donor sodium nitroprusside (SNP) and/or ANP. We performed a detailed study on the localization and developmental aspects of the cGMP synthesizing structures in the spinal cord of the 2 weeks and 3 months old rat. In addition, we attempted to characterize these structures in terms of neurotransmitters involved, or cell-specific marker molecules.

Materials and Methods

Tissue preparation

Lewis rats aged 2 weeks and 3 months were used. Rats were bred and housed at the University facility for experimental animals. All experiments were approved by the committee on animal welfare according to Dutch governmental regulations.

At each age, at least six rats were examined. After decapitation, the cervical spinal cord was taken out and cut into 400 μm slices using a McIlwain tissue chopper [8]. Individual slices were immediately placed in aerated Krebs-Ringer bicarbonate buffer, kept at 25°C. Subsequently the temperature was raised till 35°C and the slices were incubated in the same buffer for 30 minutes at 37°C under an atmosphere of 95% O_2 and 5% CO_2 and in the presence of 1mM isobutylmethylxanthine (IBMX) to inhibit phosphodiesterase activity.

In each experiment the slices were further incubated for another 10 minutes with either 100nM ANP or 0.1 mM of the NO donor sodium nitroprusside to activate pGNC and sGNC respectively. NMDA (0.1 mM) was present for 2 min.

To block NOS activity, some slices were incubated with 100 mM N⁶-nitro-L-arginine methyl ester (L-NAME) from the start of the incubation.

The incubations were terminated by adding ice-cold fixative solution (final concentration 4% paraformaldehyde, 10% sucrose, pH 7.4). Fixation was continued for 2hrs, followed by a 30 min wash in ice-cold 0.1 M phosphate (pH 7.4) containing 10% sucrose. Subsequently, cryostat sections (10µm) were cut and thawed on to chrome-alum/gelatin-coated slides and processed for immunochemistry.

Immunocytochemistry

All antibodies dilutions were made in Tris-buffered saline (TBS; pH 7.6) containing 0.3% (v/v) Triton X-100 (TBS-T). Sections were incubated overnight at 4°C with the primary antibodies in the dilutions indicated below. Primary antibodies were visualized with the secondary antibodies as indicated.

cGMP-immunoreactivity was visualized using sheep anti-cGMP antiserum (1:4000) in combination with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep antiserum at a 1:150 dilution, or a rabbit anti-cGMP antiserum (1:300) in combination with a CY-3 conjugated donkey anti-rabbit antiserum (1:800). To investigate nNOS immunoreactivity a sheep anti-nNOS antiserum (a gift of Piers Emson, Babraham Institute, Cambridge, UK) (1:750) was used in combination with a FITC-conjugated donkey anti-sheep antiserum (1:150) [21]. A mouse monoclonal antibody against GFAP was used (1:30) visualized with a CY-3 conjugated donkey anti-mouse antiserum (1:300). The acetylcholine transporter molecular (VAChT) was visualized using a rabbit antibody raised by the late Dr. Ichikawa [23]. The neuronal glutamate transporter molecule (EAAT1) was demonstrated using a rabbit antibody (1:300) (a gift by J. Rothstein, NIH, USA) [15]. Parvalbumin was visualized with a rabbit antiserum (1:1000) provided by P.C. Emson (Babraham Institute, Cambridge, UK). The mouse monoclonal antibody against tyrosine hydroxylase (1:30) was a gift from Prof. A.C. Cuervo (McGill University, Montreal, Canada).

Photography

Sections were examined with an Olympus AX70 microscopy equipped with a narrowband MNIBA-type FITC filter, or a MNG filter for CY fluorescence (Chroma Technology). These filters made it possible to photograph FITC or CY-3 fluorescence in the complete absence of cross-over fluorescence. As demonstrated previously, this filter combination is adequate to visualize two markers in the same section [5].

Confocal Microscopy

Confocal laser scanning microscopy was performed using a Leica TCS NT (Leica Microsystems, Germany). The Argon-Krypton laser was used for excitation at 488 nm (FITC) and 568 (CY-3). The fluorescence emission was separated with a 580 dichroic beam splitter. Thereafter, FITC fluorescence was selected by a 530(/30)-nm band pass filter, and CY-3 fluorescence was selected by a 590-nm-long pass filter. Cross-over fluorescence was negligible. Each optical section (0.5 μ m) was averaged twice and a series of 8 optical sections (FITC and CY3 fluorescence separately) was made from one tissue section. A representative optical section from the series was selected for photography. FITC fluorescence and CY-3 fluorescen are depicted either separately or combined using the integrated software of the Leica TCS NT.

Materials

Atriopeptin III was from Sigma; sodium nitroprusside and L-NAME were from Fluka; GFAP antiserum was from Innogenetics; FITC-conjugated donkey anti-sheep antiserum was from Alexia; CY-3 conjugated donkey anti-rabbit and donkey anti-mouse antisera were from Jackson; NMDA was from RBI.

Results

cGMP-immunocytochemistry on sections from spinal cord slices incubated *in vitro* in the absence of a nonspecific phosphodiesterase inhibitor like IBMX yielded no results, similar to the situation described for most areas of the brain and peripheral nervous system [8, 47, 67]. Incubation of the rat spinal cord slices at PN14 in the presence of 1 mM IBMX resulted in intense cGMP immunostaining (cGMP-IS) in the superficial layers of the dorsal horn. This finding indicates the presence of high phosphodiesterase activity. Therefore, all incubations were performed in the presence of 1 mM IBMX. Reference in the text to basal cGMP levels refers to cGMP levels observed after incubation of the slices in the presence of 1 mM IBMX only.

In Table 1 we present a summary of the experiments. In this table, we indicate that, although the majority of the responses of the slices to the pharmacological treatments was consistent, there was some inter-experimental variation. Thus, basal cGMP-IS in the spinal cord slices at the age of 3 months, was generally absent (even in the presence of 1 mM IBMX). Nevertheless, in 2 experiments a very slight basal cGMP-IS was observed. In addition there was an inter-experimental variation in the extent of cGMP-IS in the slice after stimulation of the respective cyclases, which was most prominent with ANP (compare Figs. 1 and 3).

Table 1

Summary of the experiments performed with rat spinal cord slices at PN14 and at 3 months of age.

Numbers represent the number of experiments

Age		Conditions								
		Basal	SNP	ANP	NMDA	L-NAME	SNP + L-NAME	ANP + L-NAME	NMDA + L-NAME	
PN 14	absence cGMP-IS	0	0	0	0	5	0	0	3	
	cGMP-IS	11	11	8	6	0	6	5	0	
	weak cGMP-IS	0	0	3 ^a	0	1	0	0	0	
3 months	absence cGMP-IS	20	0	0	10	10	0	0	4	
	cGMP-IS	0	24	16	0	0	5	5	0	
	weak cGMP-IS	3	0	3	0	0	0	0	0	

^a Weak cGMP-IS was observed in the laminae V-X

cGMP synthesis in the cervical spinal cord at PN14

At 2 weeks after birth, in the absence of stimulators of either pGNC or sGNC added to the slices (basal conditions), cGMP-IS was observed in lamina I, II and III of the dorsal horn and in some ramifying, isolated cells which might be observed in each layer (Fig. 1a). Incubation of the slices in the presence of 0.1 mM L-NAME abolished this basal cGMP-IS (Fig. 1b), indicating that active NOS was involved in this cGMP synthesis. Treatment of the slices with 0.1 mM SNP for 10 min resulted in the accumulation of NO induced cGMP in cells and fibers in the white and grey matter (Fig. 1c). This effect of SNP on cGMP-IS was not sensitive to L-NAME (Fig. 1d). Incubation of the slices in the presence of 0.1 mM NMDA increased cGMP-IS in lamina I and II (Fig. 1e). The involvement of NO in the NMDA-mediated increase in cGMP-IS was demonstrated by the absence of cGMP-IS in slices incubated in the presence of NMDA together with 0.1 mM L-NAME (Fig. 1f). Stimulation of pGNC with 100 nM ANP resulted in an increase in cGMP in lamina I and II, the areas where also basal cGMP synthesis is ongoing (Fig. 1g). Therefore, the effect of ANP was revealed convincingly in the presence of 0.1 mM L-NAME (Fig. 1h). The effect of ANP was consistently strong in the lamina I and II and a variable effect was noted in white matter areas.

At 2 weeks after birth NOS-immunostaining was observed in structures which were mainly located in laminae I, II and X (particularly in the dorsal commissure), the lateral cervical nucleus and in neuronal cell bodies diffusely present in grey and white matter. In general the nNOS immunostaining was in agreement with previous published data (see Section 4). We found a good complementary overlap between the NO-mediated cGMP-IS and nNOS throughout the slice, with an occasional colocalization between NO-mediated cGMP and nNOS (Fig. 2a and b, arrowheads). In all layers of the cervical spinal cord we found accumulation of cGMP in astrocytes which were characterized by GFAP-IS. However, as shown in Fig. 2c - f), not all GFAP-positive structures were cGMP-positive as well.

Figure 1. cGMP-immunostaining in slices from the 2-week-old rat cervical spinal cord incubated *in vitro* under the following conditions: (a) basal situation; (b) in the presence of 0.1 mM L-NAME; (c) in the presence of 0.1 mM SNP; (d) in the presence of 0.1 mM SNP and 0.1 mM L-NAME; (e) in the presence of 0.1 mM NMDA; (f) in the presence of 0.1 mM NMDA and 0.1 mM L-NAME; (g) in the presence of 100 nM ANP; (h) in the presence of 100 nM ANP and 0.1 mM L-NAME. Photographs were taken with a 4x objective using extended exposure times up to 5 minutes (g and h). Bar is 100 μ m for all pictures.

Figure 2. Rat cervical spinal cord at the age of 2 weeks. Double immunostaining of cGMP with nNOS (a and b) or GFAP (c - f): (a) cGMP-IS in a section from the dorsal horn of a slice incubated in the presence of 0.1 mM SNP; (b) same section as described under (a) immunostained for nNOS; arrowheads point to colocalization of cGMP and nNOS. In (c) and (e) cGMP-IS is shown in the dorsal (c) and ventral (e) part of a slice incubated in the presence of 0.1 mM SNP. The same section was also immunostained for GFAP (d and f). Arrowheads point to a colocalization between cGMP and GFAP. For cGMP-IS in (a) we used the rabbit anti-cGMP antiserum and for cGMP-IS in (c) and (e) we used the sheep anti-cGMP antiserum. Although both antisera immunostain exactly the same structures (verified using double immunostaining for cGMP using both antisera), the sheep anti-cGMP antiserum performs superior to the rabbit anti-cGMP antiserum in sections from spinal cord slices. The reason for this disparate performance of the antisera is not known. (see also Figs 3d, f, and h). Bar is 50 μ m for all pictures.

Figure 3. Characterization of cGMP-IS (a, b, d, f, and h) in sections from rat spinal cord at the age of 3 months. (a) basal conditions; (b, d, f, and h) in the presence of 0.1 mM SNP; (c) and (d) double immunostaining of the same section for nNOS and cGMP (dorsal horn); (e and f) double immunostaining of the same section for nNOS and cGMP (ventral horn); (g and h) double immunostaining for serotonin (5-HT) and cGMP in the ventral horn. Arrowheads in (c) through (f) point to colocalization of nNOS and cGMP. See the note in the legends of Fig. 2 on the performance of rabbit anti-cGMP antiserum and the sheep anti-cGMP antiserum. Bar is 100 μ m in (a) and (b) and 50 μ m in the other pictures

cGMP synthesis in the cervical spinal cord at 3 months

At the age of 3 months, using low power magnification, no cGMP-IS could be observed in the cervical spinal cord slices incubated under the basal conditions (Fig. 3a). Nevertheless, at high magnification, using a confocal laser scanning microscope, we were able to demonstrate cGMP-IS in some experiments (Fig. 7d) (see also the summary of the results in Table 1). Similarly as found at PN14, NO-responsive GNC activity in the 3 months old spinal cord (incubation in the presence of 0.1 mM SNP) was found in neuronal fibers and cells in all lamina (Fig. 3b). Discrete and dispersed activity was found in cell somata and fibers of the grey matter and in fibers extending into the white matter. The NO-mediated effect of NMDA on cGMP-IS was completely abolished in the 3 months old spinal cord slice. On the other hand, activation of pGNC at this age proved to be generally similar as at PN14, as ANP-mediated cGMP synthesis was found in isolated astrocytes (see below) in both grey and white matter, and also in fibers in lamina I and II of the dorsal horn which showed a clear punctate staining pattern (Fig. 4a and d). No effect of L-NAME on the ANP-responsiveness was observed at this age.

Figure 4. Double immunostaining of cGMP and GFAP in sections from slices of the 3 months old rat spinal cord. Slices were incubated in the presence of 100 nM ANP (a, b, and d) or 0.1 mM SNP (f). GFAP immunostaining in (c), (e) and (g) has been done on the same sections as shown in (b), (e), and (g) respectively. (b) is a two times enlargement of the upper right hand part of (a). Arrowheads point to sites of colocalization between cGMP and GFAP. Bar is 50 μ m for (a) and 25 μ m for all other pictures.

Figure 5. Confocal laser scan microscopy of a section from a slice, incubated in the presence of 0.1 mM SNP, of the ventral horn of the rat spinal cord at the age of 3 months. (a) cGMP-IS; (b) V_{Ach} ; (c) combination of (a) and (b); summation of eight single color sections (eight scans for each color) each taken at a different depth in the section. Yellow color shows the colocalization between the two markers. Bar is 20 μ m for all pictures.

Double-immunostaining of cGMP-IR structures

nNOS-immunostaining in the spinal cord at the age of 3 months was found in varicose fibers throughout all layers and in several cell groups. Many small nNOS-immunopositive cells were observed in the lamina II and III (Fig. 3c). The same pattern as was observed at the age of 2 weeks, however, less intense. Similarly, a good complementary overlap was found between NO-mediated cGMP and nNOS-immunostaining, and an infrequent colocalization of sGNC activity and nNOS was observed in neuronal cells or fibers (Fig. 3 c - f).

In all layers of the 3 months old spinal cord some, but certainly not all, astrocytes (GFAP-positive cells) were found to be cGMP-IR in the basal, NO- or ANP-stimulated condition (Fig. 4).

There was no accumulation of cGMP-IR, after stimulation with either NO or ANP, observed in serotonergic (Fig. 3g and h) or dopaminergic (not shown) fibers. Similarly, no NO or ANP stimulated cGMP production was found in fibers which were immunopositive for the presynaptic glutamate transporter (EAAC1) (not shown). Using confocal laser microscopy, we were able to demonstrate NO-mediated cGMP synthesis in large cholinergic terminals situated on or near the motor neurons in the ventral horns (Fig. 5). In addition, we found a colocalization between ANP-stimulated cGMP synthesis and parvalbumin in cells and fibers of the lamina II and III (Fig. 6a - c). We found extensive colocalization between parvalbumin and cGMP in all lamina of the spinal cord (Fig. 6d - f dorsal horn, and Fig 7 a - c ventral horn). Even in spinal cord slices incubated under basal conditions an occasional colocalization between parvalbumin and cGMP-IS could be demonstrated (Fig. 7d).

Figure 7. Confocal laser scan microscopy from the ventral horn in sections of slices of the spinal cord of the 3-month-old rat. Slices were incubated in the presence of 0.1 mM SNP (a - c) or without additions (basal) (d). (a) cGMP; (b) parvalbumin; (c) combination of (a) and (b). d) combination of cGMP (green) and parvalbumin (red). Colocalization between parvalbumin and cGMP shows yellow. Bar is 10 μ m for all pictures.

← Figure 6. Confocal laser scan microscopy from the dorsal horn in sections of slices of the spinal cord of the 3-month-old rat. Slices were incubated in the presence of 100 nM ANP (a - c) or 0.1 mM SNP (d - f). (a) parvalbumin; (b) cGMP; (c) combination of (a) and (b). (d) combination of (e) and (f); (e) parvalbumin; (f) cGMP. Colocalization of parvalbumin and cGMP is shown in yellow. Bar is 10 μ m for (a) - (c), and 20 μ m for (d) - (f).

Discussion

The expression of the various molecular entities of the cGMP signal transduction cascade shows a strong developmental profile in the CNS of different species [8, 13, 34, 55, 57]. As there is little information available on the cGMP response in the spinal cord of the rat, we studied the developmental aspects of this pathway, in combination with a characterization of the cellular structures responsive to NO and/or ANP. From our results we may conclude that, similar to the brain, NOS and sGC have an extensive distribution in the rat spinal cord and that both enzymes are distributed in overlapping structures. Again similar to the rat brain, colocalization between NOS and sGC appears to be an exception. NO-mediated cGMP synthesis was found in parvalbumin-positive (GABAergic) neurons and in isolated cholinergic synaptic terminal in the ventral horn. We could not demonstrate the presence of NO-mediated cGMP synthesis in other neurotransmitter systems. In contrast to the situation in the rat brain, the properties to activate sGC (by NO) and pGC (by ANP), appeared to be fully developed at PN14. In contrast, the glutamatergic activation of NOS by NMDA was still in a developmental phase at this age.

We compared the effects of a number of NO donor compounds from which NO is released through different mechanisms on cGMP-IR in spinal cord slices of the 14-day-old rat (Fig. 8) [11, 14]. As demonstrated, the effects of SNP, S-nitrosopenicillamine (SNAP) and S-nitroglutathione (SNOG) on cGMP accumulation in the spinal cord slices was qualitatively similar. SNP has been reported to antagonize the NMDA receptor [12]. However, this antagonism was just significant at 0.1 mM SNP only under magnesium free conditions. This indicates that, considering our experimental conditions, the antagonism of the NMDA receptor by SNP is not relevant for our results. In addition, our condition of 0.1 mM SNP for 10 min results in an NO concentration of 50 nM or lower, a concentration which is considered to be in the physiological range [46, 50, 51]. Also, considering the costs, we took SNP as the NO donor compound for this study.

NOS in the adult spinal cord

The localization of NOS is studied routinely using two different approaches, i.e. immunocytochemistry using antibodies against NOS or NADPH-diaphorase histochemistry [2, 22, 60]. The pattern of distribution of NOS-positive neurons in the spinal cord was qualitatively similar across four species, i.e. the rat, mouse, cat and squirrel monkey [10, 12]. Several studies of the adult rat spinal cord showed that distinct groups of neurons express NOS: groups of neurons around the central canal, in preganglionic sympathetic neurons in the intermediolateral horn, throughout the deeper laminae of the dorsal horn and in the ventral horn [9, 35, 40, 53, 56, 62]. Our findings using nNOS immunocytochemistry are in agreement with these previous studies.

Compared to the continuous NADPH-diaphorase/NOS expression in the above described groups of neurons, somatic motor neurons in the rat spinal cord may show a more transient or biphasic diaphorase activity, which may be species dependent [4]. Wetts et al. observed a transient expression of NOS in motor neurons, which is in accordance with the observation made by Bruning et al in mouse spinal cord [3, 64]. In adult rats, no NOS in motor neurons could be found, while in aged rats, with increasing age the number of NADPH-d positive motor neurons increased [9, 28, 40, 54]. Wu et al. found an upregulation of NADPH-d in motor neurons before they died after spinal root avulsion [66]. Motor neuron death could be prevented by nitroarginine, a nonspecific inhibitor of NOS, and it was suggested that induction of NOS expression may be involved in the neuronal death after traumatic injury [66]. In addition the maturation of motor neurons could be blocked by NOS antagonists [27]. We did not find nNOS expression in motor neurons at both ages studied. Therefore, it is clear that the expression of NOS in the spinal cord is complex and depends on age, cell type, topography and state of maturation.

Developmental aspects of NOS expression

The pattern of expression of nNOS in the rat spinal cord at different ages was shown to be different for the various groups of nNOS containing spinal neurons, not only in a horizontal plane but also in a rostral to caudal direction [3, 31, 61, 64]. Thus, cells around the central canal expressed NADPH-diaphorase already very early during development, whereas cells in laminae I and II express this enzyme activity relatively late during development. Similarly, it has been reported recently that the rat embryonic motor neurons contain eNOS and, in addition, it was suggested that at this stage of development, motor neurons were capable of NO-mediated cGMP synthesis [12]. We never observed nNOS-IS, nor NADPH-diaphorase staining or cGMP-IS in motor neurons at the age of 2 weeks or 3 months. This difference in maturation probably reflects the changing capabilities of the developing animal. Our results show that at 14 days after birth, the location and sensitivity of sGNC, as the target for NO, is already similar to the adult situation.

This contrasts with the developmental pattern in the activation of NOS by NMDA receptors which is evident at PN 14 and absent in the adult spinal cord slices. This latter finding is in agreement with the results of Liuzzi et al who described an adult pattern of NOS localization as late as the end of the third postnatal week [31].

cGMP synthesis in the spinal cord: particulate guanylyl cyclase

Little information is available about the localization of both ANP- and NO-mediated cGMP synthesis. In the neonatal rat, the ANP-stimulated cGMP synthesis could only be demonstrated in the presence of L-NAME (Fig. 1). In the adult rat we found ANP-responsive structures in laminae I-II with a clear punctate non-somatic staining and, in addition, in cell somata (Fig. 6). Information about the localization of ANP receptors and ANP-containing structures in the spinal cord are scarce. Immunoreactive ANPs were found in porcine and rat spinal cord, respectively [58, 68]. Nevertheless, ANP binding sites were not observed in the rat,

guinea pig, cat and human [33, 38]. However, Saper et al demonstrated immunostaining for brain ANP of sensory afferent fibers innervating lamina I and IIa, the regions where we localized the ANP-responsive, cGMP-producing cells [42]. In addition Simonnet et al reported the presence of ANP receptors at non-neuronal cells, i.e. astrocytes and epitheloid cells, of the mouse spinal cord [49]. In accordance with these observations and in agreement with our results obtained in the rat brain, we were able to localize ANP-stimulated cGMP synthesis in astrocytes [7]. Presently the function of the ANP-mediated cGMP synthesis in the spinal cord is unknown.

Figure 8. cGMP immunostaining in slices from the 2-week-old rat cervical spinal cord incubated in vitro for 10 min with three different NO donor compounds: (a) in the presence of 0.1 mM SNP; (b) in the presence of 0.1 mM S-nitropenicillamine (SNAP); (c) in the presence of 1 mM S-nitrosoglutathione (SNOG). Photographs were taken with identical exposure times and after development of the film the exposure times were kept constant. Bar is 25 μ m for all pictures.

cGMP synthesis in the spinal cord: sGNC

Morris et al described the NO-mediated cGMP response in immature spinal cord, and our results confirm these initial observations [35]. Using IBMX as a PDE inhibitor, we found that in the neonatal spinal cord slices there is a certain amount of age related ongoing NO synthesis. This 'spontaneous' endogenous NO synthesis is absent in the adult spinal cord slices. As already discussed above, we found an age related decline of NMDA-stimulated and NO-mediated cGMP response in the spinal cord. On the other hand, our results did not show qualitative changes in the effects of SNP on cGMP synthesis during maturation. A close correspondence in location was observed between the NO-mediated cGMP response and the nNOS in the spinal cord. This correspondence is generally complementary, with occasional colocalization between cGMP-IR and NOS. These findings are in agreement with previous findings in the rat brain [5, 52].

Double immunolabeling of cGMP: colocalization studies

Our double immunolabeling studies showed that NO-mediated cGMP synthesis in the rat spinal cord is found primarily in parvalbumin-containing cells and fibers. About 60 - 70% of the parvalbumin-immunoreactive neurons in lamina I and II were described as being GABAergic neurons whereas in the layers III and IV this percentage of colocalization was considerably lower [1]. Nevertheless, as GABA is found in all gray matter layers, the parvalbumin-immunopositive fibers observed in layers V-VIII could arise from interneurons situated in layers VII and VIII or belong to dendritic trees of cells in layers III and IV [32]. These findings raise some questions about structure and function of the NO - cGMP signal transduction pathway in the spinal cord. First, the complementary nature of the location of NO-synthesis and the NO-effect (cGMP synthesis) as discussed above, supports the concept of an intercellular messenger role for NO [17]. In this concept NO is often thought to act as a retrograde messenger and to enhance excitatory neurotransmission [17, 18, 29, 65]. Secondly, it has been reported that there is some degree of colocalization of NOS and GABA in the lamina II islet cells and the

laminae II-III border cells [30, 59]. On the basis of the absence of colocalization between cGMP-IR and NOS and the presence of colocalization between cGMP-IR and parvalbumin, we conclude that cGMP synthesis does not take place in the GABAergic neurons which also contain NOS. Thirdly, cGMP-IS was found in large boutons which were positive for the acetylcholine transporter molecule (Fig. 5). These boutons were found in the ventral horn and adjacent to the large motor neurons. The significance of this observation is presently unclear. Apart from the motor neurons, cholinergic neurons are found in lamina III and these cells have been reported to possess also NOS [54]. According to our observations these cells do not contain sGC as an NO target. This disparity in NO-responsiveness of the cholinergic somata and fibers has also been observed in the rat brain (De Vente et al, submitted). Our finding of NO-mediated cGMP accumulation in parvalbumin-positive fibers and some cholinergic fibers does not encourage interpretation of the effect of NO as a retrograde messenger molecule inducing long-lasting changes in excitatory synaptic transmission. It is not clear what the effect of a raise in cGMP level in the GABAergic terminal will have on GABAergic neurotransmission. As there is good evidence that NO and cGMP are involved in nociceptive signalling, it might also be that NO decreases GABAergic neurotransmission along a cGMP-dependent pathway [16, 26, 41, 48]. Therefore, our data support the concept, as has been reported for the rat brain, that NO may mediate both excitatory and/or inhibitory functions depending on the site of activation of NOS in specific neural networks [19, 39, 45]. Our results are in agreement with a recent concept that during development NMDA-receptor mediated activation of NOS in the ventral horn may be involved in the arborization of motor neuron dendrites [25]. In addition, our findings show that it is not possible to generalize one concept of NO-action in the spinal cord. Depending on the layer of the spinal cord, Figs 8 and 9 summarize tentatively the NO-cGMP pathways in the dorsal horn and the ventral horn respectively.

Figure 9. Schematic representation of the organization of sources and targets of NO in the dorsal horn (a) and ventral horn (b) of the cervical spinal cord slice. NOS is activation by glutamate and NO diffuses of the range indicated by the sphere (the diameters of the spheres in (a) and (b) bear no relation to the actual diffusion range of NO). cGMP synthesis (dark gray) is stimulated in parvalbumin immunopositive structures in the dorsal horn (a) and in the ventral horn (b). Large synaptic terminals which are immunostaining for cGMP and V_{Ach} are found in the ventral horn (dark grey). Similar synaptic terminals are found which are staining for V_{Ach} (white) or cGMP (black) but not for both markers. No cGMP-IS was observed in glutamatergic fibers. The asterisk (*) in (a) indicates a passing fiber not responding to NO; this might be a glutamatergic, serotonergic, or dopaminergic fiber. Contrary to the *in vivo* situation in our study, cGMP synthesis is stimulated only in that segment of the parvalbumin fibers, which is situated within the diffusion distance of NO.

Conclusions

In conclusion, our data show the presence of an abundant NO · cGMP signaling system in all layers of the spinal cord. The NO-mediated cGMP synthesis takes place in parvalbumin (GABAergic) neurons and in the ventral horn in axon terminals probably connecting to the motor neurons. In addition cGMP synthesis can be found in a subpopulation of glial cells either through activation of pGNC and/or sGNC. Using cGMP-IS as a read-out for NO-effector structures, no evidence was found for a role for NO as a retrograde messenger in glutamatergic presynaptic terminals in the adult cervical spinal cord.

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Abstract

We investigated developmental apoptosis in the white matter of the neonatal rat cervical spinal cord at postnatal day 2, 5, and 8. Apoptotic cells were labeled using TUNEL and caspase-3 immunostaining. Apoptotic cells were diffusely distributed throughout the white matter of the spinal cord. The total amount of apoptotic cells in the cervical spinal cord white matter was related to postnatal age, with the lowest at P2 (mean 7.9 , SD 5.6) and the highest at P8 (mean 109, SD 21.4). Using double immunostaining for ED-1 and O4, apoptotic cells were identified as microglia and oligodendrocytes.

Cell death during normal development has been described in nearly all neuronal cell types in the central nervous system (CNS) and peripheral nervous system (PNS) as well as in the cerebral gray and white matter [8, 11]. In contrast to a large number of studies in brain tissue, only few studies described cell death in the spinal cord. The main focus of these studies was cell death through apoptosis or necrosis of motoneurons after trauma, infection or ischemia of the spinal cord [6, 12]. For developmental cell death, a wave of apoptosis in the gray matter of the embryonic and postnatal spinal cord of the rat has been reported [4, 13]. A distribution of apoptotic cells was found from a ventral to a dorsal extension, with a high total number of cells in the ventral horn at embryonic day 16 and high counts in the dorsal horn postnatally. No developmental apoptosis has been described in the white matter of the spinal cord of normal rats [4,7].

The present study demonstrates apoptosis in the white matter of the developing spinal cord of normal postnatal rats. This observation was made coincidentally in a project studying the effects of perinatal asphyxia on the developing spinal cord in rats.

Wistar rats were used to investigate developmental apoptosis in the spinal cord. The rats were anaesthetized and sacrificed by transcardial perfusion fixation with 4 % paraformaldehyde at postnatal day 2 (P2), 5 (P5) and 8 (P8). For the labeling of apoptotic cells we used a modified TdT-mediated dUTP-biotin nick end-labeling (TUNEL) technique in which we used methacarn (methanol : chloroform : acetic acid = 66:33:1) in stead of proteinase K [2]. TUNEL positive cells were counted in 6 successive cervical spinal cord sections per rat at level C3-C4.

TUNEL- positive cells were detected in the spinal cord white matter (figure 1). These cells were distributed diffusely throughout the whole white matter (both cervical and lumbar), with no obvious preference for a subscribed region. The total amount of apoptotic cells in the cervical spinal cord white matter differed with age, being the lowest at P2 (mean 7.9, SD 5.6), median at P5 (mean 76.2, SD 41.3) and the highest at P8 (mean 109, SD 21.4).

To confirm apoptosis, we investigated co-localization between TUNEL and activated caspase-3 (BD Biosciences, Europe), and detected co-localization of TUNEL and caspase-3 (figure 2a, 2b) in nearly all TUNEL stained cells. With concern to gray matter apoptosis our findings are in agreement with the findings of Lawson et al. However, in contrast to Lawson et al. we also observed apoptosis in the white matter [4].

To identify the type of cells which were TUNEL positive, different (immuno-) cytochemical markers were used. Negative controls were performed by omitting the primary antibody. No co-localization was seen using neuron specific enolase (NSE) as a marker for neurons, or glial fibrillary acidic protein (GFAP) as a marker for astrocytes. The observations of the lack of co-localization between TUNEL and GFAP are in accordance with the findings of Lawson et al. [4]. Similarly, using vimentin, a marker for immature astrocytes, no co-localization with TUNEL was observed.

Co-localization was observed between TUNEL and ED1 (figure 2g, 2h, 2i), a specific marker for reactive microglia or macrophages, in both the white and gray matter of the cervical and lumbar spinal cord. Approximately 5% of ED1 positive cells were also TUNEL positive, while 18% of TUNEL positive cells could be identified as microglia or macrophages.

TUNEL positive cells could also be identified as immature oligodendrocytes in both the white and gray matter, using the specific marker O4 (Chemicon international, Temacula) (figure 2c, 2d, 2e, 2f) [9]. With the use of methacarn (methanol) in the TUNEL technique, O4 immunoreactivity could still be observed. Co-localization with O4 could be detected in 32% of TUNEL positive cells. This most probably is an underestimation due to the influence of methanol on O4 immunoreactivity.

To our knowledge no studies have reported developmental apoptosis in the spinal cord white matter. One study describing myelination patterns in the anterior funiculus of the lumbar spinal cord of newborn rats reported electron dense material in immature oligodendrocytes [14]. However, these features were

attributed to phagocytosis and not to apoptotic cell death. More recently, apoptosis in the spinal cord white matter has been described in genetically altered mice or myelin-deficient rats [5, 12]. These models are not fully representing the normal developmental process in the spinal cord. In addition, apoptotic features in oligodendrocytes in the white matter have been described after trauma, ischemia or radiation [1, 6, 10]. In these studies cell death was induced in adult rats, again a process not representative for normal developmental apoptosis.

Figure 1.
(a) Localization of TUNEL positive (red) cells in the cervical spinal cord white matter at post natal day 5 of a normal rat (blue, Hoechst, Sigma). These images were taken using an Olympus Ax70 microscope equipped with a cooled CCD camera (F-view). Images were taken with a CY3 (U-M 41007a) filter (red, b) for TUNEL processed sections and with a DFR (U-M 61000) filter for the Hoechst staining. Using the Image Analysis System (SIS), gray scaled images were directly converted into artificial colors. This representative low power microphotograph clearly indicates TUNEL positive cells within the white matter. Due to the low magnification, the typical combination of red and blue only appears very moderate in the TUNEL positive cells, a higher magnification is shown in the insert (Magnification 40x, GM; gray matter, WM; white matter, VR; ventral root).

Figure 2.

Photomicrograph illustrating developmental apoptosis in white matter of the P5 cervical spinal cord (magnification 100x for all images). The cells shown in (a), (d) and (g) all show the characteristic nuclear fragmentation observed during apoptosis (Hoechst, Sigma , blue). (a) TUNEL positive cell double labeled for the apoptotic marker caspase-3 (red, b). (c) O4 immunoreactivity without the use of methacarn. (d) oligodendrocyte (O4 positive, green, f) also staining positively for TUNEL (red e). (g) an ED1 positive microglia/macrophage (green, i) double labeling for TUNEL (red, h). The images were taken using an Olympus AX70 microscope equipped with a Sony color CCD power HAD.

Not all the TUNEL positive cells could be identified in our study. The remaining cells most probably are astrocytes, although no co-localization between TUNEL and GFAP, or vimentin was detected. Identifying filamentary proteins during development of neural tissue cannot always be done because of difference of developmental profile of the various markers. This may be the case in this study with both vimentin and GFAP.

Future studies will focus upon the functional relationship between (inter-) neural and oligodendrocyte apoptosis, as our findings imply a role for apoptosis in the spinal cord white matter in the development of normal locomotor behavior and possibly of locomotor abnormalities (e.g. spasticity) after perinatal asphyxia [3].

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Abstract

The aim of our study was to investigate the effect of perinatal asphyxia on developmental apoptosis in the cervical and lumbar spinal cord in the neonatal rat. Perinatal asphyxia was induced by keeping pups at term in utero after hysterectomy in a water bath at 37° C for 20 min, followed by resuscitation. Effects of this treatment on developmental apoptosis were studied at postnatal day 2, 5 and 8 using TUNEL and caspase-3 staining. TUNEL positive cells were identified using double immunostaining.

On postnatal day 2 an increase of 215 % in TUNEL positive cells was detected ($p = 0.005$) in laminae IV-VII of the lumbar spinal cord of rats which underwent perinatal asphyxia compared to controls. An increase of 55 % compared to controls ($p = 0.03$) was seen in laminae I-III of the lumbar spinal cord at postnatal day 8. TUNEL positive cells could be partly identified as microglia cells (ED1 positive) and oligodendrocytes (O4 positive).

The effect of perinatal asphyxia on programmed cell death in the neonatal rat spinal cord was mainly observed in the intermediate zone and dorsal horn of the lumbar spinal cord.

It is concluded that perinatal asphyxia has a pronounced effect on the survival of cells in a specific region of the spinal cord and thus may have a profound effect on the development of motor networks.

Introduction

Perinatal asphyxia leading to cerebral palsy is the most common cause of mortality and morbidity in childhood, with a prevalence of 1.5 to 2.5 per 1000 live births [18]. Although cerebral palsy is not due to birth injury alone, between 8 and 20 percent of children with cerebral palsy suffered some degree of asphyxia during birth [28, 30].

In the first description of cerebral palsy by Little in 1861, the assumption was made that chronic spasticity after asphyxia is referred to lesions in the spinal cord [21]. In a more recent study, histological evidence of hypoxic-ischemic injury of the spinal cord gray matter was present after neuropathological examination of asphyxiated neonates [3].

Perinatal asphyxia can lead to mental retardation, epilepsy and motor abnormalities such as spasticity [13]. Seizures and mental retardation are obvious clinical signs of an impaired cerebral function. However, the locomotor abnormalities of cerebral palsy may not be solely derived from cerebral dysfunction but could also partly be caused by dysfunction of the spinal cord neural network. Spasticity, a velocity dependent increase in tonic stretch reflexes, is a common locomotor abnormality after perinatal asphyxia [19].

The pathophysiological mechanisms in spasticity are not completely understood. Yet, in spastic cerebral palsy, evidence for hyperexcitability of spinal alpha motor neurons due to abnormal supraspinal inhibition or abnormal segmental control of spinal motor neuron output exists [9, 15, 34]. An emphasis on spinal inter-neuron damage has been made by Harrison as a possible cause of local spinal disorganisation and spasticity [15].

In recent studies by Lawson and Lowrie developmental programmed cell death of spinal interneurons in the rat has been described [20, 24]. A wave of apoptosis in the grey matter of the lumbar spinal cord in late embryonic and early postnatal rats was observed. By means of immunocytochemical labelling for neuronal markers and the anatomical distribution of apoptotic cells, they

concluded that most apoptotic cells were interneurons and not motoneurons, astrocytes or oligodendrocytes.

Although neural networks within the spinal cord are involved in the development of normal and abnormal locomotion no studies have been done addressing the influence of perinatal asphyxia on (programmed) cell death in the spinal cord.

The aim of our study was to investigate the effect of perinatal asphyxia on developmental apoptosis in the cervical and lumbar spinal cord in the neonatal rat.

Experimental Procedures

Inducing asphyxia

Severe perinatal asphyxia was induced as described in detail by Loidl et al., using time pregnant Wistar rats at the last day of gestation [23].

Rats were bred by Charles-River company and housed at the University Maastricht facility for experimental animals. Water and food were given ad libitum. All experiments were approved by the committee on animal welfare according to Dutch governmental regulations and all efforts were made to minimize the number of rats used and their suffering.

The rats were decapitated and hysterectomized after their first pup was allowed to be delivered vaginally (control, CVD). Both the uterus horns were placed in a water bath at 37°C for 20 minutes (severe perinatal asphyxia, SPA). After 20 minutes the uterus horns were opened, the pups removed, resuscitated and allowed to recover in an incubator at 37°C. The pups were placed with a surrogate mother and sacrificed at either postnatal day 2 (PN2), 5 (PN5) or 8 (PN8). The overall mortality rate after 20 minutes of severe perinatal asphyxia was approximately 40 percent and similar to previously reported mortality rates using the above described model for inducing perinatal asphyxia [23].

Tissue preparation

The rat pups were anaesthetized by an intra peritoneal injection of sodium pentobarbital (Sanofi, 60 mg/kg) and transcardially perfused with a flush of Tyrode solution (pH 7.4, 37°C) followed by 4% paraformaldehyde, in 0.1 M phosphate buffer (pH 7.4, 37°C). The total spinal cord was dissected and post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. The spinal cord was cut into parts of two segments, and embedded in warm 10% gelatin in 0.1 M phosphate buffer.

The gelatin cups were kept on ice for 1 hour, and fixed in 4% paraformaldehyde in 0.1 M Phosphate buffer for 2 hours. The gelatin blocks containing the spinal segments were cryoprotected in 15% sucrose in Tris-buffered saline (TBS, pH 7.4) overnight at 4°C, and frozen in liquid-nitrogen cooled 2-methylbutane. Subsequently cryostat sections (10 µm) were cut (Leica CM3050) and thawed onto chrome-alum/gelatin coated slides, stored at -80 °C and processed for immunocytochemistry.

Immunocytochemistry and histochemistry

Cervical and lumbar segments of the spinal cord slices were used for the various staining techniques. Three methods were used to study the presence of apoptotic nuclei. First presence of apoptotic nuclei was studied using a chemical hematoxylin and eosin (HE) staining. Secondly, a modified terminal deoxynucleotidyl transferase mediated biotinylated-14-dUTP nick end labelling (TUNEL) technique was used and thirdly a caspase-3/TUNEL double staining was performed using an antibody against activated caspase-3 (BD Biosciences, Europe). TUNEL technique was performed according to Gavrieli [12].

Slides were washed in TBS 3 x 5 min, thereafter permeabilisation of the tissue was done with methacarn (methanol : chloroform : acetic acid = 66:33:1 v/v/v) during 10 min and a final wash in TBS during 10 min. Slides were incubated in a humidified box at 37°C for 90 min using 20 µl reaction solution per slide consisting of; 0.4 µl terminal deoxynucleotidyl transferase (0.5 U/µl); 2 µl cobalt chloride (2.5

mM); 4 µl TdT reaction buffer; 0.8 µl biotin-dUTP (2 nM); 12.8 µl MilliQ, Roche, Germany). To stop the incubation, slides were washed with SSC for 4 x 5 min and TBS for 2 x 5 min. Slides were incubated with streptavidine Cy3 (1:2000, Jackson ImmunoResearch Laboratories Inc) in TBS containing 0.3 % Triton X-100 (TBS-T) and thereafter washed with TBS for 2 x 5 min, after which slides were incubated with Hoechst (1:500, Sigma) in TBS-T. The procedure was finished by washing with TBS for 10 min and enclosure with TBS:glycerol (1:3).

Double staining

In order to characterize the TUNEL-positive cells a number of double(immuno) stainings were performed. To identify astrocytes we used an antibody to monoclonal anti-glial fibrillary acidic protein (anti-GFAP) (1:1600, Sigma) and mouse anti-vimentin antiserum (undiluted) (Zymed, San Francisco, California, USA). Neurons were identified with a polyclonal antiserum against neuron specific enolase (NSE antiserum)(undiluted, Immunotech, Marseille, France) and a mouse monoclonal anti-neuronal nuclei (NeuN) antibody (1:50, Chemicon International, Temacula). For identification of activated microglia we used an anti ED1 antibody (1:5, gift from J. Damoiseaux, University Maastricht, The Netherlands). Oligodendrocytes were detected with a monoclonal antiserum against 2', 3'-cyclic nucleotide phosphodiesterase (anti-CNPase) (1:4000, Sigma) and anti O4 antiserum (1:50, Chemicon International, Temacula). Negative controls were performed by omitting the primary antibody. After counting TUNEL positive cells, glass covers of the slides were removed and the slides were washed with TBS 3 x 5 min. After application of the primary antibodies as described above, slides were kept overnight at 4°C. After repeated washes with TBS and TBS-T, the appropriated secondary antibodies were added and enclosure with TBS:glycerol (1:3) was done.

Part of the apoptotic cells in the spinal cord gray matter could be inhibitory interneurons, i.e. GABAergic cells. We tried to demonstrate apoptosis in GABAergic cells by combining TUNEL with markers for GABAergic cells using rabbit antibody to glutamic acid decarboxylase (GAD_{65/67})(1:200, Biotrend), sheep anti-parvalbumin

antiserum (1:1500) and anti-calbindin antiserum (1:2000, gifts of Dr. P.C. Emson).

Analysis

TUNEL-positive cells were counted in 6 consecutive sections of the cervical and lumbar intumescence of each rat at PN2, PN5 and PN8. Counting was done by two investigators (AdL and HPS), blinded for the experimental groups. Inter observer variation between the two investigators using the Pearson correlation test was 0.755 and significant at the 0.01 level.

In counting cells in the spinal cord, a distinction was made between the number of TUNEL positive cells in the cervical gray and white matter. In the lumbar region, counting was only performed in the gray matter as the small size of the white matter at the investigated ages prevented reliable counting in this region. Counting was done separately in the dorsal horn (Rexed's laminae I-III), the intermediate zone (Rexed's laminae IV-VII), the ventral horn (Rexed's laminae VIII and IX) and the area surrounding the central canal (Rexed's laminae X) [32]. Means and the standard error of mean (SEM) were calculated and statistically analysed using a one way ANOVA. A p value < 0.05 was taken as a significant difference. Microphotographs were taken using an Olympus AX-70 microscope. Details on the photography are supplied in the legends of the figures.

Results

For both the asphyctic and control group at each age (PN2, PN5 and PN8) at least 5 spinal cords were analysed. Light microscopic analysis of HE stained sections showed no large morphological differences (e.g. lesions) between SPA and CVD at both the cervical and lumbar level. However, many cells displayed features of apoptosis (condensation, shrinkage and fragmentation) in spinal cord sections of both asphyctic and control rats in all age groups (Figure 1a, 1b and 1c). These cells were diffusely distributed throughout the gray and white matter.

Developmental apoptosis, quantification of TUNEL positive cells

In the control group we detected developmental apoptosis in both the gray and white matter of the cervical and lumbar spinal cord. In the white matter, TUNEL-positive cells were diffusely distributed with no obvious preference for a subscribed region [6]. The results of the counting of TUNEL-positive cells in the gray matter of asphyctic and control rats are summarized in Table 1. In Figure 1d, TUNEL-positive cells are shown in the dorsal horn at PN5.

In the cervical dorsal horn (Rexed's laminae I-III) of control rats a statistically significant increase in apoptotic cells was observed between PN2 and PN5 from 11 ± 3 to 29 ± 5 ($p = 0.016$). Also in the lumbar dorsal horn a statistically significant increase was evident between PN2 and PN5 from 15 ± 4 to 47 ± 7 ($p = 0.006$) and a decrease between PN5 and PN8 to 20 ± 4 ($p = 0.003$).

Table 1. Number of TUNEL positive cells in the different spinal cord regions for both the asphyctic (SPA) and control (CVD) group at PN2, PN5 and PN8. The numbers are given as means $\pm$ SEM

		post natal day 2		post natal day 5		post natal day 8	
		CVD	SPA	CVD	SPA	CVD	SPA
laminae I-III	cervical	11 (3)	18 (4)	29 (5)	41 (8)	29 (6)	28 (5)
	lumbar	15 (4)	12 (2)	47 (7)	55 (3)	20 (4)	31 (3)
laminae IV-VII	cervical	70 (14)	119 (19)	12 (3)	7 (2)	5 (1)	7 (1)
	lumbar	70 (13)	151 (13)	20 (4)	17 (3)	5 (2)	5 (1)
laminae VIII + IX	cervical	18 (5)	22 (3)	4 (1)	7 (1)	9 (2)	11 (2)
	lumbar	18 (5)	29 (3)	7 (2)	11 (1)	5 (1)	6 (1)
laminae X	cervical	12 (1)	11 (2)	10 (2)	12 (3)	8 (2)	9 (1)
	lumbar	13 (3)	16 (5)	29 (5)	29 (3)	12 (1)	12 (2)
white matter	cervical	8 (3)	13 (3)	76 (17)	75 (23)	109 (10)	96 (11)

Figure 1. Apoptosis in the normal neonatal rat cervical spinal cord at PN2. a: HE staining (4x) showing the spinal cord with the dorsal root ganglia. The region indicated by the square is shown enlarged in fig 1b. b: HE staining (20x) showing apoptotic cells that are enlarged in fig c at 100 x magnification. Apoptotic cells (arrows) show nuclear fragmentation. d: TUNEL positive cells at 20 x magnification (red). The area in the white square is shown enlarged at the top right corner. Image a, b and c were made with an Olympus AX70 microscope using a Kodak Ektachrome professional Colour Reversal film. Images were digitalized using a Polaroid Sprints can 35. Image d was taken using an Olympus AX70 microscope equipped with a Sony colour CCD power HAD camera. Except for the insert in image d, no digital manipulations were made.

Perinatal asphyxia

Although the number of TUNEL-positive cells the gray matter areas in the cervical spinal cord of asphyctic rats was generally higher than in the corresponding regions of control rats, the differences were statistically not significant. Moreover, no significant difference in the number of apoptotic cells

could be detected in the cervical white matter of control and asphyctic rats.

In the lumbar spinal cord, a statistically significant increase in TUNEL-positive cells was detected from 70 ± 13 (controls) to 151 ± 13 ($p = 0.005$) on PN2 after perinatal asphyxia (Figure 2). This increase in apoptosis by 215 % was only observed in the intermediate zone (Rexed's laminae IV-VII). On PN8 a increase from 20 ± 3 (controls) to 31 ± 4 ($p = 0.03$) was seen in the dorsal horn (Rexed's laminae I-III) of the lumbar spinal cord of asphyctic rats.

Figure 2. Increased number of TUNEL positive cells after perinatal asphyxia. Localization of TUNEL positive (red) cells in the lumbar spinal cord at PN2 in asphyctic (SPA) and control (CVD) rats (blue, Hoechst, Sigma). These images were taken using an Olympus Ax70 microscope equipped with a cooled CCD camera (F-view). Images were taken with a CY3 (UM 41007a) filter (red, b and d) for TUNEL processed sections and with a DFR (UM 61000) filter for the Hoechst staining (a and c). Using the Image Analysis System (SIS), gray scaled images were directly converted into artificial colors. In all images the contrast was digitally enhanced by 50% to improve clarity.

Double staining

We used a double staining technique of TUNEL and activated caspase-3 immunocytochemistry in order to confirm apoptosis in TUNEL-positive cells. In both asphyctic and control spinal cord sections the localization of caspase-3 could be detected in nearly all TUNEL-positive cells. Nevertheless, the intensity of the caspase-3 immunostaining differed considerably between TUNEL positive cells (Figure 3).

No co-localization of TUNEL-staining was observed using NSE , NeuN or GFAP. Similarly, vimentin (a marker for immature astrocytes) immunostaining showed no co-localization with TUNEL staining in control or asphyctic rats [4].

ED1 immunostaining, a specific marker for reactive microglia or macrophages, colocalized with TUNEL-staining to some extent in both the white and gray matter of the cervical and lumbar spinal cord. Based on co-localization, cell morphology and soma size, we identified 18% of TUNEL-positive cells as microglia or, this population being approximately 5% of ED1-positive cells.

TUNEL-positive cells could also be identified as immature oligodendrocytes in both the white and gray matter, using the specific marker O4. Co-localization with O4 could be detected in 32% of TUNEL-positive cells in the control rats. Double staining in cervical and lumbar sections of both control and asphyctic rats with anti-calbindin, anti- parvalbumin or anti-GAD did not result in localization of these markers in TUNEL-positive cells.

Discussion

Several models for cerebral asphyxia have been described in the literature. Most of these models are arterial occlusion models in combination with an hypoxic environment, most probably more representative for local ischemia and not for the asphyxia as we have used in our perinatal asphyxia model [10, 16]. We used a model for perinatal asphyxia described by Loidl et al which is a more

representative model for the clinical situation of perinatal asphyxia [23]. In this model we studied the influence of perinatal asphyxia on apoptosis in the developing spinal cord.

Figure 3. Co-localization of TUNEL and caspase-3 Localization of TUNEL positive cells in the cervical spinal cord at PN5 of a control rat (blue, Hoechst, Sigma). These images were taken using an Olympus AX70 microscope equipped with a cooled CCD camera (F-view). Images were taken with a CY3 (UM 41007a) filter (red, a', b' and c') for TUNEL processed sections and with a DFR (UM 61000) filter for the Hoechst staining (a, b and c). Using the Image Analysis System (SIS), gray scaled images were directly converted into artificial colors. a and a': magnification 20x, Apoptotic cells (a, arrows) double immunostained with caspase-3 (a', arrows). b and c: magnification 100x, TUNEL positive cells shown in image a. Note the difference in caspase-3 immunoreactivity from evident (b') to hardly detectable (c'). For reasons of clarity the contrast in all images was digitally altered.

In our study, we showed that perinatal asphyxia increases apoptotic cell death superimposed on normal (developmental) cell death in a specific region within the gray matter of the lumbar spinal cord at a specific stage of development: i.e. at PN2 we observed an increased number of TUNEL- positive cells in the intermediate zone (laminae IV-VII).

The findings in our experiment on developmental apoptosis in the spinal cord gray matter of control rats are well in accordance with the findings of Lowrie and Lawson [24]. In their study, as in ours, no evidence was found for astrocytic apoptosis using GFAP as a marker. Moreover, we could not detect double staining between TUNEL and vimentin, a marker for immature astrocytes [4, 40].

In addition, we found no evidence for motoneuron related apoptosis, neither during normal development nor after asphyxia. In the study of Oppenheim [29], no postnatal motoneuron death in the spinal cord of the rat was detected. Nevertheless, it has been well established in different species that motoneurons undergo apoptosis during embryonic development [39]. Other studies have demonstrated postnatal apoptosis of motoneurons after ischemia, infection or trauma [16, 22, 25]. In our study we used NSE and NeuN as neuronal markers. No double labelling between these neuronal markers and TUNEL was observed. This seems surprising compared to the study by Lawson et al. in which post natal apoptosis of neurons in the spinal cord has been described [20]. However, the immunocytochemical techniques used by Lawson were different from the TUNEL technique used in our study. This difference in techniques could account for the differences in the detection of neuronal apoptosis. In addition, although NSE and NeuN are neuron specific markers, this does not imply that all neurons will be stained with these antibodies.

To our knowledge, no detailed reviews have been published on the comparability of central nervous system (CNS) maturation at birth between humans and rats. Nevertheless, it is assumed that CNS maturation at PN day 7-9 in the rat is comparable with the CNS development in a full-term human neonate [1, 27].

In our model of perinatal asphyxia the hypoxic/ischemic injury was introduced after an à term pregnancy in the rat, corresponding with a pre-term human neonate.

The clinical effects of pre-term neonatal injury in humans have been well described. Prematurity as the only cause of diplegia (spastic paraplegia of the lower extremities) was found to be likely in 41 % of children with cerebral palsy [5, 14, 38]. In à term perinatal asphyxia, a quadriplegic cerebral palsy (spastic paralysis of all four extremities) is more likely to occur [41]. Postmortum studies on perinatal hypoxic/ischemic spinal cord injury are rare. In a study by Sladky and Rorke [35], necropsies were performed on 21 asphyxiated human neonates. In this study, six of nine infants with pathological features of spinal cord infarction were premature and lesions were found to be more severe at lumbosacral levels. Moreover, in a study by Takahashi et al[36], the dendrites of lumbar motoneurons were found to be shorter and less elaborated in rats with a neonatal hypoxic insult than in controls. In their study, no significant difference between hypoxic and control rats was observed in cervical motoneurons. The observed clinical features of spastic diplegia in pre-term human neonates corresponds with the findings of increased cell death in the lumbar spinal cord in our model.

We were able to identify a proportion of the apoptotic cells in the spinal cord as activated microglia or macrophages using the specific marker ED1. Apoptotic microglia were diffusely distributed throughout the spinal cord white and gray matter, without an obvious preference for a described region. Using the marker O4, a population of apoptotic cells could be identified as immature oligodendrocytes in the white and gray matter of both the control and the asphyxiated spinal cord [8, 11].

The finding of oligodendrocytic apoptosis has been described after radiation and trauma in the adult spinal cord [8, 22, 33]. Besides our recent findings, oligodendrocytic apoptosis in the developing rat spinal cord has not been reported [6]. Disturbance of the developing myelinisation pattern in the spinal cord could have a profound effect on locomotor abilities.

Not all TUNEL-positive cells could be characterized in our study. Part of the remaining cells might be astrocytes, although no co-localization between TUNEL and GFAP, or vimentin was detected. Identifying filamentary proteins during development of neural tissue cannot always be done because of difference of developmental profiles of the various markers [40]. In this study this may be the case with both vimentin and GFAP.

Considering the anatomical localization of the increased number of apoptotic cells in the intermediate zone at PN2 and the dorsal horn at PN8 after perinatal asphyxia, the abundant presence of interneurons in these zones and the uncertainties concerning the staining properties of marker antibodies as described above, it can be speculated that part of the apoptotic cells are interneurons [7, 17, 31]. Spinal interneurons mediate an inhibitory effect upon motoneurons [37]. Unfortunately, all immunocytochemical markers for GABAergic neurons did not co-localize with the TUNEL staining. In a recent study evidence was found for suppression of interneuronal pathways with Ia afferent input after a disruption of the descending system [2]. After muscle stretch, primary afferent Ia fibers are excited resulting in a monosynaptic excitatory action on the agonistic muscle. The Ia afferents also monosynaptically connect with inhibitory interneurons that project directly to the antagonist motoneuron. In spasticity, this connection is impaired in favour of the excitatory action [26, 34]. Considering this mechanism, our data imply that an increased cell death of lumbar spinal inhibitory interneurons could be contributing to the development of spasticity in pre-term perinatal asphyxia.

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Abstract

The neurotransmitter gamma-aminobutyric acid (GABA) is thought to have a controlling action on spinal locomotor networks. In spasticity, spinal locomotor networks are thought to play a role. A well known drug in the treatment of spasticity is the GABA_B agonist Baclofen. In a previous study we demonstrated atrial natriuretic peptide (ANP)-mediated cyclic GMP (cGMP) synthesis in GABAergic neurons and fibres in the rat cervical spinal cord. In this study we investigated the effect of Baclofen on the cGMP synthesis in the spinal cord.

An immunocytochemical technique was used to visualize cGMP synthesis in the cervical spinal cord of 2-week old Lewis rats. Spinal cord slices were incubated in Krebs buffer with or without the presence of Baclofen. Slices were further incubated with the nitric oxide (NO) donor sodium nitroprusside (SNP) to activate soluble guanylyl cyclase (sGNC) or ANP to activate particulate guanylyl cyclase (pGNC).

We found an inhibitory effect of Baclofen on the ANP-mediated cGMP synthesis in the superficial dorsal horn (laminae I-III) of the rat cervical spinal cord. This inhibitory effect of Baclofen could not be detected after incubation with the NO donor SNP.

As a neurotransmitter, GABA has an important role in presynaptic inhibition of sensory motor input [2]. It is known that GABA depresses excitatory synaptic transmission in the spinal cord, mainly in the dorsal horn (laminae I-III) and in lesser extend in laminae IV-VI and the ventral horn. In addition, in the spinal cord, GABA is associated with interneurons in both the laminae I-III and the ventral horn [10]. During development GABA is thought to have a controlling action in the formation of spinal locomotor networks [2].

Recently we reported that atrial natriuretic peptide (ANP) stimulates cGMP accumulation in GABAergic structures of laminae I-III in the rat cervical spinal cord [17]. Although the presence of natriuretic peptides (NP) and NP receptor has been described several years ago, there is no report on the functional significance of NP stimulated cGMP synthesis in the spinal cord [19].

The GABA_B agonist Baclofen is a widely used drug in the treatment of spasticity [12]. Spasticity may be viewed as the outcome of a disorder of developing spinal networks [4]. In this study we investigated the effects of Baclofen on cGMP synthesis in the developing spinal cord

A total of 12 male Lewis rats aged 2 weeks were used. For biochemical measurements of cGMP five rats were used and for cGMP immunocytochemistry we used seven rats.

All experiments were approved by the committee on animal welfare according to Dutch governmental regulations. Rats were bred and housed at the University facility for experimental animals.

Rats were decapitated without anaesthesia and the cervical spinal cord was taken out while cooled in ice-cold Krebs buffer, desheated and cut into 400µm slices using a McIlwain tissue chopper in the cold room at 4°C [14]. Individual slices were immediately placed in aerated Krebs-Ringer bicarbonate buffer containing 1 mM isobutylmethylxanthine (IBMX) to inhibit phosphodiesterase (PDE) activity. Subsequently the temperature was raised till 35°C and the slices were incubated in the same buffer for 30 minutes under an atmosphere of 95 % O₂ and 5 % CO₂.

In each experiment the slices were further incubated for another 10 minutes

with either 100nM ANP or 0.1 mM of the NO donor sodium nitroprusside to activate particulate (plasma membrane bound) guanylyl cyclase (pGC) or soluble guanylyl cyclase (sGC), respectively. Incubations were done in the presence or absence of 10 μ M Baclofen. The incubations were terminated by: (a) transferring slices into ice cold 5 % TCA for biochemical measurement of cGMP levels using a radioimmunoassay (RIA) as described in detail [16]; and (b) adding ice-cold fixative solution (final concentration 4% paraformaldehyde, 10% sucrose, pH 7.4) for slices used for cGMP immunocytochemistry. Fixation was continued for 2 h, followed by a 30 min wash in ice-cold 0.1 M phosphate (pH 7.4) containing 10% sucrose. Cryostat sections (10 μ m) were cut and thawed on to chrome-alum/gelatin-coated slides and processed for immunocytochemistry. All antibodies dilutions were made in Tris-buffered saline (TBS; pH 7.6) containing 0.3% (v/v) Triton X-100 (TBS-T). Sections were incubated overnight at 4°C with sheep anti-cGMP antiserum (1:4000). Primary antibody was visualized with a fluorescein Alexa 488-conjugated goat anti-sheep antiserum at a 1:100 dilution (Molecular Probes).

Sections were analysed using an Olympus AX70 microscope equipped with an Olympus Digital Video-Camera F-View, a cooled CCD camera. Fluorescence intensity was measured and converted into 255 gray value classes (0-255 high to low). Images were recorded and subsequently analysed with analySIS software. Laminae I-III and laminae IV-X were selected as the region of interest and the mean gray value of the selected region was calculated. The effect of ANP with or without Baclofen on cGMP levels was calculated from the measurement of the immunofluorescence intensity, after subtracting the values obtained from slices incubated without ANP, setting the effect of ANP alone as 100% effect. The validity of this approach in assessing cGMP levels in slices has been described in detail [16]. The data were statistically analysed using a paired sample T-test. A p-value of < 0.05 was considered to be statistically significant. Protein level was measured according to Lowry et al. [9].

Biochemical measurement of cGMP in the spinal cord slices showed no statistically significant difference between ANP stimulated and ANP/Baclofen stimulated slices (Table 1). Similarly, stimulation with SNP and SNP/Baclofen did

not result in a significant difference in cGMP level. However regional differences within a segment of the spinal cord can not be detected using this approach. Therefore, in order to analyze the effects of Baclofen at the segmental level, we turned to cGMP immunocytochemistry.

condition	n	mean	SD	significance
basal	5	5.14	0.98	p = 0.284
basal/Baclofen	4	5.86	0.86	
SNP	5	98.69	26.85	p = 0.402
SNP/Baclofen	6	112.88	26.46	
ANP	6	15.39	1.85	p = 0.364
ANP/Baclofen	13	13.59	4.5	

Table 1. cGMP content in cervical spinal cord slices using a radioimmunoassay. In the basal condition measurements were done without incubation with either sodium nitroprusside (SNP) or atrial natriuretic peptide (ANP). The values represent the mean level of cGMP in pmol per mg protein. (SD, standard deviation; n, number of experiments).

In cervical spinal cord slices of 2 weeks old rats, the cGMP immunostaining in the absence of stimulators of either pGNC or sGNC (ANP respectively SNP) was low and only present in laminae I-III. These findings on basal cGMP immunoreactivity are well in accordance with earlier findings [17]. Surprisingly, after stimulation of cGMP synthesis by 100 μ M ANP in the presence of 10 μ M Baclofen fluorescence intensity in laminae I-III decreased to 77 $\pm$ 4% (mean $\pm$ SEM) of control values (Fig. 1a,b). This decrease was highly significant ($P < 0.0001$). Under these conditions, the apparent decrease of cGMP immunofluorescence intensity in laminae IV-X proved to be non significant (87 $\pm$ 8, $P = 0.01$).

Stimulation of soluble GC with SNP resulted in strong cGMP immunoreactivity in all laminae of the cervical spinal cord. However, incubation of slices in the combined presence of SNP and Baclofen did not result in a statistically significant decrease of cGMP immunoreactivity; 87 $\pm$ 9% ($P = 0.474$) for laminae I-III and

94% $\pm$ 6.5% ($P = 0.703$) for laminae IV-X of the mean gray value obtained after stimulation with SNP alone (Fig. 1c,d).

Figure 1. cGMP immunoreactivity in cervical spinal cord slices, (a) and (b) were obtained after incubation with atrial natriuretic peptide (ANP) in the absence (a) or presence (b) of Baclofen (BAC). (c) and (d) incubations were done with the NO donor sodium nitroprusside (SNP) with (d) or without (c) Baclofen. Bar represents 50 μ m.

In our study we demonstrated the inhibitory action of Baclofen on ANP stimulated cGMP formation in laminae I-III of the cervical spinal cord of rats, using semiquantitative immunofluorescence analysis. Because of the localized effect of Baclofen on cGMP synthesis in the spinal cord slices we were unable to demonstrate a decrease of cGMP level using a radioimmunoassay.

The functional significance of ANP stimulated cGMP synthesis in the spinal cord is not known. Nevertheless, low density ANP-binding sites have been described in the dorsal horn and ventral horn [14, 19]. Specific ANP-binding sites in the rat spinal cord were detected on neurons and astrocytes [8]. Similarly, using autoradiographic analysis, a high density of GABA_B receptors in laminae I-IV has been described [3]. This localization of GABA_B receptors corresponds with the location where in our study we observed that Baclofen decreased ANP stimulated cGMP immunoreactivity.

From a structural point of view the inhibitory effect of Baclofen on ANP-induced activation of pGC is surprising. Nevertheless, isatin(indole-2,3 dione) which is also structurally unrelated to ANP has been described as an inhibitor of pGC [7]. Recently it has been demonstrated that isatin inhibits pGC independent of the occupation of the ANP receptor [11]. It is known that phosphorylation of the ANP receptor is required for optimal catalytic activity [5]. activation of GABA_B receptors might result in activation of phospholipase A and potentiation of cAMP formation [18]. Thus, the effect of Baclofen might involve triggering of a phosphorylating/dephosphorylating cycle of the intracellular part of the ANP receptor and subsequently downregulation of the pGC activity [13].

From a functional point of view, little is known about the role of cGMP in the spinal cord. NO-mediated cGMP is involved in nociception in the spinal cord, an increased level of cGMP in the dorsal horn of the spinal cord is associated with hyperalgesia through activation of cGMP dependent protein kinase Ialpha [1, 6, 15]. Data on ANP-mediated cGMP synthesis in the spinal cord is almost lacking completely [17].

We conclude that stimulation of the GABA_B receptor by Baclofen leads to inhibition of cGMP synthesis in laminae I-III in the rat cervical spinal cord at the age of two weeks.. The clinical effect of Baclofen on the reduction of pain and spasticity draws attention to a possible role of ANP-induced cGMP synthesis in these clinical conditions.

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Abstract

Spasticity is a locomotor abnormality which can be observed after perinatal asphyxia. The GABA_B agonist Baclofen is a widely used drug for the treatment of spasticity. In this study we investigated the effects of Baclofen on the ANP and SNP mediated cGMP synthesis in the rat cervical spinal cord after perinatal asphyxia.

Using a slice experiment and immunocytochemical techniques, we observed ANP mediated cGMP synthesis in astroglial cells after perinatal asphyxia. In normal rats, a decreasing effect of Baclofen on ANP mediated cGMP synthesis was observed, while an increasing effect on NO mediated cGMP synthesis was observed after perinatal asphyxia.

Our results suggest that the effects of Baclofen in the spinal cord are at least partially NO-cGMP mediated.

Introduction

Perinatal asphyxia (PA) is the most common cause of mortality and morbidity in childhood, with a prevalence of 1.5 to 2.5 per 1000 live births [12]. PA can lead to mental retardation, epilepsy and motor abnormalities such as spasticity [7]. The pathophysiological mechanisms in spasticity after PA are not completely understood. Yet, in spastic cerebral palsy, evidence for hyperexcitability of spinal alpha motor neurons due to abnormal supraspinal inhibition or abnormal spinal inhibitory control of spinal motor neuron output exists [4, 8, 18].

Recently, we reported that atrial natriuretic peptide (ANP) and sodium nitroprusside (SNP), a nitric oxide (NO) donor, stimulated cGMP accumulation in GABAergic structures of laminae I-III of the rat cervical spinal cord at the age of 2 weeks [20]. Furthermore, we observed an inhibitory effect of the GABA_B agonist Baclofen, a well known and widely used drug in the treatment of spasticity, on ANP mediated cGMP synthesis in the 2 weeks old rat dorsal horn [2].

In the central nervous system (CNS), the second messenger cGMP is synthesized by guanylyl cyclases. Two classes of guanylyl cyclases can be distinguished, a soluble (sGC) and a membrane bound or particulate guanylyl cyclase (pGC). Activation of sGC is NO mediated while activation of pGC is mediated by natriuretic peptides [21]. In the rat brain, ANP mediated cGMP immunoreactive (cGMP-IR) cells have been partly identified as being astroglial cells [3]. Moreover, specific ANP-binding sites were detected on cultured neurons and astrocytes from the rat spinal cord [9].

In the spinal cord, cyclic nucleotides like cGMP contribute to spinal hyperalgesia [6]. Recent studies provide evidence for a contribution of the NO-cGMP pathway in the development and maintenance of central sensitization of spinothalamic tract neurons by reducing the effectiveness of spinal inhibition [13]. Furthermore, cyclic nucleotides are thought to be involved in the guidance of neural growth cones during development [1]. As spasticity after PA may be viewed as a symptom of decreased spinal inhibitory control and cGMP is thought to influence the effectiveness of spinal inhibition [13], we studied the effects of PA on

ANP and SNP mediated cGMP synthesis in the adult rat spinal cord, especially in relation to the effects of the spasmodic drug Baclofen on the ANP and SNP mediated cGMP synthesis after PA.

Materials and methods

Animal experiments

Time pregnant Wistar rats at the last day of gestation were obtained from Charles-River company and housed at the University Maastricht facility for experimental animals. Water and food were given ad libitum. All experiments were approved by the committee on animal welfare according to Dutch governmental regulations and all efforts were made to minimize the number of rats used and their suffering. Severe PA was induced as described in detail by Loidl et al [14].

In short, the rats were decapitated and hysterectomized after their first pup had been allowed to be delivered vaginally (control, CVD). Both the uterus horns were placed in a water bath at 37 °C for 20 minutes (severe perinatal asphyxia, SPA). After 20 minutes the uterus horns were opened, the pups removed, resuscitated and allowed to recover in an incubator at 37 °C. Male pups were then placed with a surrogate mother. The mortality after severe PA was approximately 40% in our model and comparable with earlier studies using this model [14].

Tissue preparation

Six males from the SPA group and six male CVD rats were used for the slice experiments at the age of 8 weeks. After decapitation without anaesthesia, the cervical spinal cord was taken out while cooled in ice cold Krebs buffer, desheated and cut into 400µm slices using a McIlwain tissue chopper in a cold room at 4° C [19]. Individual slices were immediately placed in aerated Krebs-Ringer bicarbonate buffer containing 1 mM isobutylmethylxanthine (IBMX) to inhibit phosphodiesterase activity. Subsequently the temperature was raised till 35°C and the slices were incubated in the same buffer for 30 minutes under an atmosphere of 95% O₂ and 5%CO₂.

In each experiment the slices were further incubated for another 10 minutes with either 100 nM ANP or 0.1 mM of the NO donor sodium nitroprusside to activate pGC or sGC respectively. Incubations were done in the presence or absence of 10 μ M or 100 μ M Baclofen. The incubations were terminated by adding ice-cold fixative solution (final concentration 4% paraformaldehyde, 10% sucrose, pH 7.4) and processed for immunocytochemistry as described in detail [20].

Immunocytochemistry

All antibodies dilutions were made in Tris-buffered saline (pH 7.6) containing 0.3% (v/v) Triton X-100. Sections were incubated overnight at 4°C with sheep anti-cGMP antiserum (1:4000). Primary antibody was visualized with a fluorescein Alexa 488-conjugated donkey anti-sheep antiserum at a 1:100 dilution (Molecular Probes). In order to identify cGMP immunoreactive (cGMP-IR) cells, we used antibody to monoclonal anti-glial fibrillary acidic protein (anti-GFAP) (1:1600, Sigma) visualized with a fluorescein CY3 donkey anti-mouse antiserum (1:800, Jackson).

Analysis

Sections were analysed using an Olympus AX70 microscope equipped with an Olympus Digital Video-Camera F-View, a cooled CCD camera. Fluorescence intensity was measured and converted into 265 gray value classes (0-265 low to high fluorescence intensity). Images were recorded and subsequently analysed with analySIS software. Laminae I-III was selected as the region of interest and the mean gray value of the selected region was calculated. The effects of ANP and SNP with or without Baclofen on cGMP levels was calculated from the measurement of the immunofluorescence intensity, setting the effect of ANP or SNP alone as 100% effect. The data were statistically analysed using an ANOVA. A p-value of < 0.05 was considered to be statistically significant. All data are represented as means and standard error of means (SEM).

Results

The normal rat spinal cord

In cervical spinal cord slices of 8 week old rats, the mean gray value intensity of cGMP-IR in the absence of stimulators of either pGC or sGC (basal conditions) was 57 ± 8 for the control group and only present in laminae I-III, although IBMX was present. In the control group an increase could be detected from 57 ± 8 to 90 ± 11 ($p = 0.037$) after stimulation with SNP and an increase from 57 ± 8 to 80 ± 9 ($p = 0.032$) after stimulation with ANP.

Perinatal asphyxia

The mean gray value intensity of cGMP-IR in the asphyxia group was 62 ± 10 . An increase in the mean gray value was seen from 62 ± 11 to 79 ± 3 ($p = 0.14$) and from 62 ± 11 to 73 ± 8 ($p = 0.23$) for SNP and ANP respectively. Without the presence of Baclofen, no statistically significant differences could be detected between the control and asphyxia groups, neither under basal conditions nor after stimulation with either SNP or ANP (Table 1).

condition		n	mean	SEM
BASAL	asphyxia	6	62	11
	control	5	57	8
SNP	asphyxia	6	79	3
	control	5	90	11
SNP/Baclofen10	asphyxia	6	83	6
	control	5	78	6
SNP/Baclofen100	asphyxia	6	94	6
	control	6	72	6
ANP	asphyxia	6	73	8
	control	6	80	9
ANP/Baclofen10	asphyxia	6	67	9
	control	6	66	6
ANP/Baclofen100	asphyxia	6	74	4
	control	6	69	3

Table 1. Summary of the different experimental conditions. The mean gray value intensity of cGMP-IR (mean) for both asphyctic and control rats after stimulation with either atrial natriuretic peptide (ANP) or sodium nitroprusside (SNP). (SEM = standard error of means)

Baclofen

For assessing differences between cGMP levels in the asphyxia and control group with or without Baclofen, we subtracted the values obtained from slices incubated without Baclofen, setting the effect of ANP or SNP alone as 100 % effect.

In the control group a statistically significant decrease to 85 % $\pm$ 5 of cGMP-IR was seen in laminae I-III when cGMP was stimulated with ANP in the presence of 10 μ M Baclofen ($p = 0.04$). This decrease could not be detected for ANP stimulated cGMP in the presence of 10 μ M Baclofen in the asphyxia group (92 % $\pm$ 7, $p = 0.36$), nor in after stimulation of cGMP by SNP in the control group (88 % $\pm$ 15, $p = 0.49$).

In the presence of 100 μ M Baclofen the mean gray value intensity of cGMP-IR significantly increased in the perinatal asphyxia group (94 $\pm$ 6) compared to the control group (72 $\pm$ 6, $p = 0.024$). In laminae I-III of the perinatal asphyxia group SNP in the presence of 100 μ M Baclofen significantly increased the cGMP-IR to 118 percent ($p = 0.004$) compared to the 100% stimulation with SNP alone (Figure 1). In the control group a decrease to 81 percent was observed under these conditions ($p = 0.013$).

Figure 1. Representative photomicrographs of cGMP immunoreactivity in the cervical spinal cord after incubation with SNP alone (a) or incubation with SNP and 100 μ M Baclofen (b). Images were taken using an Olympus AX 70 microscope equipped with a cooled CCD camera (F-View). (bar = 100 μ m, magnification 10 $\times$)

Double immunocytochemical staining

Using a double immunocytochemical staining techniques with anti GFAP antiserum, a small number of the cGMP-IR cells could be identified as astroglial cells (Figure 2). Co-localization between cGMP and GFAP was most pronounced in the asphyxia group after stimulation with ANP alone and after stimulation with ANP and incubation with 100 μ M Baclofen.

Figure 2. Photomicrograph showing cGMP IR astrocytes. Images were taken using an Olympus AX70 microscope equipped with a cooled CCD camera (F-View). (bar = 20 μ m, magnification 40x in a and 10 μ m, magnification 100x in b)

Discussion

We investigated the effects of Baclofen on ANP and SNP stimulated cGMP-IR in laminae I-III of the cervical spinal cord of 8 week old rats after perinatal asphyxia. A decrease of the cGMP-IR after stimulation with ANP in the presence of Baclofen was detected in the control group. This decrease was not seen in the perinatal asphyxia group, nor after stimulation with the NO donor SNP in the control group. Using semiquantitative immunofluorescence analysis, we found an increase in NO mediated cGMP in laminae I-III after stimulation with SNP in the presence of 100 μ M Baclofen after perinatal asphyxia.

The findings of the inhibitory effect of Baclofen on the ANP mediated cGMP synthesis in the adult rat are in accordance with our earlier findings in the 2 week old rat spinal cord [2]. From a structural point of view the inhibitory effect of Baclofen on ANP-induced activation of pGC is surprising. It is known that phosphorylation of the ANP receptor is required for optimal catalytic activity [5]. Activation of GABA_B receptors might result in activation of phospholipase A and potentiation of cAMP formation [22]. Thus, the effect of Baclofen might involve triggering of a phosphorylating/ dephosphorylating cycle of the intracellular part of the ANP receptor and subsequently downregulation of the pGC activity [16].

Presently, the function of ANP mediated cGMP synthesis in the spinal cord remains unknown. Nevertheless, in an electrophysiological study the presence of ANP receptors on cultured spinal cord astrocytes was demonstrated [10]. Furthermore, in a recent study in humans an increase was observed in ANP immunoreactive astrocytes after brain ischemia [15]. In our study a small part of the ANP mediated cGMP-IR cells could be identified as astroglial cells on the basis of their GFAP immunostaining. Double immunostained cells were mainly detected in the SPA group under 2 experimental conditions: after stimulation with ANP alone and after stimulation with ANP in the presence of 100 μ M Baclofen. No colocalization between ANP mediated cGMP-IR and GFAP was observed in the control group.

The effects of NO on cGMP in the adult rat spinal cord are region specific, in

laminae I-III an inhibitory effect on spontaneous neural electric activity has been described [17]. We earlier reported an extensive co-localization between NO stimulated cGMP synthesis and parvalbumin-positive (GABAergic) neurons and fibres [20]. Activation of the GABA_B receptor in the dorsal horn is known to inhibit the release of both glutamate (excitatory) and GABA (inhibitory) in the spinal dorsal horn [11]. Using a whole cell voltage clamp recording technique, Yang et al showed a slow outward inhibitory current in dorsal horn neurons after bath application of Baclofen [23]. The findings in our present study suggest that the known inhibitory effects of the GABA_B agonist Baclofen in the spinal cord dorsal horn are at least partly NO-cGMP mediated. However, the exact mechanism by which Baclofen interacts with the NO-cGMP pathway remains unknown.

Conclusion

In spasticity after perinatal asphyxia the equilibrium between excitation and inhibition of spinal motor neurons is disturbed in favour of the excitatory activity. Inhibition of excitatory afferent input, both pharmaco-therapeutically and surgically, is one of the cornerstones in the treatment of spasticity [24].

In our study we have demonstrated that the known inhibitory effects of the GABA_B agonist Baclofen could at least partially be NO-cGMP mediated. Baclofen seems to have a variable effect on cGMP synthesis in the spinal cord dorsal horn under different conditions. In normal rats, a decreasing effect on ANP mediated cGMP synthesis was observed, while an increasing effect on NO mediated cGMP synthesis was observed after perinatal asphyxia.

Further studies on the effects of perinatal asphyxia on the GABA_B receptor in the dorsal horn could provide further insights in the pathophysiological mechanisms of spasticity after perinatal asphyxia.

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Abstract

Radiofrequency lesions of the dorsal root ganglion can be used in the treatment of pain and spasticity. To date the effects of a radiofrequency lesion has been studied in peripheral nerves and in the spinal cord dorsal horn shortly after the treatment. In our study we investigated the long- term effects of a RF-DRG treatment on the spinal cord using c-Fos immunoreactivity. Furthermore, we used different immunocytochemical markers to identify c-Fos immunoreactive (c-Fos IR) cells.

In 6 Wistar rats RF-DRG treatments were performed adjacent to 6 dorsal root ganglions, and 6 ganglions were used as sham operated controls. Tissue was investigated at postoperative day 7.

In the RF-DRG treated rats a highly significant increase in c-Fos IR cells was observed as compared to the sham operated controls. c-Fos IR cells were detected both ipsi- and contra-lateral of the treatment side. Furthermore, c-Fos IR cells could be identified as activated microglia, astrocytes and motor neurons but not as oligodendrocytes.

We conclude that the long- term effects of a RF-DRG treatment can be detected in all Rexed's laminae both ipsi- and contra-lateral after an unilateral treatment. We furthermore suggest that the clinical effects of a RF-DRG treatment in spasticity and pain are possibly due to interference with both afferent input into the spinal cord and efferent output from the spinal cord.

Introduction

Radiofrequency lesions of the dorsal root ganglion (RF-DRG) are widely used for the treatment of chronic cervical pain [16]. Besides the use of RF-DRG in the treatment of pain, a beneficial effect of RF-DRG has been described in the treatment of spasticity [5, 17].

In RF-DRG treatment, as in all neurosurgical treatments for spasticity, a reduction of spasticity is thought to be established through interruption of the afferent part of the stretch reflex in order to decrease excitation. The clinical effects of a RF-DRG lesion on spasticity and pain are not immediate but can only be detected after several weeks. An initial motor reaction of the muscles corresponding to the treated spinal level can be observed during RF-DRG treatment for spasticity.

The effects of a radiofrequency lesion have been studied on peripheral nerve fibres. An early effect of radiofrequency lesions on small A-delta and C fibres, followed by a delayed effect on fibres in the A-alpha and beta group has been described [9]. In contrast, other studies report an indiscriminating effect on all peripheral fibre types [13, 15]. We recently reported an increase of proliferation inside the dorsal root ganglion after a RF-DRG lesion adjacent to the ganglion without signs of neural tissue damage (e.g necrosis) inside the ganglion [1].

Recently, an increase of c-Fos immunoreactive cells in laminae I and II was noted 3 hours after a RF-DRG lesion [2]. The proto-oncogene c-fos is known as a cellular immediate early gene, used as a functional anatomical mapping tool to identify cells that become activated in response to various stimuli [8]. Under basal conditions c-fos expression is low [3]. The maximal level of c-Fos protein is detectable between 1 and 3 hours after a stimulus and gradually disappears from the cell nucleus by 4 to 6 hours after stimulus [8]. However, a biphasic c-fos response has been described after excitotoxic insults [18]. Fos immunoreactivity was detectable in rat dorsal horn neurons for 30 days after chronic constrictive injury to the sciatic nerve [20].

To our knowledge, no studies have been done on the long term effects of a RF-DRG lesion on the spinal cord. Taken into account the delayed clinical effects of a RF-DRG lesion in the treatment of spasticity, we investigated the long term c-Fos protein immunoreactivity in the adult rat spinal cord after a radiofrequency lesion adjacent to the dorsal root ganglion.

Materials and methods

Radiofrequency lesions

A total of 7 male and female Wistar rats (250-270 grams) were used for the experiments. In 3 rats (experimental group) a radiofrequency lesion was made adjacent to 2 dorsal root ganglia on the right side (the left side being used as control). Another 3 rats were used as sham operated controls. One rat was used as a double control, all pre- and post-operative measures were taken without performing the actual operation. Rats were bred by Charles-River company and housed at the University Maastricht facility for experimental animals. Water and food were given ad libitum. All experiments were approved by the committee on animal welfare according to Dutch governmental regulations.

Rats were anaesthetised with an i.p. injection of sodium pentobarbital (Sanofi, 60 mg/kg), the abdomen and cervical region was sheaved and the rat was placed on an inductionpad connected to a radiofrequency generator (Radionics). After identifying the cervical vertebra prominence as a landmark, a cervical hemilaminectomy was performed on the levels C5 and C6. The corresponding dorsal root ganglia on the right side were identified and a 100 mm 30G Levin chordotomy electrode (Radionics) with a 2 mm exposed tip was introduced adjacent to the dorsal root ganglion C5 and C6 using a micro-manipulator (Leitz wetzlar, Germany).

The impedance (an indicator for the type of tissue next to the cannula tip) was tested, and if high (above 1000 Ohm) the tip of the cannula was manipulated in order to obtain an impedance below 700 Ohm, indicating that the tip of the cannula was near to soft tissue (e.g. the dorsal root ganglion). After checking the

impedance, electrical stimulation was started at a rate of 2 Hz and 50 Hz and the rat was observed for muscle contractions in the corresponding muscles of the stimulated level (C5 or C6). If muscle contractions were observed the stimulation threshold was noted.

A radiofrequency current was then led through the electrode in order to increase the temperature to 67⁰ C, in line with the procedure in the clinical practice. A 60 sec 67⁰ C unilateral lesion was made adjacent to the dorsal root ganglion at level C5 and C6 on the right side. After the lesion the muscles were approximated and the skin was closed. In 3 rats the procedure was identical with omission of the radiofrequency current (sham operated controls).

Tissue preparation

At post-operative day 7, the rats were anaesthetised by an i.p. injection of sodium pentobarbital (Sanofi, 60 mg/kg) and transcardially perfused with a flush of Tyrode solution (pH 7.4) followed by phosphate buffered 4% paraformaldehyde (pH 7.4). The cervical spinal cord with the attached dorsal root ganglions was dissected and post fixed in phosphate buffered 4% paraformaldehyde overnight. Then, the spinal cord was dissected into parts of one segment, and embedded in 10% porcine gelatin. The gelatin cups were allowed to harden on ice for 1 hour, and post fixed in phosphate buffered 4% paraformaldehyde for 2 hours. The tissue was cryoprotected in 15% sucrose in Tris buffered saline (TBS) overnight at 4°C, and frozen in a liquid-nitrogen cooled bath with 2-methylbutane. Subsequently cryostat sections (10 µm) were cut (Leica CM3050, cryostat) and thawed on to chrome-alum/gelatine coated slides and processed for immunocytochemistry.

Immunocytochemistry and histochemistry

For morphological determination and verification of the actual lesion site, (e.g. next to the dorsal root ganglion and not inside) a hematoxylin and eosin (HE) staining was used. For the identification of c-Fos immunoreactive (c-Fos-IR) cells, we used an immunocytochemical fluorescent staining technique. A polyclonal rabbit anti c-Fos (4) antibody (1:4000, Santa Cruz biotechnology Inc) was used as a

primary antibody. After application of the primary antibody, slides were kept overnight at 4°C. Slides were further incubated with donkey anti rabbit Cy3 (1:800, Jackson ImmunoResearch Laboratories Inc) in TBS containing 0.3 % Triton X-100 (TBS-T) and thereafter washed with TBS for 2 x 5 min, after which slides were incubated with Hoechst (1:500, Sigma) in TBS-T. The procedure was finished by washing with TBS for 10 min and enclosure with TBS:glycerol (1:3).

Double staining

In order to characterize the c-Fos-IR cells a number of double(immuno) stainings were performed. To identify astrocytes we used an antibody to monoclonal anti-glial fibrillary acidic protein (anti-GFAP) (1:1600, Sigma). Oligodendrocytes were detected with an anti O4 antiserum (1:50, Chemicon International, Temacula). For identification of activated microglia we used an anti ED1 antibody (1:5, gift from J. Damoiseaux, University Maastricht, The Netherlands). As secondary antibody we used goat anti-mouse Alexa (1:100, Molecular Probes, USA). Negative controls were performed by omitting the primary antibody.

For the detection of (pro)apoptotic cells an activated caspase-3/c-Fos double staining was performed using a goat monoclonal antibody against activated caspase-3 (BD Biosciences, Europe).

After counting c-fos-IR cells, glass covers of the slides were removed and the slides were washed with TBS 3 x 5 min. After application of the primary antibodies as described above, slides were kept overnight at 4°C. After repeated washes with TBS and TBS-T, the appropriated secondary antibodies were added and the slides were enclosed with TBS:glycerol (1:3).

Analysis

The counting of c-Fos IR cells was done in 4 consecutive sections for each ganglion on the levels C5 and C6, both ipsi- and controlateral. Cells were counted in 4 different spinal cord gray matter regions; the dorsal horn (Rexed's laminae I-III), the intermediate zone (Rexed's laminae IV-VII), the ventral horn (Rexed's

laminae VIII + IX) and the area surrounding the central canal (Rexed's laminae X) [11].

Means and the standard error of mean (SEM) were calculated and statistically analysed using a one way ANOVA and a non parametric test when appropriate. A p value < 0.001 was taken as a significant difference.

Microphotographs were taken using an Olympus AX70 microscope. Details on the photography are supplied in the legends of the figures.

Results

Experimental procedure

Both the experimental and the sham operated rats showed no clinical signs of paralysis or sensory deficits (e.g. pain) of the limbs on post operative examination. The mean stimulation threshold was 0.65 ± 0.24 V for the sham operated rats and 0.52 ± 0.12 V ($p = 0.64$) for the RF-DRG treated rats respectively. In accordance with the clinical observation in patients, an initial motor reaction of the muscles corresponding to the treated spinal level could be observed during the first few seconds of RF-DRG treatment

Examination of the HE stained sections showed infiltration with leucocytes and macrophages in the tissue adjacent to the lesioned dorsal root ganglion in the experimental group (Figure 1). On the untreated controlateral side only mild signs of infiltration could be detected. Using HE staining, no morphological changes like degenerating cells displaying light microscopic features of apoptosis (condensation, shrinkage and nuclear fragmentation) could be detected in both the dorsal root ganglion nor the spinal cord after the radiofrequency treatment. Furthermore, using the marker for activated caspase-3, no evidence for apoptosis was observed.

Figure 1. Photomicrograph showing the tissue adjacent to the dorsal root ganglion in: a) a sham operated rat and b) a RF-DRG treated rat. After RF-DRG treatment, macrophages (arrows) were detected. (HE staining, magnification 20x).

Localisation of c-Fos IR cells

The localization and numbers of c-Fos IR cells in the spinal cord are summarized in Table 1. In both the sham operated controls and the double control rat, only few c-Fos IR cells could be detected without an obvious preference for a circumscribed region. In contrast, in the control group, abundant c-Fos IR cells could be detected in all Rexed's laminae 7 days after RF-DRG treatment, both ipsi- and contra-lateral of the lesion side (Figure 2). In the dorsal horn an increase from 0.5 ± 0.2 to 94 ± 13 ($p < 0.0001$), in the intermediate zone an increase from 3.5 ± 1.2 to 188 ± 24 ($p < 0.0001$) and in the ventral horn an increase from 0.3 ± 0.2 to 14 ± 3 ($p < 0.0001$) in c-Fos IR cells was observed.

No significant difference in the number of c-Fos IR cells could be detected between the treated and the contra-lateral side, although in all animals the higher numbers were observed in the treatment side: in the dorsal horn 94 ± 13 c-Fos IR cells, in the treatment side versus 62 ± 13 ($p = 0.107$) in the contra-lateral side, for the intermediate zone 188 ± 24 cells vs 135 ± 20 cells ($p = 0.119$) and for the ventral horn 69 ± 10 cells vs 57 ± 10 cells ($p = 0.423$).

group	n	dorsal	intermediate	ventral	central
RF-DRG	6	94 (13)*	188 (24)*	69 (10)*	14 (3)*
sham	6	0.5 (0.2)	3.5 (1.2)	1.7 (0.3)	0.3 (0.2)
control	2	0.5 (0.5)	1.5 (0.5)	0 (0)	0 (0)

Table 1. Mean number of c-Fos IR cells on the RF-DRG treated side (Mean and SEM, * $p < 0.0001$).

Identification of c-Fos IR cells

In contrast to the immediate c-Fos expression in the nucleus, c-Fos IR was observed within the cytoplasm of ventral horn motor neurons (identification of cells based on morphology and localization within the spinal cord) 7 days after a RF-DRG treatment (Figure 3).

Using double immuno-fluorescent techniques, c-Fos IR cells could partially be identified as activated microglial cells (ED1 positive) and astroglial cells (GFAP positive) (Figure 4). No co-localization was observed between the oligodendrocyte marker O4 and c-Fos IR.

Figure 2. Representative photomicrograph showing c-Fos immunoreactive cells (white) in the spinal cord ventral horn in a sham operated and in a RF-DRG treated rat (magnification 10x)

Discussion

In our study we investigated the long term effect of a RF-DRG lesion on c-Fos expression in the rat cervical spinal cord. Using double immunocytochemical fluorescent staining techniques, we observed an increased expression of c-Fos IR in motor neurons, astrocytes and activated microglia in the spinal cord gray matter, both ipsi- and contra- lateral, after a unilateral RF-DRG lesion. At post operative day 7 the expression of c-Fos protein was found to be cytoplasmic and not nuclear.

The expression of the c-fos protein is generally known to be an indicator of cellular activation in an early stage [4]. The peak of nuclear c-fos expression is reached within 3 hours after stimulation, after which a gradual decrease to total nuclear elimination is reached at 24 hours [8]. However, at day 7, a second peak in fos expression has been described [12]. The localization of c-fos during this second peak was found to be cytoplasmic instead of nuclear. Moreover, cytoplasmic expression of c-fos was evident exclusively in vulnerable regions after a NMDA agonist induced excitotoxic cascade [6].

In the CNS, c-Fos immunoreactivity has been suggested to be related to apoptotic cell death [7, 12]. After a lesion of the developing sciatic nerve apoptosis of spinal motor neurons, and dorsal horn interneurons has been observed [10]. Using the sciatic injury model in transgenic mice, c-Fos expression was observed in the dorsal root ganglia and ventral motor neurons [12]. However, in our study, no morphological features of apoptosis or induction of caspase-3 could be observed.

Induction of c-Fos in the rat spinal dorsal horn has been described within several hours after dorsal rhizotomy and after a RF-DRG lesion [2, 14]. In our study, the long term effects of a radiofrequency lesion adjacent to the dorsal root ganglion in the adult rat were not only found to be within the dorsal horn, but also on relatively remote spinal motor neurons. The observation of c-Fos expression in neurons remote from the lesion site has been described before.

Figure 3. Photomicrograph showing cytoplasmic c-Fos expression (green) in motor neurons after a RF-DRG treatment. Nuclei are stained blue (Hoechst) and astrocytes are stained red (GFAP). Images were taken using an Olympus AX70 microscope equipped with a cooled CCD camera (F-view). Using the Image Analysis System (SIS), gray scaled images were directly converted into artificial colors. (magnification in a 40x and in b 100x)

Figure 4. Photomicrograph illustrating double immunostaining for: (a) c-Fos and the activated microglial marker ED1 and (d) c-Fos and GFAP. Images of c-Fos (red, b and e) were taken using a CY3 (U-M 41007a) filter. Green staining in figures (c) and (f) represent ED1 immunoreactivity and GFAP immunoreactivity respectively. Nuclei are stained blue (Hoechst). (magnification 100x for all images).

Cytoplasmic expression of Fos immunoreactivity in cervical spinal cord motor neurons (bilaterally) has been reported after focal (unilateral) cerebral ischemia [19]. In this study it was suggested that the remote c-Fos expression was due to “transneuronal degeneration “ as a result of deprivation of afferent inputs following the death of corticospinal neurons.

We observed c-Fos IR cells both ipsi- and contralateral of the lesioned side. These findings are in accordance with the findings of bilateral c-Fos expression in dorsal horn neurons after a unilateral sciatic nerve injury [20]. Minor skin damage is known to evoke c-Fos expression. The observed bilateral c-Fos expression after a unilateral lesion could have been the result of the surgical procedure (midline incision). However, virtually no c-Fos IR cells were observed in the sham operated controls in our study, thus ruling out an operation artifact. The exact mechanism of contralateral c-Fos expression remains unknown. Nevertheless, the above described mechanism of transneuronal degeneration after a CNS lesion could at least partially play a role.

Conclusion

In our study we demonstrated delayed c-Fos expression in several cell types in the rat spinal cord after a radiofrequency treatment of the dorsal root ganglion. A striking outcome was the bilateral effect on c-Fos expression after an unilateral RF-DRG treatment, even on the more remote motor neurons. Therefore, our results suggest that the clinical effects of a RF-DRG treatment in spasticity and pain is two folded: firstly, by reducing afferent input and secondly by a delayed and remote effect on motor neurons possibly resulting in a reduction of efferent output.

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Abstract

The use of percutaneous radiofrequency lesion adjacent to the dorsal root ganglion (RF-DRG) in the treatment of pain has been established for years. A relatively novel indication for RF-DRG treatment is spasticity in children with cerebral palsy. In this article the pathophysiology and management of spasticity is discussed with an emphasis on the role of RF-DRG. In the management of spasticity, RF-DRG could prove to be a little invasive treatment option with little adverse effects.

Introduction

Cerebral palsy is the most common motor disorder in childhood. The term cerebral palsy harbors different pathological conditions of the central nervous system, all resulting in locomotor abnormalities. The pathophysiological mechanisms involved in cerebral palsy are intrauterine ischemia, intrauterine infection, perinatal asphyxia and prematurity [13, 18]. The incidence and prevalence of cerebral palsy is estimated to increase in the near future because of still changing perinatal care resulting in a lower mortality [1, 22]. Cerebral palsy can lead to mental retardation, epilepsy and motor abnormalities such as spasticity [9]. Cerebral palsy is the result of a multiple motor disorders known as the Upper Motor Neuron syndrome (UMN). The UMN syndrome is divided in positive and negative symptoms. Negative symptoms are; paresis, fatigability and loss of dexterity. The positive components of the UMN syndrome, characterized by excessive motor activity, are spasticity, hyperreflexia, dystonia and contractures [20].

Spasticity

Spasticity is defined as a sensorimotor disorder with a velocity dependent increase in tonic stretch reflexes and exaggerated tendon reflexes as a result of hyperexcitability of the stretch reflex [14]. In cerebral palsy, spasticity can be a very disabling feature: limited locomotor abilities, contractures (lasting stiffness of muscles, tendons, ligaments and joints), pain and limitations for self-care and care givers, amount to considerable healthcare costs.

The exact pathophysiological mechanisms of spasticity in cerebral palsy remain unclear. Nevertheless, it is generally accepted that the balance between excitation and inhibition is disturbed in favour of the excitatory forces.

The Ia afferent stretch reflex arc is the most basic neural circuit within the pathophysiology of spasticity [12]. Fast conducting sensory fibres (Ia afferents) relay excitatory impulses originated in muscle spindles after muscle stretch. After excitation of the primary afferent Ia fibres an excitatory action on the alpha-

motoneuron of the agonistic muscle is established. Besides monosynaptic connections on motor neurons by primary afferents, oligosynaptic and polysynaptic connections with excitatory interneurons are established [27]. The Ia afferents also connects with inhibitory interneurons that project directly to the antagonist motoneuron, known as reciprocal inhibition. In spasticity, the reflex circuit is impaired in favour of the excitatory action by an enhanced processing of afferent information within the spinal cord [15, 20]. Reducing the positive symptoms of the UMN syndrome associated with the hyperactive spinal reflex arcs form the basis of the management of spasticity [7].

Management of spasticity

The management of spasticity in children with cerebral palsy remains to be complex, although several new treatments have been developed in recent years. The management is focussed on the improvement and/or prevention of complications like contractures. Because spasticity goes beyond the scope of one specific discipline, careful evaluation of the patient in a multi-disciplinary team is required. An emphasis on functional improvement and/or facilitating rehabilitation after treatment should be made.

The management of spasticity with the aid of physical therapy in order to improve functionality and avoid painful contractures has been the only treatment option for years. Besides physical therapy, roughly three treatment options are available nowadays; oral pharmacotherapeutic, chemical denervation and surgery [7].

Drugs mostly used for oral administration are; Baclofen, Diazepam, Dantrolene and Tizanidine. All these drug enhance inhibition by inhibiting excitatory neurotransmitters or augmenting inhibitory neurotransmitters at the spinal cord level, in order to reduce spasticity. An important side effect of oral drug is sedation due to an effect on brain neurotransmitters. These side effects are a limiting factor in the use of oral pharmacotherapy.

The second therapeutical option is chemical denervation. Before the

introduction of Botulinum toxin type A (BTX-A), local injections with Phenol or alcohol were performed.

Nowadays, intramuscular injections with BTX-A made the use of Phenol as a local denervation agent almost obsolete. The beneficial effect of BTX-A on function and muscle tone has been extensively proven in randomized double blinded clinical trails [2, 8]. The major drawback of BTX is the recurrence of spasticity, the mean duration of the effect is approximately 3 months. Moreover, adverse events of repeated BTX therapy have been reported [17].

Besides orthopaedic interventions for the relief of contractures, the surgical management of spasticity is mainly focussed on selective dorsal rhizotomies (SDR) and intrathecal baclofen pumps. In all neurosurgical interventions, reduction of spasticity is established through interruption of the stretch reflex in order to decrease excitation [21].

Rhizotomy

Selective dorsal rhizotomy (SDR) for the treatment of spasticity in children with cerebral palsy has recently regained considerable interest. The first dorsal rhizotomy for the treatment of spasticity was described by Foerster in 1913 [6]. Because of complications like excessive sensory loss due to extensive sectioning of the dorsal root, the procedure fell into disgrace. The treatment has been modified since by several authors, resulting in the SRD technique used nowadays. The observation of an abnormal contraction of spastic muscles after stimulation of rootlet sections while non spastic muscles responded with a normal contraction, made it possible to selectively sectioning of the rootlets responsible for spasticity [5].

In SDR major surgery is involved. After extensive laminectomy of the levels L2 to S1, the rootlets are identified using electromyography and partially resected. The used techniques relieve spasticity without interfering with sensation. The reported results of selective dorsal rhizotomy on the relief of spasticity in a selected population of children with cerebral palsy are promising [4, 16]. The reduction in muscle tone can be detected almost immediately post operative.

Some controversy on the degree of functional improvement after SDR exists. However, functional improvement of functional abilities, especially sitting, is seen in the majority of children.

The invasive techniques used in the SDR are complicated and time consuming procedures only to be performed by highly specialized neurosurgeons. Moreover, considerable cooperation of the patient is required in order to successfully complete the post operative rehabilitation program during one year. Indications for SDR are improvement of walking in ambulatory diplegic children and improvement of care in the quadriplegic non-ambulatory child [24]. Patients must preferably have the mental capabilities of a toddler. Many children with severe cerebral palsy are too severely retarded for treatment with SDR. An alternative for the SDR is the percutaneous radiofrequency lesion adjacent to the dorsal root ganglion (RF-DRG).

Radiofrequency lesion adjacent to the dorsal root ganglion

In the 1980's several authors reported on the beneficial effects of RF-DRG on adult patients with intractable spasticity [10, 11]. In these studies recurrent spasticity was seen after 6 to 9 months, although the pre-operative level of spasticity was not reached. More recently Vles et al reported the use of RF-DRG in the treatment of flexor spasms of the hip of spinal and cerebral origin in children [25]. Accumulated clinical experience with RF-DRG has shown that it is possible to alleviate pain without clinical signs of nerve damage [23]. Furthermore, RF-DRG is a simple and safe treatment with little side effects [19].

The assumed mechanism of action of RF-DRG in the treatment of spasticity is the same as in SDR; a reduction of spasticity is established through interruption of the stretch reflex in order to decrease excitation. The exact mechanism of action of a RF-DRG lesion itself remains unknown. Nevertheless, an increase in nitric oxide synthetase (the key enzyme, regulating NO concentration) and motor neuron death was observed after root avulsion in the postnatal rat [26]. Moreover, we recently reported an increase of proliferation inside the dorsal root ganglion after a RF-DRG lesion adjacent to the ganglion without signs of neural tissue damage (e.g

necrosis) inside the ganglion [3]. At the moment a study is conducted to investigate the effects of a RF-DRG lesion on the dorsal root ganglion and the spinal cord.

Recently, in a pilot study, we investigated the effects of RF-DRG on spasticity and pain of the lower extremities in children with cerebral palsy (unpublished data). In this study we treated children with RF-DRG on multiple lumbar levels after BTX had failed to give improvement. Although the studied group was small, we found a beneficial effect on both muscle tone and care- giving. Furthermore, RF-DRG improved pain associated with the spasticity, a feature not treated with BTX. No adverse effects or treatment complications were reported. All treatments were performed on an outpatient basis under general anaesthesia.

In our pilot study the follow up was 4 weeks. We did not investigate the long term outcome of RF-DRG treatment in spasticity. A randomized blinded clinical trial comparing BTX, RF-DRG and physical therapy alone in the treatment of spasticity has been started, with an emphasis on both short and long term outcome.

Conclusion

The treatment of spasticity in children with cerebral palsy remains to be a problem, despite several new treatment options. RF-DRG could prove to be a little invasive treatment option with little adverse effects. Further studies should focus on the long term effects.

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Abstract

The treatment of spasticity in children with cerebral palsy remains to be a problem. The selective dorsal rhizotomy as a treatment was shown to be effective, although major surgery is involved and adverse events have been reported. Percutaneous radiofrequency lesion of the dorsal root ganglion (RF-DRG) could be a simple and safe alternative treatment. We investigated the effects of RF-DRG on muscle tone and daily activities of life and found a beneficial effect.

Introduction

Selective dorsal rhizotomy (SDR) for the treatment of spasticity in children with cerebral palsy (CP) has recently regained considerable interest. The reported results of SDR on the relief of spasticity in a selected population of children with CP are promising [5]. Nevertheless, the invasive techniques used in the SDR are complicated and time consuming procedures. Moreover, considerable cooperation of the patient is required in order to successfully complete the post operative rehabilitation program.

An alternative for the SDR is the percutaneous radiofrequency lesion of the dorsal root ganglion (RF-DRG), a treatment for pain since 30 years [9]. In the 1980's several authors reported on the beneficial effects of this method on adult patients with intractable spasticity [2, 3]. More recently, in a case report, the use of RF-DRG in the treatment of flexor/adductor spasms of the hip in children was reported [12]. The aim of this pilot-study was to investigate the effects of RF-DRG on spasticity in children with CP.

Materials and methods

Inclusion

Children ($n = 6$) included in this study were seen in a multi-disciplinary spasticity management team. Individual treatment goals were determined after a careful assessment of the etiology, functional ability and associated impairments as a result of the spasticity. During the last 3 years 137 patients were seen. Inclusion criteria for RF-DRG consisted of CP with spasticity of the lower extremities, with severe hip flexor/adductor spasms accompanied by pain or caregiving difficulties. Children were not eligible for the RF-DRG treatment if no functional improvement was to be expected due to severe contractures. Patients characteristics are summarized in Table 1, all children suffered from mental retardation and failed to respond on oral medication and intramuscular Botulinum toxin type A treatment.

patient#	M/F	age (years)	ethology	classification	treatment goals	lumbar levels treated			total Ashworth score	
						left	right	before	4 weeks	6 months
1	F	11	asphyxia	quadriplegia	1	L2 · L4	L2 · L4	12	5	8
2	F	15	prematurity	quadriplegia	1,2,3		L1 · L3	23	15	13
3	F	13	asphyxia	quadriplegia	2,3	L1 · L4	L1 · L4	50	23	35
4	M	10	prematurity	quadriplegia	1,3	L1 · L4		15	19	15
5	F	12	trauma	quadriplegia	1,2,3	L1 · L4	L1 · L4	12	6	4
6	F	6	asphyxia	diplegia	2	L1 + L2	L1 + L2	14	8	9

Table 1. Summary of patients treated with RF-DRG. Treatment goals 1=pain, scissoring=2, care problems=3

The study was approved by the ethical committee of the academic hospital Maastricht, according to Dutch governmental regulations. Children were included in this study after a written informed consent by the parents.

RF-DRG procedure

All patients were treated in our outpatient clinic. After general anaesthesia, the patients were placed in a prone position on a operating table. The procedure was performed in tunnel vision technique, this term is assigned to a technique for entering the electrode in the direct vision of the X-rays. Therefore a mobile C-arm of a fluoroscopic apparatus (Philips bv 25) was positioned in such a way that the entry point on the skin was found just under the transverse proces and slightly ventral of the inferior articular proces of the relevant level. A 100 mm 22G SMK cannula (Radionics) with a 5 mm exposed tip was introduced at this entry point. Through slight manipulation, the tip of this electrode was placed in the craniodorsal part of the foramen. This position was confirmed with the C-arm in a lateral view (Figure 1a).

Then the C-arm was changed in an Anterior- Posterior position and the needle was advanced until the tip projected over the middle of the facetal column. The location of the cannula was tested using radio-opaque contrast (Omnipaque 0.5 ml) (Figure 1b). Then a radiofrequency probe (Radionics) was introduced. After checking the impedance (an indicator for the type of tissue next to the cannula tip), electrical stimulation was started at a rate of 2 Hz and the corresponding muscles were observed for contractions. Radiofrequency current was then led through the electrode in order to increase the temperature to 67° C for 60 seconds. After full recovery from the anaesthesia, the patients were discharged at the same day.

Study outline

After inclusion, a baseline assessment was performed by a physical therapist (LP). The modified Ashworth scale was used for the assessment of changes in muscle tone [7]. In the Ashworth scale the muscle tone is scored on a 5 point

scale, in which "0" represents no hypertonia or increase of muscle tone and "4" represents severe hypertonia and severe stiffness of the extremities. In each patient hip flexion and adduction, knee flexion and extension and dorsal and plantar flexion in the ankle were tested in both legs.

Functional improvement was assessed using the Gross Motor Function Measure (GMFM), a widely used scale for evaluating treatment of spasticity in children with cerebral palsy consisting of 5 different locomotor domains [4, 8]. Furthermore, we developed a questionnaire for care-givers based on a visual analog scale (VAS). Besides items on pain, several items of daily activities of life, like dressing, undressing and bathing, were investigated. Parents were asked to mark the items on a 11 point scale in which "0" represents a poor performance and "10" represents a good performance.

Short term (4 weeks) and long term (6 months) evaluation of the treatment was done by the same physical therapist using the instruments described above. Data were statistically analysed using a students T-test and a Wilcoxon signed rank test. A p value of < 0.05 was considered to be statistically significant. All data are represented as means and standard error of means (SEM).

Figure 1. Photomicrograph showing the position of a RF electrode a: lateral view and b: Anterior-Posterior view, after injection of Omnipaque.

Results

All RF-DRG procedures were performed without complications. No side effects like dysaesthesia or excessive weakness in the treated limbs were reported. An initial motor reaction, on the unilateral side, corresponding with the treated level could be observed during the first few seconds of the RF-DRG treatment in all patients. No significant differences were observed between the different lumbar levels for both the impedance and the stimulation threshold.

A significant improvement of muscle tone after RF-DRG could be detected on both the short (4 weeks) and long (6 months) term using the Ashworth scale. The mean overall Ashworth score (all evaluated joints included) before treatment was 21 ± 6 , at 4 weeks after treatment the mean total score was 13 ± 3 ($p = 0.046$) and after 6 months the mean total score was 14 ± 4 ($p = 0.043$). Especially the right hip adductors showed improvement on the Ashworth score from 2.2 ± 0.5 before RF-DRG to 0.5 ± 0.2 ($p = 0.031$) 4 weeks after RF-DRG and 1.0 ± 0.4 ($p = 0.02$) 6 months after RF-DRG.

The GMFM showed no improvement after RF-DRG treatment. At baseline a mean total score including all 5 domains of $17.2 \% \pm 6$ was observed. After 4 weeks the score was $17.3 \% \pm 7$ (not significant) and after 6 months the score was $16.2 \% \pm 6$ (not significant).

Using the care givers questionnaire, an improvement in daily activities of life was detected. Although statistically not significant, in four patients an improvement of pain in the lower extremities was detected, both at 4 weeks and at 6 months. In one patient there was no change in pain and in another patient pain increased at 4 weeks but improved at 6 months as compared to the baseline. The mean total score of 5 items concerning dressing improved from 33 ± 2.7 to 39 ± 2.7 on a total score of 50 ($p = 0.024$). Especially putting on trousers was more easy after a RF-DRG treatment, on a 10 point scale improvement was detected from 5.5 ± 1 to 7.5 ± 0.7 ($p = 0.042$).

Discussion

The management of spasticity in children with cerebral palsy remains complex. In the past decade BTX and SDR have been shown to be effective therapies in the treatment of spasticity. The major drawback of BTX is the recurrence of spasticity, the mean duration of the effect is approximately 3 months. Moreover, adverse events of repeated BTX therapy have been reported [6].

One model of spasticity is the hypothesis that locomotor abnormalities are the result of hyperexcitability of spinal interneurons involved in the spinal stretch reflex [13]. The reduction of spinal input through deafferentation is the basis of SDR. Although the results of SDR are promising in a selected population of children with CP, major surgery is involved and several adverse events have been described [11]. Modulating the spinal stretch reflex through partial deafferentation by RF-DRG could be a less invasive alternative for SDR.

We observed improvement in muscle tone and care giving 4 weeks and 6 months after the RF-DRG treatment. The main treatment goal in all patients was reduction of scissoring due to adductor spasm of the hip. At 6 months we could still observe a reduction in hip adductor tone. No functional improvement could be detected using the GMFM. This most probably is due to the severity of cerebral palsy in our treatment group and the small number of treated children.

In a study by McLaughlin et al improvement in function using the GMFM could be detected in a randomized clinical trial investigating the efficacy and safety of SDR, however no differences with the control group were detected [5]. Beneficial effects of RF-DRG in the treatment of spasticity in adults have been reported [2, 3]. In these studies recurrent spasticity was seen after 6 to 9 months, although the pre-operative level of spasticity was not reached. The results obtained in children in our study point in the same direction.

The mechanisms of action of RF-DRG remain largely unknown. In a recent study in the goat we demonstrated that RF-DRG increased proliferation inside the dorsal ganglion without causing damage (i.e. necrosis) of the neurons inside the ganglion [1]. Moreover, accumulated clinical experience with RF-DRG has shown

that it is possible to alleviate pain without clinical signs of nerve damage or signs of motor denervation [10].

We found that RF-DRG is an effective treatment for spasticity in children. The advantages are the less invasive and safe character of RF-DRG compared to the SDR. Furthermore, FR-DRG is an outstanding treatment for pain, a feature not treated with BTX. A relative disadvantage of RF-DRG could be the recurrence of spasticity, although the treatment can easily be repeated. In order to investigate the long term outcome and efficacy of RF-DRG, a randomized clinical trial comparing BTX and RF-DRG as a treatment for spasticity in children with CP has been started.

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Although the origin of the locomotor impairment in children with cerebral palsy is embedded in the term *cerebral* palsy itself, the spinal cord is at least partially responsible for some of its clinical features. It is commonly accepted that cerebral palsy is the result of different pathological conditions during pre- and postnatal development [21]. Among these pathological conditions, perinatal asphyxia is responsible for approximately 10 to 20 percent of the cases of cerebral palsy [24]. There are numerous animal studies on the effects of perinatal asphyxia on the developing brain, using different models (see introduction) Surprisingly, virtually no studies have been done on the effects of perinatal asphyxia on the (developing) spinal cord, whereas the spinal cord is indispensable in controlling locomotion.

The results of our animal studies show abnormal development of the spinal cord after perinatal asphyxia and support a role for the spinal cord in the pathophysiological mechanisms of spasticity.

Apoptosis

Besides normal developmental apoptosis, we observed an increase in apoptotic cell death in the intermediate zone of the lumbar spinal cord after perinatal asphyxia. The observed increase in apoptotic cells in the lumbar spinal cord after perinatal asphyxia, most probably will have an effect on the normal development of local spinal cord networks at this level.

Besides an increase in apoptotic cells in the spinal cord, apoptotic neuronal

loss in the neonatal rat striatum after perinatal asphyxia has been detected [32]. In the human neonatal brain, perinatal asphyxia is known to cause brain lesions in several regions. The location of the lesions is dependent on the severity of the asphyxia and the developmental stage of the neonate (e.g. full-term or pre-term) [7].

Cerebral lesions, as all CNS lesions, are known to inflict damage on remote cells within the CNS through a process called transneuronal degeneration. In transneuronal degeneration, the target cells atrophy and/or die after disruption of the major inputs from the afferent cell [14]. The observed increase of apoptotic cell death in the lumbar spinal cord after asphyxia in our study, could to our opinion not be the result of transneuronal degeneration, because corticospinal axons originating from sensorimotor cortex neurons only reach their target at the mid-lumbar level at the fifth postnatal day [13]. The lumbar gray matter targets are not yet reached at postnatal day two, thus the observed increase in apoptotic cell death most probably is not secondary to cerebral lesions.

We demonstrate apoptosis in the white matter of the developing spinal cord of normal postnatal rats. Apoptotic cells could be identified as oligodendrocytes and activated microglia. This observation was made serendipitously while studying the effects of perinatal asphyxia on the developing spinal cord in rats. To our knowledge no studies have previously reported developmental apoptosis in the rat spinal cord white matter. These findings imply a role for apoptosis in the spinal cord white matter in the development of normal locomotor behavior.

The increase in apoptosis after perinatal asphyxia was mainly observed in the lumbar intermediate zone (Rexed's laminae IV-VII) at postnatal day 2. In the spinal cord intermediate zone abundant numbers of interneurons have been described [4]. Therefore, we speculated that part of the apoptotic cells observed in our study could be interneurons. There are some other studies on apoptosis of spinal interneurons during normal development which supported apoptosis of interneurons [16, 19]. In these studies, as well as in our study, no direct evidence was found for interneuron apoptosis by means of double immunostaining techniques. To date no reliable marker for interneurons has been developed.

The functions of spinal interneurons are to mediate an inhibitory effect upon motor neurons [30]. In spasticity, it has been suggested that these interneurons do not function normally, probably by making false connections [8, 37].

The concept of spinal interneuron involvement in the pathophysiological mechanisms of spasticity has mainly been studied by measuring changes in reflex excitability using neurophysiological techniques [31]. Our studies in an animal model for perinatal asphyxia provide additional direct evidence for spinal cord involvement. In the studies described in this thesis, no functional outcome parameters were assessed after perinatal asphyxia. Future studies on the effect of perinatal asphyxia on the spinal cord should involve neurophysiological techniques like sensory evoked potentials and EMG in order to clarify the functional implication of the observed increase in interneuron apoptosis.

Cyclic GMP

When starting the studies on the effects of perinatal asphyxia on the spinal cord, little was known about the localization and developmental changes of NO or ANP mediated cGMP synthesis in the rat spinal cord. We observed abundant NO-mediated cGMP synthesis in all laminae of the rat cervical spinal cord. Moreover, NO- cGMP synthesis was observed in laminae I-III in 14 -day-old rats, which activity was nearly absent at the age of 3 months. Using double immunostaining techniques, NO-mediated and ANP mediated cGMP immunoreactive (cGMP-IR) cells in both the gray and white matter could partially be identified as astrocytes and GABAergic (parvalbumin positive) neurons.

The observation of NO mediated cGMP synthesis in GABAergic cells in laminae I-III of the rat cervical spinal cord raised the question whether GABA could activate nNOS to synthesize NO, which, as a retrograde messenger molecule, would activate cGMP synthesis in GABAergic terminals. Therefore we investigated the effects of Baclofen on cGMP synthesis. The GABA_B agonist Baclofen is a widely used drug in the treatment of spasticity [38].

In normal rats we found an inhibitory effect of Baclofen on the ANP-mediated cGMP synthesis in laminae I-III of the cervical spinal cord at the age of two weeks

and two months. This inhibitory effect of Baclofen could not be detected after the incubation with the NO donor SNP. From a structural point of view the inhibitory effect of Baclofen on ANP induced activation of particulate guanylyl cyclases is surprising. Nevertheless, isatin (indole- 2,3 dione) which is also structurally unrelated to ANP has been described as an inhibitor of pGC [5]. Recently it was shown that isatin inhibits pGC independent of the occupation of the ANP receptor [20]. Activation of GABA_B receptors might result in activation of phospholipase A and potentiation of cAMP formation [6]. Thus, the effect of Baclofen might involve triggering of a phosphorylating/dephosphorylating cycle of the intracellular part of the ANP receptor and subsequently downregulation of the pGC activity [23].

In the study describing the effects of perinatal asphyxia on the ANP and NO mediated cGMP synthesis, a small part of the ANP mediated cGMP-IR cells could be identified as astroglial cells on the basis of their GFAP immunostaining. Double immunostained cells were mainly detected in the SPA group under 2 experimental conditions: after stimulation with ANP alone and after stimulation with ANP in the presence of 100 μ M Baclofen. No colocalization between ANP mediated cGMP-IR and GFAP was observed in the control group.

To date, the role of natriuretic peptides in CNS physiology and pathology is still rather unclear. In a recent study in humans an increase was observed in ANP immunoreactive astrocytes after brain ischemia [22]. Furthermore, in an electrophysiological study the presence of ANP receptors on cultured spinal cord astrocytes was demonstrated [11]. Nevertheless, the findings of both the decreasing effects of Baclofen on ANP mediated cGMP synthesis in the normal rat spinal cord and the observed ANP mediated cGMP synthesis in astroglial cells after perinatal asphyxia, remain difficult to explain.

In addition, we found an increase in NO mediated cGMP in laminae I-III after stimulation with SNP in the presence of 100 μ M Baclofen after perinatal asphyxia. Activation of the GABA_B receptor in the dorsal horn is known to inhibit the release of both glutamate (excitatory) and GABA (inhibitory) in the spinal dorsal horn [12]. Using a whole cell voltage clamp recording technique, Yang et al showed a slow outward inhibitory current in dorsal horn neurons after bath application of

Baclofen [36]. Our results suggest that the inhibitory effects of Baclofen are at least partly NO-cGMP and/or ANP-cGMP mediated. A possible future study to elucidate the effects of Baclofen on the NO and ANP mediated cGMP synthesis should involve cell cultures of spinal astrocytes.

Rat model for perinatal asphyxia

We used an animal model for perinatal asphyxia described by Loidl et al, a representative model for the clinical situation of perinatal asphyxia [18]. Regarding our studies, the major drawback of this model is the lack of clinical features of spasticity in the rat after perinatal asphyxia. In rats, spasticity has only been described in the so called mutant Han-Wistar rats [34].

A parallel between perinatal asphyxia in rats and the human situation during birth is difficult to make. A substantial difference between CNS maturation at birth exists between the two species [1]. Studies describing the effects of perinatal asphyxia on the spinal cord of human neonates are rare. In the few published studies on the effects of perinatal asphyxia on the human spinal cord, signs of ischemia and necrosis have been described [2, 25].

Knowing the results obtained in animal experiments and their limitations, it seems inevitable to further investigate the effects of perinatal asphyxia on the brain and spinal cord using postmortem tissue of human neonates.

Radiofrequency lesion adjacent to the dorsal root ganglion

In the last three chapters of this thesis the use of a radiofrequency lesion adjacent to the dorsal root ganglion (RF-DRG) for the treatment of spasticity in children with cerebral palsy is described. In RF-DRG treatment, as in all neurosurgical interventions for spasticity, reduction of spasticity is established through interruption of the stretch reflex in order to decrease excitation of spinal neural networks [28]. The beneficial effects of a RF-DRG in the treatment of spasticity in adults has been demonstrated in the 1980's [9, 15]. Whereas the clinical effects of a RF-DRG lesion in the treatment of pain have been well described, the exact mechanism of action of the RF-DRG lesion itself remains

unknown [33].

In order to investigate the effects of a RF-DRG on the dorsal root ganglion and the spinal cord, we developed a rat model. We firstly intended to perform a percutaneous RF-DRG in the rat model, parallel to the technique used in the patient. Due to the small diameter of the ganglion in the rat and the relative large diameter of the radiofrequency probe, we did not succeed in producing a reproducible lesion. In a larger animal this would not have been a problem [3].

Nevertheless, for logistic reasons, we choose to perform a laminectomy in the rat in order to have a direct view on the dorsal root ganglion. Based on the lack of c-Fos IR immunoreactivity (c-Fos IR) in the sham operated rats and the morphological localization of the treatment site (outside the ganglion) in both the sham and the RF-DRG treated rats, we believe that the open surgical model is a good alternative for the percutaneous model.

Seven days after radiofrequency treatment of the dorsal root ganglion we observed an increased expression of c-Fos IR in motor neurons, astrocytes and activated microglia in the spinal cord, both ipsi- and contra- lateral, after a unilateral RF-DRG treatment. At post operative day 7 the expression of c-Fos protein was found to be cytoplasmic and not nuclear.

Induction of c-Fos in the rat spinal dorsal horn has been described within several hours after dorsal rhizotomy and after a RF-DRG lesion [10, 29]. In our study, the long term effects of a radiofrequency lesion adjacent to the dorsal root ganglion in the adult rat were not only found to be within the dorsal horn, but also on relatively remote spinal motor neurons. The observation of c-Fos expression in neurons remote from the lesion site has been described before. Cytoplasmic expression of Fos immunoreactivity in cervical spinal cord motor neurons (bilaterally) has been reported after focal (unilateral) cerebral ischemia [35]. In this study it was suggested that the remote c-Fos expression was due to "transneuronal degeneration " as a result of deprivation of afferent inputs following the death of corticospinal neurons.

To date, the effects of a radiofrequency treatment of the dorsal ganglion were thought to be merely by reducing afferent input on spinal cord neurons. In our

study we demonstrated that the clinical effects of a RF-DRG treatment in spasticity seems to be two folded. Firstly, by reducing afferent input and secondly by a remote effect on motor neurons possibly resulting in a reduction of efferent output.

A future direction of the studies on the effect of RF-DRG lesions in the rat, both in normal rats and after perinatal asphyxia, should involve neurophysiological parameters like electromyography, in order to investigate the functional implications of the lesion, especially related to the effects on efferent output.

A second future direction on the effects of RF-DRG on the rat spinal cord and dorsal root ganglion will focus on different techniques to perform a RF-DRG lesion. In our study we used a continuous radiofrequency current at 67° C. In a recent study no difference could be observed in the outcome of pain treatment between a 67° C and a 40° C lesion [26]. Pulsed radiofrequency (PRF), a non destructive method of exposing tissue to RF electric fields at 42° C with potentially lesser side effects, was recently proposed by Sluijter [27]. A study comparing the effects of a RF and a PRF lesion on the spinal cord and the dorsal root ganglion is conducted at the moment.

The preliminary results obtained in our study on the effects of a RF-DRG lesion in the treatment of spasticity, are promising. However, only a small group of children was included in this pilot study. Moreover, no data was available on the long term outcome after treatment. In earlier studies on RF-DRG treatment of spasticity in adults, recurrent spasticity was seen after 6 to 9 months, although the pre-operative level of spasticity was not reached [9, 15]. Momentarily, a randomized clinical trial comparing Botulinum toxin A, RF-DRG and physical therapy alone in the treatment of spasticity has been started, with an emphasis on both short and long term outcome.

Final remarks

In 1861, Little presented his magnum opus on the locomotor changes after difficult birth [17]. With the statement that the features of spasticity are (partially) related to lesions of the spinal cord, he proved to be a visionary. Little by little we have started to investigate the effects of perinatal asphyxia on the developing spinal cord.

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Summary

The present thesis was designed to investigate the effects of perinatal asphyxia on the spinal cord in order to better understand the pathophysiological mechanisms of spasticity in children with cerebral palsy. Moreover, based on the findings in an animal model, an attempt is made to introduce a treatment option for spasticity.

Chapter 1

In Chapter 1, an introduction is given on the present knowledge of the pathophysiology of perinatal asphyxia, cerebral palsy and spasticity in relation to the current treatment options and the morphological and biochemical concepts used in this thesis. Furthermore, the general aims of the studies in this thesis are introduced.

Chapter 2

In Chapter 2 an immunocytochemical technique was used to study the localization and developmental aspects of GMP-synthesizing structures in the cervical spinal cord of 2 weeks and 3 months old Lewis rats in response to the nitric oxide (NO) donor sodium nitroprusside (SNP) and/or atrial natriuretic peptide (ANP). By using cell-specific markers, the cell structures involved were investigated. NOS-mediated cGMP synthesis was observed in the cervical spinal cord in laminae I, II and III in 14 days old rats, which activity was mainly absent at the age of 3 months. Using confocal laser microscopy, NO-mediated cGMP synthesis was observed in large cholinergic terminals nearby motor neurons in the ventral horn. An extensive co-localization between NO-stimulated cGMP synthesis and parvalbumin-positive (GABAergic) neurons and fibres was observed in all laminae. In the ANP-stimulated condition a colocalization with parvalbumin structures was found in laminae II and III.

In conclusion, our data show the presence of an abundant NO-cGMP signalling

system in all layers of the spinal cord. The NO-mediated cGMP synthesis takes place in parvalbumin (GABAergic) neurons and in the ventral horn in axon terminals probably connecting to the motor neurons. In addition cGMP synthesis can be found in a subpopulation of glial cells either through activation of pGNC and/or sGNC.

Chapter 3

In Chapter 3 we investigated developmental apoptosis in the white matter of the neonatal rat cervical spinal cord at postnatal day 2, 5, and 8. Apoptotic cells were labelled using TUNEL and caspase-3 immunostaining. Apoptotic cells were diffusely distributed throughout the white matter of the spinal cord. The total amount of apoptotic cells in the cervical spinal cord white matter was related to postnatal age, being the lowest at P2 (mean 7.9 , SD 5.6) and the highest at P8 (mean 109, SD 21.4). Using double immunostaining for ED1 and O4, apoptotic cells could be identified as microglia and oligodendrocytes.

Chapter 4

In Chapter 4, we investigated the effect of perinatal asphyxia on developmental apoptosis in the cervical and lumbar spinal cord in the neonatal rat.

Perinatal asphyxia was induced by keeping pups at term in utero in a water bath at 37° C for 20 min, followed by resuscitation. Effects of this treatment on developmental apoptosis were studied at postnatal day 2, 5 and 8 using TUNEL and caspase-3 staining. TUNEL positive cells were characterized using double immunostaining.

On postnatal day 2 an increase of 215% in TUNEL positive cells was detected ($p = 0.005$) in laminae IV-VII of the lumbar spinal cord of rats that underwent perinatal asphyxia compared to controls. TUNEL positive cells could be partly characterized as microglial cells (ED1 positive) and oligodendrocytes (O4 positive).

As the effect of perinatal asphyxia on programmed cell death in the neonatal rat spinal cord was mainly observed in the intermediate zone and dorsal horn of the lumbar spinal cord, it is concluded that this may have a profound effect on the

development of motor networks.

Chapter 5

In Chapter 5 we report an inhibitory effect of Baclofen on the ANP-mediated cGMP synthesis in the superficial dorsal horn (laminae I-III) of the rat cervical spinal cord. This inhibitory effect of Baclofen could not be detected after incubation with the NO donor SNP. Baclofen is the drug of choice in treating spasticity, therefore these results point to a possible role of ANP in controlling spinal locomotor networks.

Chapter 6

In Chapter 6 we investigated the effects of perinatal asphyxia and the influence of Baclofen on the ANP and/or SNP mediated cGMP synthesis in the adult rat cervical spinal cord. In normal rats, a decreasing effect of Baclofen on ANP mediated cGMP synthesis was observed, while an increasing effect on NO mediated cGMP synthesis was observed after perinatal asphyxia. Our results suggest that the effects of Baclofen in the spinal cord are at least partially NO-cGMP mediated.

Chapter 7

In Chapter 7 we investigated the long-term effects of a RF-DRG treatment on the spinal cord using c-Fos immunoreactivity. Furthermore, we used different immunocytochemical markers to identify c-Fos immunoreactive (c-Fos IR) cells.

In 6 Wistar rats a laminectomy was performed. RF-DRG treatments were performed adjacent to 6 dorsal root ganglions, and 6 ganglions were used as sham operated controls. Moreover, one rat was used as double control. Tissue was investigated at postoperative day 7.

C-Fos IR cells were detected both ipsi- and contra-lateral of the treatment side. Furthermore, c-Fos IR cells could be identified as activated microglia, astrocytes and motor neurons but not as oligodendrocytes.

We conclude that the long-term effects of a RF-DRG treatment can be detected

in all Rexed's laminae both ipsi- and contra-lateral after an unilateral treatment. We furthermore suggest that the clinical effects of a RF-DRG treatment in spasticity are possibly due to interference with both afferent input into the spinal cord and efferent output from the spinal cord.

Chapter 8

In Chapter 8, in a review, the present pathophysiological mechanisms of spasticity and its treatment options are reviewed. Furthermore, the use of radiofrequency lesions of the dorsal root ganglion in the treatment of spasticity in children with cerebral palsy is reviewed.

Chapter 9

In Chapter 9 we investigated the effects of a radiofrequency lesion of the dorsal root ganglion in the treatment of spasticity in children with cerebral palsy on muscle tone and daily activities of life. We found a beneficial effect 4 weeks and 6 months after treatment without signs of side effects.

Chapter 10

In Chapter 10, the morphological and biochemical findings in the spinal cord after perinatal asphyxia in the rat are discussed. Furthermore, an attempt is made to put the findings in the rat model in a clinical perspective. Especially for the use of the radiofrequency treatment of the dorsal root ganglion as a treatment for spasticity in children with cerebral palsy. Moreover, in this chapter, future studies on the role of the spinal cord in the pathophysiological mechanism of spasticity are described

With the studies that form this thesis we "little by Little" have started to investigate the effects of perinatal asphyxia on the developing spinal cord.

Samenvatting

Het leven van een kind met cerebral palsy kan ernstig beperkt zijn. Deze beperkingen worden ondermeer veroorzaakt door de gevolgen van spasticiteit, zoals verminderde mobiliteit, pijnklachten en verzorgingsproblemen. Het exacte pathofysiologische mechanisme van spasticiteit bij cerebral palsy is nog niet geheel bekend. Tot op heden wordt de oorzaak van cerebral palsy vooral gezocht in de hersenen, zoals het woord *cerebral* in cerebral palsy al aangeeft. De invloed van de hersenen op het klinisch beeld van cerebral palsy is ongetwijfeld groot, alhoewel niet alle klinische verschijnselen zijn te verklaren door hersenschade. Met name de beperkingen in motoriek zouden ook een oorsprong kunnen hebben op een lager niveau van het centrale zenuwstelsel namelijk het ruggenmerg. Deze klinische constatering vormt de basis van de studies waaruit dit proefschrift bestaat.

Op dit moment bestaat er nog geen behandeling waarmee we spasticiteit kunnen voorkomen. De huidige behandeling richt zich dan ook vooral op het verminderen van bovengenoemde problemen. Een beter inzicht in de pathofysiologie van spasticiteit kan mogelijk leiden tot meer rationele behandelmethoden.

Het klinisch beeld van cerebral palsy wordt veroorzaakt door uiteenlopende aandoeningen welke voor een deel onbekend zijn. Bij een deel van de kinderen is perinatale asphyxie de oorzaak van cerebral palsy. In dit proefschrift worden de effecten van perinatale asphyxie op het ruggenmerg van de rat onderzocht, om een beter inzicht te krijgen in het pathofysiologische mechanisme van spasticiteit bij kinderen met cerebral palsy. Daarnaast wordt de radiofrequente laesie naast het dorsale ganglion als mogelijke behandeling voor spasticiteit bij kinderen geïntroduceerd.

Hoofdstuk 1

Hoofdstuk 1 bestaat uit een algemene introductie van de onderwerpen die in de verschillende hoofdstukken aan de orde komen. De huidige inzichten in de pathofysiologische mechanisme van perinatale asphyxie, cerebral palsy en

spasticiteit worden besproken in relatie tot de morphologische (geprogrammeerde celdood) en biochemische (NO-cGMP signaal transductie) concepten die voor de verschillende studies worden gebruikt. Daarnaast worden de doelen van de verschillende studies beschreven.

Hoofdstuk 2

In hoofdstuk 2 hebben we de expressie van cGMP in reactie op stimulatie met de NO donor sodium nitropursside (SNP) en /of atrial natriuretic peptide (ANP) in het ruggenmerg gedurende de normale ontwikkeling van de rat bestudeerd. Met behulp van immunocytochemische technieken en celspecifieke markers hebben we de betrokken celstructuren onderzocht. Op de 14^e postnatale dag wordt NOS gemedieerde cGMP synthese waargenomen in laminae I-III van het cervicale ruggenmerg, deze activiteit is vrijwel geheel verdwenen op de leeftijd van 3 maanden. Met behulp van confocale laser microscopie wordt NO gemedieerde cGMP synthese gezien in grote cholinerge axon-uiteinden op motor neuronen in de ventrale hoorn. In alle laminae hebben we een overvloedige co-lokalisatie waargenomen tussen NO gemedieerde cGMP synthese en parvalbumine (GABAerge) positieve neuronen en vezels. Na stimulatie met ANP wordt deze co-lokalisatie tussen cGMP en parvalbumine waargenomen in laminae I en II.

Concluderend laten onze resultaten een overvloedige aanwezigheid van het NO-cGMP signaal transductie systeem in alle laminae van het cervicale ruggenmerg zien. Bovendien vindt de NO-gemedieerde cGMP synthese plaats in parvalbumine positieve (GABAerge) neuronen en in axon-uiteinden die mogelijk uitkomen op motor neuronen in de ventrale hoorn. Daarnaast wordt cGMP synthese waargenomen in een subpopulatie van gliale cellen na stimulatie van zowel particulated guanylaat cyclase (ANP) of soluble guanylaat cyclase (SNP).

Hoofdstuk 3

In hoofdstuk 3 wordt de aanwezigheid van apoptose in de witte stof van het ruggenmerg van neonatale ratten gedurende de normale ontwikkeling beschreven. Door middel van TUNEL en caspase-3 immunocytochemische kleuringen worden

apoptotische cellen gezien in de gehele witte stof van het ruggenmerg van de rat. Het totale aantal apoptotische cellen in de witte stof is het laagste op de tweede dag post nataal en het hoogste op de achtste dag postnataal. Met behulp van dubbelkleuringen hebben we een deel van de apoptotische cellen geïdentificeerd als microgiale cellen (ED1 positief) en oligodendrocyten (O4 positief).

Hoofdstuk 4

In hoofdstuk 4 wordt het effect van perinatale asphyxie op apoptose gedurende de ontwikkeling in het cervicale en lumbale ruggenmerg van de rat beschreven. Perinatale asphyxie wordt geïnduceerd door à terme rattenpups in utero bloot te stellen aan onderdompeling in water van 37° C gedurende 20 minuten, gevolgd door resuscitatie. De effecten van deze behandeling zijn bestudeerd door middel van TUNEL en caspase-3 immunocytochemische kleuringen op dag 2, 5 en 8 postnataal. We hebben de TUNEL positieve cellen geïdentificeerd met behulp van dubbelkleuringen.

Na perinatale asphyxie zagen we op dag 2 postnataal een toename in TUNEL positieve cellen in laminae IV-VII van het lumbale ruggenmerg van 215% ($p = 0,005$). De TUNEL positieve cellen hebben we gedeeltelijk kunnen identificeren als microgiale cellen (ED1 positief) en oligodendrocyten (O4 positief).

In de pathofysiologie van spasticiteit spelen spinale interneuronen een belangrijke rol. Aangezien in deze studie de meeste toename van apoptotische celdood na asphyxie is waargenomen in gebieden waar zich veel interneuronen bevinden, vermoeden wij dat deze toename een effect kan hebben op de ontwikkeling van spinale locomotor netwerken.

Hoofdstuk 5

In hoofdstuk 5 rapporteren wij een remmend effect van baclofen op de ANP-gemedieerde cGMP synthese in de dorsale hoorn (laminae I-III) van het cervicale ruggenmerg van de rat. Het remmende effect van baclofen kan niet worden geobserveerd na incubatie met de NO donor SNP. Aangezien Baclofen een veel gebruikt medicijn voor de behandeling van spasticiteit is, wijzen onze resultaten

naar een mogelijke rol van ANP in de controle van spinale locomotor netwerken.

Hoofdstuk 6

In hoofdstuk 6 hebben we het effect van perinatale asphyxie in combinatie met baclofen op de ANP- en SNP-gemedieerde cGMP synthese in het ruggenmerg van volwassen ratten onderzocht. In normale ratten zagen we een remmend effect van baclofen op de ANP-gemedieerde cGMP synthese, terwijl na asphyxie een stimulerend effect van baclofen op de NO-gemedieerde cGMP synthese is gezien. Onze resultaten suggereren dat de effecten van baclofen op het ruggenmerg na perinatale asphyxie ten minste gedeeltelijk via het NO-cGMP signaaltransductie systeem verlopen.

Hoofdstuk 7

In hoofdstuk 7 hebben we de lange termijn effecten van een RF-DRG behandeling op het ruggenmerg bestudeerd met behulp van c-Fos immunoreactiviteit. Daarnaast zijn verschillende immunocytochemische markers gebruikt om de c-Fos immunoreactieve (c-Fos IR) cellen te identificeren.

We hebben bij 6 Wistar ratten een cervicale laminectomie verricht. De RF-DRG behandeling is toegepast naast 6 dorsale ganglions, 6 ganglions zijn behandeld met een sham operatie en 1 rat is gebruikt als dubbele controle. De weefsels zijn zeven dagen na de ingreep onderzocht. We zagen een hoog significante toename in c-Fos positieve cellen na RF-DRG behandeling in vergelijking met de sham geopereerde ratten. We hebben na een unilaterale ingreep zowel contra- als ipsi-lateraal c-Fos positieve cellen waargenomen. Bovendien hebben we de c-Fos positieve cellen kunnen identificeren als geactiveerde microglia en astrocyten, maar niet als oligodendrocyten.

We concluderen dat de lange termijn effecten van een RF-DRG behandeling in alle Rexed's laminae waarneembaar zijn, zowel ipsi- als contra- lateraal. Daarnaast zijn de klinische effecten van een RF-DRG behandeling voor spasticiteit mogelijk het gevolg van een effect op zowel de afferente input als de efferente output van het ruggenmerg.

Hoofdstuk 8

In hoofdstuk 8 worden de pathofysiologische mechanismen van spasticiteit en de huidige behandelopties besproken. Daarnaast wordt het gebruik van de RF-DRG voor de behandeling van spasticiteit bij kinderen met cerebral palsy besproken.

Hoofdstuk 9

In hoofdstuk 9 hebben we de klinische effecten van de RF-DRG op spasticiteit bij kinderen met cerebral palsy onderzocht. Met behulp van de Ashworth schaal vinden we een verbetering van de spasticiteit op zowel 4 weken als 6 maanden na behandeling zonder dat er bijwerkingen zijn gezien. Daarnaast zien we een verbetering van de verzorging na RF-DRG behandeling.

Hoofdstuk 10

In hoofdstuk 10 worden de morfologische en biochemische bevindingen in het ruggenmerg na perinatale asphyxie bediscussieerd. Daarnaast worden deze bevindingen in het rattenmodel in een klinisch perspectief geplaatst, met name voor de RF-DRG behandeling. Tevens worden de toekomstige studies over de rol van het ruggenmerg voor de pathofysiologische mechanismen van spasticiteit beschreven.

We zijn stukje bij beetje (little by Little) begonnen met het bestuderen van de effecten van perinatale asphyxie op het ruggenmerg.

Dankwoord

Dit proefschrift is tot stand gekomen vanuit een gezamenlijke inspanning van een aantal mensen van een zeer divers pluimage. De initiator en naar mijn inziens geestelijk vader van het onderzoek naar de effecten van perinatale asphyxie op het ruggenmerg en spasticiteit bij kinderen met cerebrale palsy is professor doctor Vles. Zonder zijn gave om relatief laat in zijn levensloop vanuit een klinische vraagstelling terug te gaan naar het basale onderzoek, zou er in Maastricht geen onderzoek over het effect van perinatale asphyxie op het ruggenmerg zijn geweest. Ondermeer daarom, Hans, beschouw ik je als mijn mentor (en getuige) in de breedste zin van het woord. Hiervoor mijn dank en respect.

Professor doctor Troost, Jaap, ik ben je veel dank verschuldigd voor het gestelde vertrouwen. Jij hebt er uiteindelijk voor gezorgd dat ik in staat ben gesteld om dit onderzoek uit te voeren.

Professor doctor Steinbusch, Harry, bedankt voor je begeleiding bij het perfectioneren van de manuscripten

Doctor de Vente, Jan, ik bewonder je beschouwelijke, bescheiden, altijd ter zake doende inbreng in ons project. Het maakt je tot een groots wetenschapper en mijn "basale" leermeester.

De technische vaardigheden die nodig zijn om cellen te "verven" zijn bij mij maar summier ontwikkeld. Hellen, ik dank je voor de prettige samenwerking en je waardevolle technische inbreng. Het was goed om te zien dat je steeds enthousiaster werd over ons onderzoek. Daarnaast wil ik Marianne bedanken voor de ondersteuning en de samenwerking.

De asphyxie groep vormt de broedkamer van ons laboratorium onderzoek, beste Wilma, Arjan, Danillo, Tom en Carlos, bedankt voor de samenwerking en discussie.

Professor doctor van Kleef en "collega" van Zundert, beste Maarten en Jan, zonder jullie inzet zou het onderzoek naar de effecten van de RF-DRG behandeling, zowel in de kliniek als in de rat, niet op dit peil zijn gekomen. Ik zie uit naar de toekomstige samenwerking.

Zonder onderwijs geen onderzoek, ik dank Judith en Haike voor hun formidabele inbreng als student(assistent). Ik heb veel van jullie geleerd.

Speciale dank gaat uit naar Theo van de Nagel, je vakgroep overstijgende hulp was van onschatbare waarde.

Zoals de asphyxie groep de broedkamer van het basale onderzoek vormt, is de werkgroep spasticiteit de spil waar de behandeling van kinderen met spasticiteit om draait. Ik heb grote bewondering voor de specifieke kundigheid en inzet van alle leden. Wat betreft het klinisch onderzoek gaat mijn dank met name uit naar Liesbeth en Margriet voor hun niet aflatende organisatie. Co-pro-io Kessels (Kroes, 2002) en Lisette van Raak wil ik danken voor de statistiek en hulp bij het protocol.

De vakgroep waarbinnen ik werkzaam mag zijn heb ik altijd als zeer plezierig ervaren. In een hechte (assistenten) groep komt iedereen tot bloei. Er liggen nog vele jaren in het verschiet. De betrokkenheid van Desiree bij de studies is niet aflatend, waarvoor dank.

Het bestuur van de Dr. W.M. Phelps stichting wil ik speciaal bedanken. Het getuigt van grote visie om als stichting voor spastici het blikveld te verruimen en zowel ons klinisch onderzoek als het basale onderzoek te financieren. Daarbij past ook een woord van dank voor Dr. J. Carpay, voor het inzicht dat een promovendus geen serie publicaties hoeft te hebben voordat hij aan zijn onderzoek mag beginnen.

Peter en Wilbert, niet voor niets paranimfen

Tenslotte, een warm gezin is een voortreffelijk geschenk en het begin van alles.

Dank dat liefde liefde is.

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Curriculum Vitae

Anton de Louw werd geboren op 14 november 1968 te Schayk. In 1987 voltooide hij zijn HAVO opleiding. Na een kort intermezzo op de HBO-Verpleegkunde te Breda, begon hij in 1990 aan de studie Geneeskunde aan de Universiteit Maastricht. In mei 1997 behaalde hij het artsexamen en werkte aansluitend als AGNIO bij de vakgroep neurologie van het academisch ziekenhuis Maastricht. Vanaf november 1999 werd deze aanstelling omgezet in een AGIKO aanstelling, welke werd gestart met een periode van 2 jaar onderzoek in het laboratorium van Prof. dr. H.W.M. Steinbusch onder de leiding van Prof. dr. J.S.H. Vles. In januari 2002 is hij gestart met de opleiding tot Neuroloog in het academisch ziekenhuis Maastricht.