Translational research on spinal cord injury and cell-based therapies : a focus on pain and sensorimotor disturbances

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Translational research on spinal cord injury and cell-based therapies; a focus on pain and sensorimotor disturbances

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht, op gezag van de Rector Magnificus, Prof dr. Luc Soete, volgens het besluit van het College van Decanen, in het openbaar te verdedigen op vrijdag 14 maart 2014 om 10.00 uur door

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Personal foreword

I became interested in Neuroscience and in particular Spinal Cord Injury (SCI) research during the first years of my medical study at the University of Maastricht and I was further stimulated during my internship at the department of Pain Management and Anesthesiology. From this point onwards it became clear to me that chronic neuropathic pain is a major issue for many SCI patients. Pain, however, is clearly not the only problem after SCI, and multiple sensory and motor modalities are usually altered by the injury (e.g. walking, spasticity and/ or the perception of heat and/or touch). Unfortunately, the gross similarity between all modalities stops here. While pain, for example, is marked by a relative over-activation in the nociceptive/sensory system, the motor system is often troubled by inactivity. Any SCI treatment strategy will, therefore, face a major challenge due to this dualistic aspect, which is even more difficult to tackle considering that the nociceptive/sensory system and motor system are anatomically intertwined. What is clear is that one should not investigate the effect of the treatment at hand on one sole modality while ignoring the other. The above explains why I choose the main theme of this thesis to be SCI treatment, keeping a special focus on SCI-related pain, but not letting non-pain related consequences of SCI get out of sight.

I. Introduction

Dissertation introduction

The incidence of SCI is relatively low, with incidence rates from 1 to 8 per 100,000 and prevalence rates up to 80 per 100,000 (Wyndaele 2006). Due to the fact that most SCIs have a traumatic cause related to sports, car/sports accidents, or violence, the patient population with new SCIs is relatively young. Fortunately, modern medicine enables most SCI patients to have long life expectancies. The ancient Greek physician Hippocrates (~400 B.C.), founder of Western medicine, deemed most SCI patients were destined to die, and it was only by the mid-20th century, a time at which SCI care revolutionized, that this view changed. The improvement in SCI care was, ironically, mostly thanks to World War II, as army hospitals at the time were one of the first to establish SCI care units (encouraged by physician Donald Munro; 1898-1978). The dogma that most SCIs are “an ailment not to be treated”, which can even be traced back about 5000 years to the writings of an Egyptian physician (most likely Imhotep), was finally broken. This change was also fueled by recent medical discoveries, the most important of which was the discovery of penicillin (Alexander Fleming; 1881–1955), resulting in the fact that infections like pyelitis were no longer a major cause of death in SCI patients. In current times, most SCI persons, at least in highly developed Western world countries, achieve a fairly independent societal integration and long-term survival with nearly normal life expectancies. This sets the scene for the SCI research community, as the secondary complications of SCI, like pain and spasticity, are becoming more and more relevant issues for SCI patients. For instance, SCI patients with significant pain are generally willing to trade a chance of recovery or loss of certain physiologic functions (sexual, bowel-bladder control) for the relief of pain (Nepomuceno 1979). In addition, the intensive and chronic aspects of SCI care is making this debilitating condition expensive, yielding high costs for highly developed western societies (estimated to approach \$7 billion each year in the United States) and, at the same time, causing it to remain unavailable in developing countries (Burns 2012, McDonald 2002).

Current SCI treatment options are limited

Modern treatment of SCI can be divided into two phases; one timed to the immediate/acute phase and the other timed to the post-acute phase. In the acute phase, the treatment aims at prevention of further damage to the spinal cord. This is the reason why trauma patients are immobilized (e.g. by a backboard and rigid collar) as soon as possible (Bernhard 2005). Next, the spine is stabilized either by surgical intervention, a rigid brace, and/or axial traction. In the early 1990's a steroid anti-inflammatory drug, methylprednisolone (MP), was systematically added to the acute phase treatment as it was believed to suppress the deteriorating effects of immune cells near the site of injury and reduce the damage these cells inflict on the spinal cord. The efficacy of MP treatment is,

however, debatable (Hurlbert 2000). The three large randomized controlled trials with MP in SCI patients show only very modest effects, if any, on gain of neurological status (Bracken 1985, Bracken 1993, Bracken 1998). Furthermore, this therapeutic effect does not seem to result in any functional improvement, raising the question whether the risks for adverse effects due to high dose MP should be taken at all (Matsumoto 2001). In the Netherlands, MP is not recommended for the treatment of SCI (Vandertop 1998). Treatment timed to the post-acute phase, is initiated by a rehabilitation team through physical, communicational, and emotional support as well as education. In a follow-up, this multidisciplinary treatment is further extended through additional vocational and recreational therapy, all together providing opportunities for socialization and normal lifestyles. Secondary complications of SCI, such as a variety of pain problems, spasticity, bladder/bowel control function, and autonomic dysreflexia are also intensively addressed in the post-acute phase, but treatments are few and if available often with poor efficacy. For instance, currently available drug treatments for persistent pain and spasticity after SCI have limited therapeutic effect and are often badly tolerated (Teasell 2010). In conclusion, current SCI treatments can, at best, offer a partial prevention of further loss of function after the initial trauma and a limited improvement in secondary complications, but major health problems remain.

SCI pathophysiology

A great deal of SCI research focuses on the question whether normal function of an injured spinal cord can be restored. Unfortunately, the development of SCI regenerative treatments has proven to be extremely difficult. The adult mammalian spinal cord shows an intrinsic poor regenerative capacity. SCI also results in the formation of scar tissue and cavities (cysts/syringes) which are impediments for regeneration of severed axons. Since countless fibers, which project to a wide variety of structures, run intertwined through the spinal cord, reconnecting and retraining of neural circuits is an extremely difficult task (Deumens 2005). A further complicating factor in the design of SCI treatments is the fact that loss of spinal cord tissue and the formation of cysts and scar tissue is not merely the result of the primary injury, but is also due to a series of secondary pathophysiological processes which occur from minutes up to months after injury. These processes are triggered by the hemorrhages and swelling after the vascular damage, which is, together with necrosis and disruption of axons, one of the components of the primary injury. Secondary damage is the consequence of ischemia, lack of nutrients, oxidative stress, excitotoxicity (cell death due to an excessive excitatory drive due to massive release of the neurotransmitter glutamate), electrolyte imbalance of intra- vs. extracellular compartments, and inflammatory reactions regulated by both resident immune cells and leukocytes infiltrating the spinal cord parenchyma via a breach in the blood-brain barrier (Alexander 2009, Hagg 2006). In response to the injury, spinal cord support cells

shield off the injury site by creating an impenetrable barrier that is mostly made up of extracellular matrix molecules like collagen-based proteoglycans intermingled with reactive astrocytes and microglial cells; i.e. the glial scar (Bruce 2000, Fawcett 1999, Gris 2007, Silver 2004, Thuret 2006). The scar inhibits a further expansion of the secondary damage zone and eventually forms the lining of the cavity/-ies that form at the lesion epicenter (Faulkner 2004). Although restriction of further expansion of the lesion is basically an important protective mechanism, the cavities and scars, at the same time represent a mechanical and chemical barrier for axonal growth and, thus, prevent regeneration. Furthermore, while poorly understood, progressive enlargement of the cavity, a condition called (progressive) posttraumatic syringomyelia is known to be a fairly common additional complication (Brodbeck 2003). This condition forms a risk for further deterioration of patient, including the gradual onset/worsening of secondary complications, especially pain (Wang 1996).

SCI pain: what does it entail and how does it work?

Albeit still challenging it is generally clear what is needed for spinal cord repair; not only neuroprotection but also promoting regrowth of severed axons and replacement of lost cells followed by retraining of preserved or newly formed neural circuits. What is needed for treatment of persistent pain after SCI is, unfortunately, much less evident. This starts from a relatively poor understanding of the underlying pathophysiological mechanisms of chronic pain after SCI. Moreover, experimental findings obtained in animal studies are often difficult to translate the clinical arena, partially due limitations in systematically investigating pain in (rodent) animal models. The lack of verbal communication about perceived pain by animals makes analysis of pain even more difficult than in humans. In the experimental pain field the golden standard and most frequently used to test and assess pain in rodents involves measurement of evoked pain-like responses such as paw withdrawal following mechanical or thermal stimulation of the skin (Christensen 1996, Hao 1991). In this respect, below-level SCI pain (pain perceived/projected in areas below the level of injury) is considered to be reflected by reductions in withdrawal thresholds to hindpaw stimulation in animal models of contusion or hemisection of the mid/low thoracic spinal cord (Bennett 2000b, Christensen 1996, Hains 2006). With the use of this technique, many types of SCI models are characterized by reductions in the threshold of hindpaw withdrawal following mechanical or thermal stimulation, indicating the involvement of hyperexcitable neuronal circuits (Hains 2005). Hyperexcitability can be due to several processes including the formation of new dorsal horn circuitries (Christensen 1997), imbalances in inhibitory systems of the spinal cord (Kim 2010), and other mechanisms (Gwak 2011a). From a clinical point of view it is known that a partial preservation of the large ascending spinothalamic tract (located in the ventrolateral spinal white matter) is related to hyperexcitability

and, thus, required for an augmentation of evoked pain (Garcia-Larrea 2002). However, concerns are raised that paw withdrawal responses are not necessarily related to increased pain sensitization after SCI, as such responses often do not coincide with pain behaviors requiring higher brain centers (e.g. expected escape behavior to painful contexts) and even occur in spinalized animals (Baastrup , Yezierski 2010). It has, therefore, been proposed that these responses more closely relate to the spastic syndrome (a collection of symptoms related to exaggerated spinal reflexes induced by CNS lesions). Only a few studies/research groups have yet used the more appropriate escape/avoidance behavior test as a measure of pain in animal models of SCI (Baastrup 2010b, Vierck 1995).

Animal studies have shown that sprouting of nerve fibers contributes to neuronal hyperexcitability, not only in the nociceptive system, but also in systems that relate to motor and autonomic functions, thereby triggering spasticity and autonomic dysreflexia (Brown 2012). In this perspective, the occurrence of inhibitory cues like scar tissue and cysts are not necessarily bad, as they would to some extent prevent neuronal hyperexcitability. On the other hand, nerve growth/regeneration of injured motor axons is desired for regaining motor functionality. It is vital to realize that the structural plasticity in nerve fibers, with the formation of new neuronal connections, creates a risk for hyperexcitability, also in the nociceptive/sensory system (Deumens 2008), and thus a potential increase of secondary complications (see **figure I-1**). With respect to central pain (pain caused by a lesion or disease of the central somatosensory nervous system; definition of the International Association for the Study of Pain; IASP), mechanisms of hyperexcitability seem to be at least involved in the hypersensitivity to stimuli (the evoked pain component). Whether the same concepts of hyperexcitability are also relevant for the spontaneous (non-evoked) pain component of central pain, is less evident. By definition, spontaneous pain is pain due to changes that are internal and self-contained with respect to the somatosensory system (Bennett 2012). Then, spontaneous pain has no (apparent) trigger, shunting a role of hyperexcitability in this phenomenon. Alternatively, spontaneous pain might be related to a yet unknown ‘trigger’ or source. In support of this latter possibility, it was recently reported that SCI patients with an incomplete injury showed signs of neuronal hyperexcitability (heat evoked hyperpathia, dynamic mechanical allodynia), which often preceded the onset of spontaneous pain (Zeilig 2012).

Figure I-1: Abstract drawing of a section of an injured spinal cord, after formation of an injury cavity and glial scar. Shown are severed axons which (endogenously) attempt sprouting/regrowth(+) after injury. However, the latter is inhibited(-), mainly due to the glial scar**. Also shown are the primary afferents~ which are also sprouting in response to the injury and gain increased connectivity to neurons projecting to supraspinal levels and interneurons of the reflex loop*, resulting in hypersensitivity and hyperreflexia#, respectively. In addition, reflex loops (with variable complexity) typically lose inhibitory tone^ when located at/below the injury, causing reflexes to become easily activated, resulting in a variety of hyperreflexia#-related features (i.e. the spastic syndrome).**

Hence, processes of neuronal hyperexcitability, as yet identified in animal models, may also play a role in spontaneous pain, but the source of the spontaneous generator, or actual pain generator, remains to be identified. Some studies claim a role for the spinothalamic tract as residual function and activation of this tract seems to play a role in spontaneous pain even with clinically complete SCIs (Hari 2009, Wasner 2008). Others have emphasized that patients with complete interruption of the spinothalamic tract can also develop spontaneous pain, suggesting that the pain generator (and hyperexcitability) ought to reside present somewhere above the level of injury (Finnerup 2003a, Melzack 1978).

So far, studies investigating differences in characteristics between SCI patients with and without pain remain inconclusive, as studies typically report different findings (Finnerup 2012a). However, these differences in findings might be due to the distinct characteristics of studies, a fact that is also reflected in the high level of heterogeneity between SCI pain prevalence studies (Dijkers 2009). The causes for this heterogeneity might uncover strong determinants for pain after SCI, but are yet poorly investigated.

Advances in SCI treatment: what is needed?

What is clear from the above is that, when considering treatments to promote regrowth and regeneration following SCI, one should at least prevent the further induction of neuronal hyperexcitability which worsens secondary outcomes, like pain and the spastic syndrome. Already some time ago, it was found that injured axons of CNS neurons could grow into peripheral nerve grafts implanted into the injured spinal cord, and it was found that the CNS itself contained inhibitory signals for axonal growth (David 1981). Later, inhibitory signals/molecules present in the injury site and scar were identified and targeted; for the most studied ones being proteoglycans (chondroitin sulfate proteoglycans (McKeon 1995)) and molecules associated with myelin/myelin debris (Nogo, myelin associated glycoprotein, and oligodendrocyte myelin glycoprotein (Fournier 2001, Mueller 2005)). The balance within the CNS favoring inhibition over promotion of neurite outgrowth might be reversed by applying growth/neurotrophic factors to the injury site (e.g. Nerve growth factor (NGF), Glial derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3)) (Fortun 2009, Houweling 1998, Koelsch 2010). Furthermore, cell-based therapies can also provide beneficial effects through an array of mechanisms. For instance, immature astrocytes may be transplanted into the injured spinal cord as these cells are able to produce neurotrophic factors, scavenge excess/excitotoxic neurotransmitters, and limit immune responses after SCI (Krenz 2000, Okada 2006, Rothstein 1996). Treatments based on the above mentioned application of neurotrophic factors, however, carry the risk of worsening secondary outcomes of SCI by unspecific increase in

neuronal plasticity, which may lead to hyperexcitability. Indeed, it has been shown that, at least in animals, treatment with Nerve growth factor or transplantation of (certain types of) astrocytes can lead to increased neuronal excitability (Davies 2008, Gwak 2003, Hofstetter 2005). Besides these risks, the transplantation of immature, neurite-outgrowth promoting astrocytes into the lesion gap of SCI rats has in some cases even been found to fail in stimulating regrowth of severed axons or functional recovery (Deumens 2006b, Joosten 2004). Hence, selection of the appropriate cells for transplantation may be of utmost importance as cell-based therapies are often regarded as one of the most promising treatments for SCI considering that grafted cells can be used to both stimulate regrowth of severed axons *and* replace lost cells.

Olfactory ensheathing cells

One cell-based therapy of particular interest in the field of SCI and repair is the transplantation of olfactory ensheathing cells (OEC). OEC are derived from the olfactory system as these cells reside in the olfactory bulb, olfactory nerve, and even in the olfactory mucosa, supporting the growth of axons all the way from the nasal epithelium into the CNS throughout the life of mammals. The extraordinary neurite outgrowth promoting capacities of these glial-like cells (Deumens 2006c), and the fact that they can be used in an autologous transplantation strategy while requiring minimal pre-grafting *in vitro* manipulations make these cells a prime candidate for SCI repair. Another major characteristic of (transplanted) OEC is their ability to intermingle with reactive astrocytes and migrate through scar tissue, thereby transforming the neurite outgrowth inhibitory scar into a permissive area (Raisman 2007). Moreover, OEC form channel-like structures that serve as a substrate for regrowth of severed central axons through the growth restrictive injury site, which then may result (after remyelination) in functional improvement (Li 1997, Lopez-Vales 2006, Ramon-Cueto 2000). In addition, no signs of neuronal hyperexcitability have been noted below the level of injury after OEC treatment in rodent models (Lopez-Vales 2007, Ramon-Cueto 2000). OEC specifically acquired from the lamina propria (instead of the olfactory bulb), however, have shown to induce autotomy behavior in areas below the level of injury, which suggests the occurrence of pain (Richter 2005). The use of such interventions in human patients is, however, still made difficult as human injuries are often characterized by large cavities which are mainly absent in standard animal models of SCI (Bunge 1993). A further issue is potential adverse effects of OEC transplantation. In an experimental model where OEC were injected into the injury site in rodent SCI models neuronal hyperexcitability has been noted at the level of injury (Bretzner 2010), an effect that might be due to the release of neurotrophic factors by OEC (Woodhall 2001). Unfortunately, OEC transplantation in repair of experimental spinal lesions with large cavities was up till now not very successful. The use of poly(D,L)-lactide matrices carrying aligned OEC on their surface into large spinal lesion cavities

(dorsal hemisection T13-L1) of adult rats has only marginal effects on severed axons (i.e. corticospinal axons were found more closely to the lesion site, but not cross the lesion). This lack of axon regeneration could, however, be related to the characteristics of the scaffold material; the break-down products of poly(D,L)-lactide are acidic which may be detrimental for good integration of the scaffold within the spinal cord lesion; also the matrices used only supported seeding OEC on the matrix surface leaving the grafted cells directly exposed to the toxic environment of the injured spinal cord (Deumens 2006a).

(Human) neural stem cells

Another promising cell-based therapy for repair of the injured spinal cord involves (human) neural stem cells. These cells have the potential to provide cell-replacement for both neurons and glia. However, it has been -and still can be- a main problem that neural stem cells tend to mostly differentiate into astrocytes, which, in some cases, can support neuronal hyperexcitability (Hofstetter 2005, Martino 2006, Setoguchi 2004). Neuronal and oligodendroglia differentiation would certainly lead to better therapeutic effects of neural stem cell treatment (Abematsu 2010, Cummings 2005, Keirstead 2005). Neurons derived from neural stem cells are able to fully integrate into the host circuitry and their oligodendrocytes effectively remyelinate axons (Englund 2002, Keirstead 2005). Human fetal spinal cord derived neural stem cells (HSSC) grown in a monolayer, have shown to give rise to neurons, astrocytes, and oligodendrocytes in a rodent SCI model (Yan 2007). Furthermore, these HSSC were shown to be able to reduce spasticity (and improve locomotor function) after intraspinal grafting in a rodent SCI model of spasticity, which was attributed to their differentiation in inhibitory (γ -aminobutyric acid-ergic (GABA) and glycinergic neurons (Cizkova 2007). Lack of inhibition might be an important mechanism for neuronal hyperexcitability underlying pain and spasticity after SCI (Gwak 2011b). Furthermore, by improving the inhibitory tone in the dorsal horn it was shown that also injury-induced aberrant sprouting of sensory nerves was reduced in a rodent SCI model (Liu 2004). Hence, transplantation of HSSC holds promise to induce functional recovery and reduce/prevent secondary complications after SCI. One concern with the use of stem cells, however, is that they might continue to proliferate and fail to differentiate, thereby forming tumors (Amariglio 2009). Another concern with the assessment of the efficacy of human stem cells in rodent SCI models is graft rejection. Successful translation of cell replacement therapies which utilize xenogeneic or allogeneic cell grafts depends on the design of safe and effective immunosuppression protocols which will permit long-term survival and maturation of grafted cells. Current clinical and experimental immunosuppression protocols typically use single or combined immunosuppressive drug regimen with drugs delivered orally, intraperitoneally, intravenously, or subcutaneously in a single daily dose or divided into multiple daily doses (Barraclough 2011,

MacGregor 1995). While in human clinical settings a targeted plasma concentration of a variety of immunosuppressive drugs can be effectively achieved through drug dose titration, the accomplishment of a comparable consistency in targeted plasma levels in animal studies remains a major challenge. Identifying a consistent slow-release formula, such as liposomes, biodegradable microspheres, or biodegradable pellets, would aid tremendously as an effective, safe and simple method for immunosuppression in laboratory animals.

Validity of functional outcome measures

Furthermore a valuable preclinical assessment of cell-based therapies not only depends on adequate cell survival in laboratory animals, but, as with other therapeutic assessments, also on the validity of functional outcome measures in these experimental animals. Ideally, one would like to use tests which perfectly correlate with the situation in human SCI-patients and are validated in such a way, but serious limitations exist. First, the trauma causing the SCI in humans differs often, if not always, from experimental models as human SCI are highly variable, if not unique, among individual patients whereas animal models basically comprise standardized reproducible lesion types. Second, no pharmacological treatment has yet given consistent results in human SCI patients, despite several positive outcomes reported in experimental SCI-models. It is still remarkable that the few large randomized controlled trials of promising (pharmacological) treatments (judged on positive preclinical studies) all resulted in a negative clinical outcome (Tator 2006). Thus, apart from the inherent difficulty of developing an effective SCI treatment, the common ways of assessing therapy efficacy in SCI models does not optimally correspond with the clinical problem in human SCI patients. As mentioned above, widely used sensory/pain outcome measures in preclinical SCI models are heavily criticized for low translational value towards sensory/pain problems in human patients (Baastrup 2010b, Hofstetter 2003); it is hypothesized that paw withdrawal after SCI correspond better with the spastic syndrome. The spastic syndrome in humans basically consists of positive (i.e. gained, as opposed to lost) motor signs of the upper motor neuron syndrome, such as spasticity (the occurrence of velocity dependent reflexive increase in muscle tone upon passive muscle stretch), hyperreflexia (exaggeration of normal reflexes), spasms (involuntary muscle contractions, often due to some kind of stimulation), and clonus (automatic repetitions of reflex responses); negative signs are decreases in muscle tone, weakness, loss of voluntary control). The spastic syndrome, however, is typically deemed less prevalent in rodent models of SCI than in human SCI (Bennett 1999), although it is possible that the spastic syndrome is merely less evident in rodents instead of less prevalent (Baastrup 2010b, Bennett 1999). In human SCI patients, the spastic syndrome occurs when negative signs of the upper motor neuron syndrome are obvious; i.e. walking becomes a problem, however these 'spastic syndrome-related' paw withdrawal responses are seen in adequately walking

rats (Christensen 1996). This difference between species could be a reason why symptoms of the spastic syndrome are overlooked in rodents, as the overall clinical image is very different (walking rats vs. paraplegic humans). It would, thus, be interesting to know whether the ‘paw withdrawals spasms’ also occur in the acute phase after SCI in experimental SCI pain models. During this phase motor function is still very poor and any motor activity would indeed be indicative for the onset of spasm-like reflexes. This could then be further evidenced by the use of animals with severe SCI, which are known to recover minimally and show poor voluntary movement, even during the post-acute phase. This approach may allow studying if such spasm-like reflex behavior can remain for longer times after injury. As spasticity (one sign of the spastic syndrome) is defined as ‘a velocity-dependent increase to tonic muscle stretch’, this aspect of the spastic syndrome might, next to ‘paw withdrawal spasms’, simply not have been adequately assessed yet in the experimental SCI-field (Baastrup 2010b). In this context, one might expect a decrease in escape behavior to stimuli causing paw withdrawals after SCI in more severely injured animals when compared to uninjured animals.

It is unmistakably clear that major interspecies differences exist with respect to the spastic syndrome and its interpretations as a discrepancy between positive and negative signs of the upper motor neuron syndrome seem obvious. Indeed, the spastic syndrome goes unnoticed in most animal models of SCI, while in the clinic the term ‘spastic syndrome’ is often used almost synonymously for the upper motor neuron syndrome (Ivanhoe 2004). The difference in extent of negative motor signs after SCI might be due to a high spinal autonomous motor function in animals, when compared to humans. Spinalized laboratory quadrupeds (like rodents) are renowned for their ability to show impressive walking patterns, as long as their weight is supported for most of the time (Grillner 1973). However, primates are likely to have lost this spinal autonomy of hindlimb locomotion (Eidelberg 1981). This poses a further problem on widely used functional motor outcome assessments in SCI models (mostly rodent models), as the sensitivity of such tests to detect reconnection of supraspinal structures with spinal targets may be low. Indeed, only relatively severe injuries achieve some chronic locomotion deficits, suggesting regenerative effects can be easily missed using such functional tests (Basso 1995, Basso 1996). Furthermore, although spinal autonomous locomotion patterns are important for rodent/quadruped SCI locomotion recovery, it is believed that primates depend more on spinal cord regeneration (Rosenzweig 2010). A serious re-evaluation of currently used functional tests is required and development of new methods to test behavioral recovery of sensory as well as motor outcome after SCI are urgently needed.

Objectives of research in this thesis are:

- 1) Explore causes for the heterogeneity in prevalence rates, and find determinants for pain after SCI,
- 2) Systematically explore tools (sensory and motor assays, and immunosuppression protocols) for the preclinical and translational evaluation of SCI treatments, and
- 3) Test the relevance and feasibility of candidate cell-based therapies (OEC and HSSC) for SCI treatment in preclinical models.

Outline and research questions of this thesis

It is evident that SCI represents a multifaceted clinical challenge. Although improvements in health care have given SCI patients a good life expectancy and autonomic position in society, major health problems and complications remain. Chronic, poorly treatable pain still is one of the most severe among the various complications, and thus of high relevance when designing SCI treatments. Unfortunately, pain after SCI is poorly understood as predictors or mechanisms for pain syndromes after SCI remain obscure. Reports suggesting an association between patient characteristics (e.g. level or cause of injury) and the onset of pain after SCI vary, and thus remain inconclusive. On the other hand, the reported pain prevalence after SCI also varies tremendously, suggesting important differences between studies. Dissimilarities in study characteristics/methodology (and patient populations) can cause differences in the relationships found between patient characteristics and the occurrence of pain. To know which between-study differences result in differences in pain prevalence can further aid in the identification of those patient characteristics which relate most to pain after SCI, and might help to isolate the mechanisms of pain after SCI. Therefore, the first research question of this thesis is:

1. Is it possible to identify which study and/or population characteristics show a relationship with the differences in reported pain prevalence rates in SCI patient populations?

Chapter 2 explores which differences between studies are causing dissimilar pain prevalence outcomes. A systematic review of studies reporting pain prevalence in populations with SCI is performed and study and (sub-) population characteristics are compared using meta-analytic methods.

Development of SCI therapies requires preclinical research, which in turn relies largely on animal models, mostly rodents. Compared to *in vitro* models, animal models offer the advantage of

assessing functional benefits of the tested therapy through behavioral tests. However, differences between animals and humans might be highly relevant when assessing SCI outcomes. The high biomechanical stability of a quadruple gait, compared to human bipedal walking, allows more locomotion control to be executed through spinal reflexes. These spinal reflexes are then difficult to distinguish from actual recovery of SCI (i.e. restoration of circuitry with supraspinal structures), destabilizing the value of animal models for SCI outcomes. This is not only relevant for locomotion assessments after SCI, but also for pain tests. The cornerstone of pain assessments in animal models relies on evoked pain-like responses. Therefore, the second research question of this thesis is:

2. Can we differentiate between spinal reflex mediated functionality and supraspinally mediated functionality in rodent SCI models?

In **Chapter 3** we explore the clinical relevance of paw withdrawal responses as observed in a widely used rat model of SCI and SCI pain: a thoracic spinal cord contusion injury, which is inflicted by a standardized weight-drop (Gruner 1992, Hains 2006). Furthermore, in **Chapter 4** we test which behavioral assay reflects recovery of supraspinally mediated locomotion functionality. However, due to the unavailability of a proven effective SCI treatment for locomotion recovery in humans (a positive control), in **Chapter 4** we investigate which behavioral test is most sensitive for locomotion changes related to different SCI severities.

Due to their regeneration promoting properties, some cell-based therapies like Olfactory Ensheathing Cells (OEC) and human Fetal Spinal Neural Stem Cells (HSSC) form a promising treatment for SCI. Olfactory Ensheathing Cells (OEC) and Human Fetal Spinal Neural Stem Cells (HSSC) are particularly interesting. OEC naturally promote axonal growth into the CNS (Ramon-Cueto 1998). Moreover, they can be used for autologous grafting, circumventing the need for immunosuppression (Munoz-Quiles 2009). These properties are useful, but SCI in humans generally results in the formation of cavities (syringes) in the spinal cord which also requires structural bridging (Bunge 1993). This problem might be overcome by the use of a multichannel, biomatrix scaffold which aligns with the severed projection axons of the host spinal cord to transfer OEC to the injured spinal cord. On the other hand, HSSC can give rise to new neurons and glia cells, and provide growth-promoting factors, providing both support and a new supply of neurons to the injured spinal cord.

3. Does transplantation of OEC or HSSC into the lesion site provide functional improvement in an experimental SCI model?

In **Chapter 5** we investigate whether OEC seeded into a multichannel, collagen scaffold can facilitate axonal regrowth and functional recovery across an cavity created at the thoracic level of the adult rat spinal cord (Christensen 1996). In this model, the cavity is created by unilateral removal of a 2mm long section of the spinal cord. In **Chapter 6** we inject HSSC into and around a spinal cavity formed after traumatic spinal cord compression. This model uses a 15 minute compression (35grams) of a lumbar segment (L3), just above the lumbar segments innervating the hindpaw/sciadic nerve. In both OEC and HSSC transplantation approaches we explore the therapeutic effects on motor function, sensory thresholds, and/or evoked paw withdrawal thresholds (below the level of injury).

Although HSSC can both provide support as well as replenish cells to the injured spinal cord, they pose a risk as they are not fully differentiated and can continue to proliferate, and therefore might form tumors in the host spinal cord (Amariglio 2009). Moreover, autologous transplantation of fetal HSSC is not possible, necessitating continuous immunosuppression of the host, especially after xenotransplantation (in laboratory animals). Therefore, the last research question of this thesis is:

4. Is long-term HSSC engraftment safe for clinical use and achievable with the use of slow-release immunosuppressive drug formulas?

Therefore, we assessed the safety of HSSC-therapy for intraspinal grafting, principally regarding their application in future clinical trials. Specific guidelines for such safety assessments of cell-based therapies are lacking. In **Chapter 7** we report on the results and the considerations of a HSSC safety study. Its design was the result of a dialog with the Food and Drug Authority (FDA; the national drug authority in the United States) and its outcome has resulted in the approval of a first clinical trial with these fetal HSSC. **Chapter 8** describes an adequate regime for long-term immunosuppression when grafting HSSC in immunocompetent rats without the need for laborious immunosuppression protocols. The latter is based on the performance of different slow-release formulas in comparison to the regularly used (twice) daily injections of immunosuppressive drugs. Both pharmacokinetics and cell-survival are used as main outcome measures to assess the quality of the immunosuppression.

II. Exploring the causes of the large variety in reported pain prevalences in spinal cord injury patient populations

Pain prevalences and their large heterogeneity after spinal cord injury: a systematic review

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Abstract

Pain prevalence studies are important as they illustrate the magnitude of pain problems in a certain patient population, such as patients living with a spinal cord injury (SCI). Strikingly, reported pain prevalence rates in SCI patients are found to vary greatly, while determinants for the differences between pain prevalence reports remain unclear. We here aim to identify determinants for the differences (heterogeneity) in pain prevalence reports through a systematic review of all SCI pain prevalence reporting studies. Literature search using MEDLINE, Cumulative Index to Nursing and Allied Health Literature (CINAHL), ISI Web of Knowledge, and Embase. Data abstraction was performed while blinded and was followed by meta-(regression)-analyses. We identified 82 studies. Study design related determinants of SCI pain prevalence reports were: pain definition strictness (mild, moderate, or high), primary study goal (pain study or not), data source (retrospective or not), and, in a limited number of cases, response/attrition rates. While correcting for latter items, populated characteristics found to determine pain prevalence rates were both proportion of patients with a depression and average time after injury (positive correlations). Between-study heterogeneity may remain even after the identification/correction of abovementioned causes of heterogeneity. Pain after SCI seems to relate to the duration of the injury and depression, yet, major causes of bias in reported pain prevalence are found to be related to the primary study goal (pain study or not), choice of pain definition, and the use of retrospective data.

Introduction

Treatment of Spinal Cord Injury (SCI) related pain is still a major problem (Finnerup 2012a, Haisma 2007, Teasell 2010). The reported prevalences of pain after SCI, however, vary considerably, with rates reported from 19 to as much as 96% (Dijkers 2009, Finnerup 2001, New 2002, Raissi 2007, Siddall 2003). In a recent, and the only published, systematic review of SCI pain prevalence studies, it was concluded that a definite statement about a pain prevalence could not be made, due to this heterogeneity of the study population (Dijkers 2009). Identifying which factors cause this heterogeneity could reveal important information, as their identification supports their role as relevant pain determinants and/or study confounders. Unfortunately, the authors of the review reported to be unable to identify such factors, yet, this was not the primary aim of their study. By using a more extensive and somewhat different approach, it might have been possible to identify factors causing the heterogeneity in reported SCI pain prevalences.

First, less stringent exclusion criteria could have been adopted. For example, studies were excluded on the basis of reporting on an unsure, or too large (>25%), portion of non-traumatic SCI. However, pain prevalence has not yet been consistently reported to differ due to SCI etiology and the influence of such factors can thus better be analyzed for their impact on heterogeneity if studies with more variation in those factors are not excluded (Ibrahim 2012, Mariano 1992, McKinley 2002b). Similarly, studies not explicitly reporting *chronic* pain problems were excluded. However, pain occurring in SCI patients is typically of a persistent nature and, regardless, the definition of 'chronic' remains ambiguous (Cruz-Almeida 2005, Purves 1998). And, again, it might have been possible to (partly) resolve the heterogeneity by analyzing the effect of the variation in pain definition strictness/sensitivity on SCI pain prevalence. Having more studies included in a systematic review might then also result in a more robust analysis of any other factors (Huedo-Medina 2006, Thompson 1994).

Secondly, quality assessment of the prevalence studies can be used by analyzing the impact of individual quality items, instead of an overall total quality scores (Stroup 2000). In this respect, it is especially important to realize that it is still uncertain which study quality items are actually relevant with respect to significantly differing (SCI) pain prevalence rates.

Therefore, in this systematic review, we wanted to further investigate which study-design or study-population characteristic(s) might have an impact on the heterogeneity of SCI pain prevalence by including previously excluded and newly published studies, and performing a detailed meta-regression analysis.

Methods

A systematic literature review was conducted on studies reporting on SCI pain prevalence (i.e. descriptive studies and observational studies). As no protocols are available for performing systematic reviews on prevalence studies specifically, we used protocols for systematic reviews of studies on treatment or exposure effects, when applicable (Dickersin 2002, 2008, Stroup 2000). The following databases were used: MEDLINE, Cumulative Index to Nursing and Allied Health Literature (CINAHL), ISI Web of Knowledge, and Embase. The search was wide and the queries comprised of the terms 'pain', 'dysaesthesia', or 'dysesthesia', and 'spinal cord injury/-ies', 'spinal cord disease/-s', or 'tetra-/quadri- or paraplegia'. The abstract and/or publication title were used to identify articles which might fit the topic. Next, the full document was requested, translated to English, and screened whether these met our inclusion criteria or any exclusion criteria. Also, the reference lists from all screened articles were hand searched for any old and/or missed articles. The major clinical research groups on SCI pain were contacted for any missing publications. Last, the major journals were hand searched (ie. Pain, Spinal Cord/Paraplegia, the Journal of Spinal Cord Medicine, the American Journal of Physical Medicine & Rehabilitation, and the Journal of rehabilitation research and development/United States Veterans Affairs).

Inclusion criteria

Studies were included if they reported on the prevalence of pain after SCI, irrespective of the type of prevalence used (i.e. at a single time point or over a certain amount of time). In one case, only an abstract was available and the data from the abstract was used (Rintala 1991).

Exclusion criteria

If more than 15% of the included patients had missing data on the occurrence of pain, the study was excluded (e.g. as in a study by Kakulas and co-workers (Kakulas 1990)). In addition, a study was excluded in any of the following conditions:

- only a subtype of SCI pain was investigated (such as neuropathic pain)
- no demographic data was available on the patients without pain
- the study population was less than fifteen subjects

Data collection method

For data abstraction, articles were blinded for journal, authors, involved institutions, dates, countries, and original language. Any references to these items were removed or replaced with dummy items to preserve story integrity. This was done to prevent subjective interpretations and predispositions becoming a source of bias. Blinding was performed in a digital document, if necessary aided by optical character recognition software (ABBYY FineReader 10). This resulted in identically formatted documents, including intact tables and graphics if those provided study results relevant to this review.

Data items

In addition to the reported pain prevalence, the following population and study characteristics were abstracted, when available (**Table II-1**). Studies which separately report on pain prevalence rates for population subgroups of patients who all either have or don't have one of the population characteristics we abstracted (e.g. a complete injury) are also split into 'sub-studies', based on these population subgroups (e.g. one sub-study only reflects the pain prevalence of the part of the study population with a complete injury). These sub-studies are used for separate meta-regression analyses of the study population characteristics. These sub-studies are replaced for their main study when their specific characteristic (e.g. prevalence of complete injury) is added to the meta-regression analysis. This way, the results are more likely to display the actual relation of this characteristic with the pain prevalence instead of a random relation.

Subgroup formation by pain definitions used

Because of the potential effect of between-study differences in used pain definitions on the heterogeneity of reported pain prevalence rates, we classified studies in subgroups based on the restrictedness of their used pain definitions. Hence, pain definitions subgroups were made on the basis of the estimated probability that SCI patients with (mostly minor) pain problems fell below the pain detection threshold. To this end, the pain definitions used per study were abstracted and presented to six pain researchers independently. The researchers were blinded to all other data from the studies (including the reported pain prevalence), and individually allocated pain definitions in three groups of increasing restrictedness. Specifically, they were asked to assign 0, 1, or 2 points for the strictness of the pain definition, indicating:

- 0: Mild strictness (Hardly excluding any pain cases)
- 1: Moderate strictness (Excluding some pain cases)
- 2: High strictness (Excluding many pain cases)

Final scores were based on consensus when unanimity was not met.

Population characteristics	Study characteristics
Proportion of patients with:	Pain assessment tool
Pain interference	Pain definition
Pain catastrophizing	Pain recall periods
Complete SCI	Primary study goal (Pain study: yes/no)
Tetraplegia	Sample size
Traumatic SCI	Attrition/Response rate
Married/living together	Sample selection method
Professional work	Population source (SCI center: yes/no)
Depression	Study method
Gender ratio	Study method uniformity
Age at study	Retrospective data
Age at injury	In- and exclusion criteria
Time since injury	Publication year
Region studied:	Study duration
Europe, Canada, and Australia	Description non-responders
vs.	Pain classification/taxonomy used;
the USA	IASP (Siddall 2000), Bryce/Ragnarsson (Bryce 2006), or Cardenas (Cardenas 2002)
	Reporting of: (each item: yes/no)
	Neuropathic or nociceptive pain prevalences
	Anatomical pain locations
	Pain descriptors

Table II-1: Data collected from included studies

Study quality

From the abstracted data, a quality score for each included study was calculated (maximal score: 18). The quality criteria used were based on those recommended by Leboeuf-Yde and Lauritsen (Leboeuf-Yde 1995), and Walker (Walker 2000). As the criteria were originally designed for studies on low-back pain, the descriptions were modified to fit the current study on SCI pain (**Table II-2**).

A. Representation of the target population	<p>1. The study must at least entail the entire source population, a random selected sample or a sample which represents the target population. (2 pts.)</p> <p>2. Either the reasons for nonresponses (to study invitation), the non-responders, a comparison of responders and non-responders, or the sample and a representative population are described. (2 pts.)</p> <p>3. Response rate $\geq 90\%$ (2 pts.) or between 70% and 90%. (1 pt.)</p>
B. Quality of the data	<p>4. Were the data from a pain prevalence study (2 pts.) or from a study not specifically designed for that purpose. (1 pt.)</p> <p>5. Data were collected on an identical fashion for all subjects. (2 pts.) Otherwise (1 pt.)</p> <p>6. Data were collected by means of a validated (3 pts.), or non-validated questionnaire/interview (2 pts.), or retrospectively by proxies or medical records. (1 pt.)</p>
C. Study method/results description	<p>7. A description was given of the target population and the setting the sample was drawn from. (2 pts.)</p> <p>8. Description of the injury (etiology, level, completeness, and timing), and patient (age, and gender). (>3 items = 1pt.)</p> <p>9. Final sample size stated. (1 pt.)</p> <p>10. Prevalence recall period should be stated. (1 pt.)</p>

Table II-2: Used quality items/criteria (adapted from Leboeuf-Yde and Lauritsen (Leboeuf-Yde 1995), and Walker (Walker 2000))

Besides analyzing the effects of between-study differences in total quality scores and scores of individual quality items (**Table II-2**) on heterogeneity, non-stratified continuous data were also analyzed when applicable (e.g. for response rates, population size; see Table 1). Moreover, in a similar way we analyzed the influence of some additional dichotomous and continuous variables possibly reflecting the study quality. These variables were: a difference found in population characteristics between participants and non-participants, study had a cohort design (yes/no), year of publication, and items indicating an extensive pain investigation: 1) the use of the IASP/Siddal, Bryce/Ragnarsson, or Cardenas SCI pain classification, 2) the report of neuropathic pain prevalence, 3) the report of musculoskeletal pain prevalence, 4) anatomical pain locations, and 5) pain descriptors.

Statistical procedures

For each study, the standard error of the prevalence rates was calculated, using the standard formula for the standard error of a proportion and the mean of the reported prevalence rates (Chang 2001). Meta-regression analyses were then used to explore sources of bias and heterogeneity. More specifically, possible reasons for (remaining) heterogeneity (I^2) were investigated using a random effects model and assessing linear correlations with reported pain

prevalence of the overall study quality and the individual study quality items, general study characteristics, and patient/population characteristics. All individual study characteristics and population characteristic were also plotted against the reported pain prevalences in order to identify possible outliers.

For comparisons of means, one way analysis of variance (ANOVA) was used (with Bonferroni post hoc test), or a *t*-test in cases where only two levels of a factor were present. Weighted means were calculated using the size of the study populations and are inversely proportional to the variance of an observation/prevalence (analytic weights). Variations of the mean are given as standard deviations, unless stated otherwise. Statistics were performed using STATA 12 (StataCorp LP, College Station, TX, USA).

Results

Based on our keywords, the electronic search resulted in 5146 articles which were screened for original SCI pain prevalence information or references. This resulted in a total of 247 articles. An additional 73 articles were found through searching the references, hand searching and contact with research groups. From the total of 320 articles, 233 articles were excluded, leaving 87 articles for analysis. Reasons for exclusion were: no pain prevalence reported, no original study conducted; only pain patients studied; a too small study population; a subgroup of pain types investigated; overview or case study article.

Figure II-1: Flow diagram summarizing systematic search and study inclusion

Individual study quality criteria

Two individual quality criteria correlated with reported pain prevalence rates, if corrected for pain definition strictness (i.e. pain definition strictness was added to the meta-regression model as a covariable; results; **Table II-3**).

First, the quality criterion ‘method used to acquire the pain prevalence (validated, non-validated questionnaires, or retrospective sources)’ was correlated with reported prevalence numbers (coef=11%, $P=0.002$). This correlation is attributed to significantly lower

prevalence rates reported by studies using retrospective data (vs. other data sources; coef=-26%, $P<0.001$, $I^2=95.6\%$, $R^2_{adj}=31\%$).

Second, the quality criterion ‘whether or not the study’s primary goal was to report pain prevalence’ was positively correlated with reported pain prevalence rates (coef=18%, $P<0.001$, $I^2=94.9\%$, $R^2_{adj}=36\%$). Hence, studies with a primary goal to report pain prevalence showed a higher number of patients suffering from pain. Moreover, this correlation was also present in all individual pain definition subgroups. From the pain definition strictness subgroup with hardly any cut-off to the ones with more strict definitions the correlation coefficients were 16% ($P=0.009$), 17% ($P=0.005$), and 18% ($P=0.016$), respectively (**Fig. II-4**). Also, the additional items ‘whether or not a study reported on neuropathic pain prevalence’ and ‘whether or not a study reported on neuropathic pain prevalence’ were found positively correlated with reported pain prevalence rates. However, the studies reporting on neuropathic and/or musculoskeletal pain prevalences are probably mostly the same studies as those with a primary study goal to report on pain prevalences. Therefore, we added the quality criterion ‘whether or not the study’s primary goal was to report pain prevalence’ as covariable to the regression model and ran the meta-regression analysis again for the variables ‘reported neuropathic pain prevalence’ and/or ‘reported musculoskeletal pain prevalence’. Neither of the latter two variables then remained correlated with pain prevalence, while the quality criterion

Figure II-3: Distribution of the overall/total study quality scores versus the reported pain prevalences. The different pain definition subgroups are grouped

‘whether or not the study’s primary goal was to report pain prevalence’ remained significantly correlated with the reported pain prevalences (coef=17%, $P<0.001$).

Correlation coefficient (%) w/ Pain Prevalence Covariable: Pain definition strictness (Mild, Moderate, or High) Coefficient $\leq -8.7\%$; $P\leq 0.003$		
Study quality items:	Coefficient (%)	I^2 (% residual variation due to heterogeneity)
None		96.2
1. Representative study population	2.8	96.1
2. Non-responders described	-0.1	96.2
3. Attrition / Response rate (continuous values) <90% vs $\geq 90\%$	-2.6 (-0.1 [†]) -3.6	96.2 (96.0 [†]) 96.2
4. Primary study goal is pain prevalence	17.6***	94.9
5. Uniform data collection	4.1	96.2
6. Data acquisition	11.1**	96.0
Other vs retrospective	-26.0***	95.6
Other vs non-validated questionnaire	7.1	96.2
Other vs validated questionnaire	5.4	96.2
7. Description of population and its source	7.9	96.2
8. More detailed description of injury	8.0	96.2
9. Final sample size stated	N/A [‡]	
10. Pain recall period stated	4.5	96.2
Additional study characteristics:		
Survey study (no vs. yes)	7.3	96.2
Year of publication	0.3*	96.1
SCI pain classification system used (no vs. yes)	11.9	96.2
Cohort study (no vs. yes)	9.2	96.1
Patients from SCI center (no vs. yes)	-3.4	96.2
Detailed pain info given (no vs. yes)		
Neuropathic pain prevalence	11.5 ^(**) [‡]	95.8
Musculoskeletal pain prevalence	12.6 ^(*) [‡]	96.0
Anatomical locations	8.2	96.1
Pain descriptors	9.8	96.2

Table II-3: Meta-regression results of pain prevalence rates and individual study quality items

[†] Studies not reporting attrition rates excluded (n=61 remaining),

[‡] All studies stated final sample size,

[‡] Correlation lost significance when “quality item 4: Primary study goal is pain prevalence” was added as a covariable to the model. * $P<0.05$, ** $P<0.01$.

The additional study characteristic ‘year of publication’ also correlated with the reported pain prevalence rates (coef=0.3%, $P=0.025$). However, after exclusion of the five studies published before 1960 (weighted prevalence: $32.6\pm SD21\%$; study quality: $8.0\pm SD1.4$), the relation between ‘year of publication’ and reported pain prevalence was lost (coef=0.0%, $P=0.928$).

Hence, studies with retrospectively acquired data and studies from before 1960 were excluded from further analyses ($n=10$ excluded). In addition, when observing the graph in which the reported prevalence rates are plotted against the response/attrition rate (Fig. II-5), three studies raised doubts on whether or not they should be interpreted as outliers (marked

Figure II-4: The relation between de primary study goal (pain study or not) and the pain prevalence reported. Retrospective and old studies (<1960) excluded and shown as interrupted circles

Figure II-5: The relation between de response rate and the pain prevalence reported. Note that the three studies with the highest prevalence rates (marked with the dotted box) are also studies which either reported a 100% response rate (mostly due to unlocatable potential participants), no response rates, and contain only small study populations. Retrospective and studies from before 1960 excluded

with interrupted/dotted rectangle). These studies were small, were reporting either no response rates or (in one case) a 100% response rate, and reported the highest prevalence rates of all remaining studies. Therefore, we decided to exclude them for further analyses ($n=3$ excluded, $n=69$ remaining). In the excluded study with a 100% response rate all 54 eligible patients who the investigators were able to get in contact with had been included, other potential study participants were not locatable (Raissi 2007).

Then, after exclusion of old (i.e. published before 1960) and retrospective studies, and correcting for the covariates '*primary study goal (pain study or not)*' and '*pain definition strictness*' ($I^2=93.1\%$, $R^2_{adj}=45\%$), both the item '*whether or not there was a representative study population*' as well as '*whether or not a study was a survey study*' correlated with the reported pain prevalences (coef=5.2%, $P=0.003$ & coef=7.6%, $P=0.011$, respectively). This remained true when both items were simultaneously loaded in the meta-regression model (coef=4.5%, $P=0.008$ & coef=6.1%, $P=0.035$, respectively). However, both or either of the latter items lost their correlation with the pain prevalence rates when adding any of the population characteristics (**Table II-1**) to the regression model, indicating a weak correlation, which made us decide to exclude them from the meta-regression model.

Next, when loading the total quality scores of individual studies to the last-mentioned meta-regression model showed that total quality scores no longer correlated with pain prevalence rates (coef=0.9%, $P=0.18$). Moreover, this was also true when the original total quality score was recalculated by subtracting the quality points assigned for the quality item '*primary study goal*' (coef=0.7%, $P=0.21$).

Possible determinants for SCI pain

With the adjustments described above (i.e. inclusion of the four covariates '*pain definition strictness*' and '*primary study goal*' in the meta-regression model, and exclusion of retrospective studies, studies from before 1960, and the three outliers on the response-rate plot) meta-regression analyses were performed in an attempt to uncover possible determinants of the reported pain prevalence by loading individual study population characteristics in to the model. The population characteristics showing a significant correlation with the reported pain prevalence were '*Time after injury*' (coef=0.9%, $P=0.03$, $I^2=95.1\%$, $R^2_{adj}=49\%$, $n=34$) and '*Depression prevalence*' (coef=0.6%, $P=0.003$, $I^2=69.7\%$, $R^2_{adj}=81\%$, $n=13$; **Table II-4**).

Then, remaining study quality items, study characteristics, or population characteristics are individually loaded in any of the two meta-regression models of the significantly correlated population characteristics, no additional correlations were identified. This was also true for the study characteristics '*representative study population*' and '*whether or not a study was a survey study*', which were earlier found to correlate with pain prevalence rates (see section above). We attempted to combine '*Depression prevalence*' and '*Time after injury*' in the meta-regression model, but due to insufficient studies reporting on both population characteristics, this was not possible.

Study Population characteristic (unit)	(sub-)studies reporting Characteristic (n)	Coefficient of study characteristic	Correlations with reported pain prevalences (%)		
			Covariates		
			Pain definition strictness (Mild, Moderate, or High)	Primary study goal (Other- vs. Pain-study)	r^2 (% residual variation due to heterogeneity)
None	69		-10***	16***	93.1
Proportion of males (%)	61	0.1	-11***	16***	92.9
	70‡	-0.0	-11***	14***	91.2
	19‡‡	-0.1	-13**	#	70.5
Average age at study inclusion (yr)	47	0.3	-10***	16***	93.8
Average age at SCI (yr)	27	-0.0	-8.0*	17**	95.6
Average time since SCI (yr)	34	0.9*	-9.4**	19***	95.1
Proportion of patients with tetraplegia (%)	62	0.0	-10***	17***	91.2
	77‡	-0.0	-9.6***	15***	93.9
	29‡‡	-0.1	#	#	91.3
Proportion of patients with complete injury (%)	42	-0.2	-11***	16***	92.1
	52‡	-0.0	-11***	16***	90.2
	20‡‡	-0.0	-8.2	#	78.5
Proportion of patients working (%)	23	0.0	-13***	15**	93.3
	25‡	0.0	-14***	16**	94.5
	# ‡‡				
Proportion of patients with traumatic SCI etiology (%)	47	-0.1	-8.7***	17***	93.5
	49‡	-0.1	-8.9***	16***	93.1
	#‡‡				
Proportion of patients with depression (%)	13	0.6**	-5.1†	23**	69.7
Retrospective study New et al 2002 re-included:	14	0.7**	-1.9	25**	79.5
Region studied: (dichotomous) EU, Can, & Australia vs. USA	62	2.8	-10***	16***	92.7

Table II-4: Meta-regression analyses of pain prevalence rates and individual study population characteristics

Retrospective studies, studies from before 1960, and response/attrition rate outliers are excluded.

‡ Studies which separately report on pain prevalence rates for population subgroups of patients who all either have or don't have the analyzed characteristic (e.g. a complete injury) are split into 'sub-studies', based on these population subgroups (e.g. one sub-study only reflects the pain prevalence of the part of the study population with a complete injury)

‡‡ Sub-studies only

* P<0.05, ** P<0.01, *** P<0.001, † P<0.10

Removed from model; most substudies fall into only one of the categories of the covariable.

After removal of ‘*pain definition strictness*’ and ‘*primary study goal*’ from the meta-regression model, ‘*Depression prevalence*’ remained significantly correlated with the reported pain prevalences (coef=0.7%, $P=0.03$, $I^2=86.5\%$, $R^2_{adj}=37\%$), which was not true for ‘*Time after injury*’ (coef=0.7%, $P=0.15$, $I^2=96.6\%$, $R^2_{adj}=3\%$). Last, after including the three previously excluded studies (based on being outliers on the response-rate plot; **Fig. II-5**) ‘*Time after injury*’ no longer correlated with the reported pain prevalence rates (coef=0.6%, $P=0.08$). Otherwise, no major differences to the values reported in **Table II-4** were noted.

Discussion

This is the first meta-analysis which systematically explored causes of the large heterogeneity in reported SCI pain prevalences. Foremost, we found that (1) pain definition strictness (stratified as mild, moderate, or high) showed a strong negative correlation with reported SCI pain prevalences (i.e. more strict definitions were associated with lower prevalence), and (2) ‘*whether or not a study was primarily a pain study*’ had a strong positive correlation with pain prevalence rates.

Furthermore, a positive correlation between pain prevalence and depression rates was found although this should be interpreted with some care, as not all of the included studies reported on depression prevalence rates ($n=13$). Only rarely official criteria for depression are used, and most studies use an arbitrary cut-off score for the amount and/or severity of depressive symptoms. Much has been written about the well-established relationship between (chronic) pain and depression (Bair 2003, Henningsen 2006, Jann 2007). A bi-directional relationship, where depression increases the risk for higher pain levels and vice-versa, seems to be present for patients suffering from a disability (Gayman 2011, Saunders 2012), although others dispute the notion that there is any relation between depression and pain in SCI patients (Hassanpour 2012). Yet, the positive correlation found between SCI pain prevalence and depression prevalence is in line with a recently published five year longitudinal study performed in SCI patients, where depression onset was related to an increase in pain (Hoffman 2011).

Time after injury also came forward as a putative SCI pain determinant in this meta-analysis, with higher pain prevalences reported in studies performed longer after the onset of the SCI. Not enough studies reported on both time after injury and depression to see if there was a correlation between these variables, to exclude co-linearity. However, average depression prevalence over the first five years after SCI was reported to change very little, suggesting little overlap between depression and time after injury in the increase in pain (Hoffman 2011). In a prospective study, pain prevalence was indeed shown to increase in time, when compared between six months and five years (Siddall 2003). This effect is mostly due to the onset of pain shortly after the six months measurement as

differences are less evident when comparing prevalence rates at two years post injury with those at six years (Jensen 2005). It is thus interesting that it was shown that, although depression and pain were independent in the acute phase after SCI (rehabilitation admission; week 2-3 post injury), a stronger relationship developed over time (Cairns 1996).

Differences in pain definitions are a clear source of heterogeneity between pain prevalence reports. It is thus unfortunate that it is impossible to predict more precisely how each difference in definition (recall period, duration, choice of words etc.) influences the reported prevalence. For meta-analyses of prevalence studies the question remains to what extent the exclusion of studies with a somewhat differing definition weighs up to the subsequent loss of statistical power (smaller size) of meta-analyses. Nonetheless, one needs to keep in mind that classification of pain definitions does not circumvent problems arising from the lack of reporting on the complete pain definitions with all its attributes as used in a study (e.g. recall period, frequency, duration, intensity, etc). Moreover, it is not always sure if reported attributes were consistently applied throughout the study.

Quality of studies to be included in systematic reviews is a major concern, but quality is not easily defined (Schulz 1995). It is doubtful that elimination of studies with below-average 'overall quality scores' prevents formulation of wrong conclusions (Moher 1998). Quality scores can be misleading, especially when designed in an ad-hoc manner (Emerson 1990, Greenland 1994). This is especially true for meta-analyses on pain prevalence studies, because no research is done on the influence of individual quality items. Strikingly, we found that higher quality studies failed to show increasingly consistent prevalence rates. In any case, it seems more important to analyze the impact of individual quality items instead of the overall quality score (Friedenreich 1994, Hasselblad 1992, Juni 1999).

In our study, only two quality items out of ten (i.e. primary study goal and method of data acquisition; retrospective or not) showed to be of main importance on the variance between reported SCI pain prevalence. First, whether or not the primary study goal was to study pain was positively correlated with reported SCI pain prevalence rates. This could mean that SCI pain prevalence studies tend to overestimate pain prevalence rates, for example, because patients without pain are less interested to participate in pain studies (Locker 1988, Rupp 2002, Schmidt 2011). Another explanation could be that the difference is a sign of publication bias (Dwan 2008). Second, retrospective studies tended to underreport pain prevalence rates. A not very surprising finding, since pain cases in retrospective studies are not actively sought and remain undetected.

The reported prevalences of complete injuries showed no trend for a relation with pain prevalence rates. Both clinical and preclinical data support the concept that specific damage and preservation of

spinal cord structures are of particular relevance to (especially below-level) SCI pain (Finnerup 2003a, Finnerup 2007, Hall 2010), but these reports are yet inconclusive due to inconsistencies (Siddall 1999, Siddall 2003, Werhagen 2004, Werhagen 2007, Zeilig 2012). Some, but limited, damage to the spinothalamic tract is suggested to be important for the onset of pain, as below-level hypersensitivity seems to be a predictor for spontaneous central neuropathic pain (Finnerup 2012b, Zeilig 2012). Surprisingly, even in patients clinically having a complete injury some remaining fibers in the spinothalamic tract might play a role in below-level pain (Garcia-Larrea 2002, Zeilig 2012). Also level of injury (i.e. percentage tetraplegia) also did not appear as a determinant for SCI pain. The largest prospective cohort study performed on SCI pain merely reported a slight difference in pain prevalence between tetra- and paraplegic patients (Siddall 2003).

With respect to our use of general/overall pain, we recognize that a sole SCI pain entity does not exist, and that determinants we identified to be (or not to be) relevant for the prevalence of 'any pain', could thus be biased due to differences in frequencies of different pain syndromes underlying our overall pain prevalences. Yet, different pain syndromes might arise from a multitude of pathophysiological processes which are not all fully understood (Siddall 2002, Widerstrom-Noga 2009). Different processes might overlap because of final common pathways, such as neuronal hyperexcitability at different levels of the central nervous system or psycho-cognitive traits (Cruz-Almeida 2009, Finnerup 2003b, Saab 2009, Zhao 2007). Therefore, determinants for any SCI pain should also be looked for in the larger general SCI pain population. Admittedly, the success of this approach may largely rely on (very) large numbers of patients, or, as done here, on meta-analyses. Of course, current clinical SCI pain classifications could also be useful to more selectively search for pain determinants, but pathophysiological overlap between these pain types might remain (Bryce 2006, Putzke 2003b, Ragnarsson 1997, Siddall 1997).

Last, we found residual heterogeneity, but one relevant method often used in meta-analyses might not be fully appropriate, at least when applied on pain prevalence studies. The standard approach to estimate within-study variation assumes that the size of the study is the most important factor for the accuracy of the prevalence estimate of an included study, which strongly determines the impact (and possible heterogeneity) of this study in the meta-analysis. This is, however, arguable in case of pain prevalence studies, as many equivocal factors come into play when one would like to accurately assess pain prevalence (attributes of pain definition, assessment method, etc). It does seem inappropriate that the within-study variation of pain prevalence rates are estimated with the same stringency and manner as for less ambiguous outcomes as, for example, mortality rates. Also, when using the standard formula for calculating the within-study variance estimate of a reported

prevalence the variance automatically gets much narrower when the prevalence approaches its extremes (i.e. 0% or 100%). However, a priori, in any random SCI patient population it is very unlikely that all patients experience pain. Hence, should we not estimate the variation of a pain prevalence differently to get a more realistic analysis of residual heterogeneity?

Conclusion

Variance in SCI pain prevalences is mostly due to the strictness of pain the definition used, whether or not the study was a pain study, but is also due to the use of retrospective data, the time since injury, and the prevalence of depression. Future SCI pain studies should clearly and more uniformly define and report pain definitions, report prevalences of depressive symptoms and time since injury and investigate them as pain determinants in SCI patients, and take special care not include bias by selecting for pain patients in pain (prevalence) studies. The identification of SCI pain determinants will be indispensable for the development of therapeutic approaches that effectively control SCI pain and ameliorate the quality of life of many SCI patients.

Table II-5: Included studies and their main characteristics

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Davis 1947	471			M	27	Y				16	8		Pain severely enough to consider active steps for its relief
Kuhn 1947	113	Y	86	M	23				71	9	8	100	Painful sensations; tingling and burning to severe agonizing, knife-like pains (rehabilitation admission)
Munro 1948	99	Y	47		34						10		Pain (admission; at discharge)
Munro 1950	228	Y		M	31				57	7	6		Pain (admission)
Botterell 1954	125				94	Y			40	16	8	100	Pain or discomfort
Kaplan 1962	44		85		56	Y					9	46	Pain (outpatient)
Burke 1973	214		100	H	45	Y					12		Continuous pain complaints and/or regularly analgesic use (admission)

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Nepomuceno 1979	200		56		80	Y				45	9	100	Painful unpleasant sensations (outpatients)
Richards 1980	75		32		77				71	37	10	88	Pain subsequent to SCI (outpatients)
Lamid 1985	64			H	44	Y					9		Significant pain and reduced activity (mostly admission)
Woolsey 1986	100			M	67	Y				48	10		Pain of which the patient is at least aware
Rose 1988	885		81	M	70	Y			42	35	12		Pain; either at or below the neurological level of injury (mostly outpatient)
Beric 1990	178				49	Y				70	8	100	Unpleasant sensations for at least three weeks, after injury
Frisbie 1990	66		100	M	73	Y	16				13	100	Altered sensations and pain
Krause 1990	347		79		64		15			57	12	100	Not no pain (mostly outpatient)
Lundqvist 1991	98		93	H	26		2.3		37	48	14	100	Pain associated with SCI (outpatient)
Rintala 1991	640			M	53	Y					8	100	Pain severely enough to impair activities of daily living (admission)
Summers 1991	54		75	M	75		12			35	12		Frequent pains (outpatient)
Brooks 1992	84		100		60			30	62	19	10		Pain problem (outpatient)
Fenollosa 1993	145		45	M	66	Y			57	26	12	83	Pain (outpatient)
Knutsdottir 1993	45		76		64				29	56	13	100	Chronic pain; a burning, sharp, aching, throbbing, or shooting, etc, sensation for > 6 months (mostly outpatient)
Craig 1994	41		87		32		0.5		74	51	13	100	Pain of >= moderate severity, after acute phase of SCI (mostly outpatient)
Stensman 1994	17		94	M	41			35	100	59	15	100	Not no pain (first admission)
Anke 1995	46			M	46	Y	0.6		39	50	13	100	>= Moderate pain in previous week (mainly at last week rehabilitation admission)

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Levi 1995	353		93	H	64	Y	11		40	43	14	100	Long-standing/chronic pain, being a significant problem/having a continuous negative impact on quality of life (mostly outpatient)
Anson 1996	348				45					56	8	91	Not no pain (first rehabilitation admission)
Cairns 1996	68		65		76	Y	0.1	46	56	34	14		Pain (outpatient)
Kennedy 1997	76		66		80	Y	0.1			32	14	100	Pain during admission (first rehabilitation admission)
New 1997	24				96	Y			37	54	9	79	Not no pain (6 weeks post injury, admission)
Stormer 1997	901			M	66	Y				58	9		Pain or distressing dysaesthesia, for >=3 months or >=6 months with remissions and relapses
Demirel 1998	47			M	61	Y			32	23	10	96	Pain related to the injury during past 4 weeks (outpatient)
Johnson 1998 & Gerhart 1992	357		100	M	28		1	2		53	14	100	Pain lasting or recurring for >= 6 months, not altered sensations (outpatient)
Post 1998	315		74		61		3.6			41	15	73	Perceived health problem: pain (outpatient)
Rintala 1998	77		92	M	75	Y	13.3			51	17	100	>= Moderate pain in previous week (during rehabilitation admission)
McKinley 1999	41	Y	84		54		0		56	20	10	100	Long-lasting pain (outpatient)
Meyers 1999	114		12	M	48		12.1	42		83	15		Any pain (of either musculoskeletal, visceral, or neuropathic origin), as established through interview, at 6 months after injury (outpatient)
Siddall 1999	100		56	M	64	Y	0.2		36	51	16	100	Pain problem (mostly outpatient)
Turner 1999	164		36		81	Y	12.1		49	47	14	72	Chronic pain problem during previous year (outpatient)
Widerstrom- Noga 1999	430		50	M	77		8.1	65	35	56	12	100	Pain (rehabilitation admission)

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Prysak 2000	91		50	M	45		17.5	22		51	11		Pain, started after SCI and persisted >= 4 months (mostly outpatient)
Ravenscroft 2000	145		67	M	79	Y	8.4		50	43	12	86	Chronic pain, during last year
Finnerup 2001	330		76		77	Y	9.3		48	36	14	75	Pain and unpleasant sensations (outpatient)
Putzke 2001	222		100	H	44				70	35	17	100	Current pain (outpatient)
Turner 2001	384		64		79	Y	12.2		44	51	14	80	Pain causing moderate daily interference during previous 4 weeks (mostly outpatient)
Klotz 2002 & Ville 2001	1668		49		74		12.9		53		12		Pain (rehabilitation admission)
McKinley 2002	117			M	60			26	33	50	10	63	Post injury pain, requiring pharmacological intervention; (first rehabilitation admission)
New 2002	134	Y	100		19			8		33	13	0	Any pain, after acute phase of SCI (mostly outpatient)
Vogel 2002	216		68		69		14.2			57	13	91	Pain problem (mostly outpatient)
Walter 2002 & Walter 2000	99		100		44			22		41	11		At least sometimes pain (mostly outpatient)
Barrett 2003	88		86		75	Y	16.7			60	15	84	Pain, ache or unpleasantness >= previous 2 weeks Or recurrent during >= 4 two-week periods during the previous year
Norrbrink Budh 2003	456		76	M	64	Y			32	46	15	75	Pain (admission)
Putzke 2003	6128		74	H	38				56	51	15	100	Significant pain that interfered with therapy (during [first] rehabilitation admission)
Siddall 2003	73		91	M	81	Y		25	38	49	16		SCI related pain
Yap 2003	40			H	70	Y			38	40	11	100	Pain of >= moderate daily interference during previous 4 weeks (mostly outpatient)

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Cardenas 2004	2433				81	Y	1		49	52	15	100	Not no pain during past 4 weeks (mostly outpatient)
Jan 2004	31				65	Y			58	39	10		Pain problems during previous year (mostly outpatient)
Rintala 2004	96		50		87	Y	10.9		44	50	15		Pain (attending pediatric tertiary care)
Saikkonen 2004	76		82	H	37			26	53	49	14	82	Pain disturbing daily life for >= 2 weeks, on 4 occasions, during previous year (mostly outpatient)
Bloemen- Vrencken 2005	454		46		55		13.3		67	34	11	79	Pain problem during previous year
Donnelly 2005	66			M	92	Y	1		55	55	13	100	Frequent pain during previous half year
Rintala 2005	348		92	M	76	Y	17.5			46	17	78	Pain (at 6 months of community living)
Meade 2006	436		57	M	38				22	55	11		Chronic pain within previous year
Jensen 2007 & Raichle 2007 & Jensen 2005	147		47		84		16.6		44	50	13	76	Not no pain (8 months after injury, mostly outpatient)
Kalpakjian 2007	65			M	66		13.7		66	46	10		SCI-related pain >= 6 months (outpatient)
Pagliacci 2007	403		85	H	32				50	37	15	100	Pain, excluding occasional headaches or menstrual cramps, >= once during previous 3 months (outpatient)
Raissi 2007	54		100		96					6	14	100	Clinical problem: pain, during previous half year (mostly outpatient)
Wollaars 2007	279		49	M	77	Y			42	57	12	63	Chronic pain, >= mild or infrequent problem, during previous 3 months (mostly outpatient)
Krahn 2009										56	14		Chronic pain, >=mild problem during previous year (mostly outpatient)
Miladinovic 2009	100			M	50	Y				0	9		Pain (start rehabilitation admission)

Article (by year)	Final sample size (n)	Retrospective study (Y=Yes)	Response rate (%)	Pain definition strictness (M=Moderate, H=High)	Pain prevalence (%)	Primary study goal (pain study; Y=Yes)	Time since injury (years)	Depression prevalence (%)	Complete injury prevalence (%)	Prevalence of tetraplegia (%)	Quality score	Prevalence of injuries with traumatic etiology (%)	Pain definition (patient setting)
Nicholson Perry 2009	47		45		77			29	45	49	14		Pain (start rehabilitation admission)
Gupta 2010	276		46	M	57	Y				0	12	89	Bothersome, frequently occurring, or periodic pain (mostly outpatient)
Hitzig 2010	344		44		58		19.1	26	35	46	12	93	Chronic pain during previous year (outpatient)
Kennedy 2010	80			H	50				31	50	12		Total of three Numerical Rating Scores scores ≥ 11 , out of 30; in last 24h, at its worst, and at its least in previous week (mostly outpatient)
Loo 2010	453		45		41		13.3		65	34	12	72	Pain, as being 1 of the most important condition (max. 5) in previous year (mostly outpatient)
Modirian 2010	1295		64		66	Y	14		99	9	7	100	$\geq$ mild pain (mostly outpatient)
Heutink 2011	279		49	M	77	Y					12		Any pain (of either musculoskeletal, spasm, visceral, or neuropathic origin), for more than 6 months duration (outpatient)
Machacova 2011	140			H	36		10.5			44	8	100	Currently seeing a doctor for pain (outpatient)
McKinley 2011	30		100		67				30	13	15	0	Pain (first rehabilitation admission)
New 2012	45	Y	100		77				25	7	13	0	Pain which received treatment or interfered with function during admission (rehabilitation admission)
Robertson 2012	115	Y	58		32				23		11	0	Pain on initial examination (rehabilitation admission)
Tan 2012	108		100	M	52				55	91	13	0	Pain which received treatment (rehabilitation admission)
Ullrich 2012	87	Y	62	M	55				52	37	13	93	≥ 4 , out of 10, on Numerical Rating Scale (rehabilitation admission)

Table II-5: Included studies and their main characteristics

III. Spinally- and supraspinally-mediated pain-like response thresholds in the thoracic spinal cord contused rat; assessment and interpretation

Translation of the rat thoracic contusion model; part 1 – supraspinally versus spinally-mediated pain-like responses and spasticity

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Abstract

Below-level sensitivity assessment in animal models of spinal cord injury is complex. Stimulus-evoked paw withdrawals can be mediated by below-level spinal cord reflexes. This makes interpretation of changes in below-level paw withdrawal response thresholds difficult, requiring both re-interpretation of prior results and novel behavioral sensory assessment methods. We here introduce a combinational-assessment design which uses the classical and (supraspinally mediated) escape-response tests for the assessment of pain-like sensitivity thresholds in acute and chronic stages of rat thoracic spinal cord contusion injury (SCI) model. As increased hindpaw withdrawal responses after SCI correlate better with the spastic syndrome (not neuropathic pain), the correlation between withdrawal and spasticity response was also investigated. The escape response test can be used to assess SCI-induced changes in below-level sensitivity thresholds. These sensitivity thresholds were found to increase soon after moderate or severe SCI, while, in parallel, hindpaw withdrawal thresholds decreased. However, the latter did not co-occur with spasticity, suggesting that SCI-induced increased withdrawal responses are probably best interpreted as a form of hyperreflexia with pathophysiological analogies of spasms and/or clonus, or a species-specific phenomenon. Decreased below-level withdrawal thresholds do not reflect pain-like hypersensitivity in rodent models of (thoracic) SCI, as hypoesthesia actually is found using our escape-response test. Paw withdrawals are inadequate to model pain, or spasticity, but could prove useful to model spasms or clonus, which are also significant clinical problems.

Introduction

Central neuropathic pain represents a severe, difficult to treat, and poorly understood aspect of SCI, which, in addition, is complex to model and evaluate in animal models. In animal SCI models, pain is investigated by determining hypersensitivity, often in dermatomes below the level of injury (below-level). Classically, a decreased hind paw withdrawal threshold to stimuli is observed and considered to reflect a central neuropathic below-level pain state. However, a reduction in the threshold for below-level withdrawal responses (BL-WRs) can present itself without any associated changes in escape/avoidance behavior to those same stimuli (Baastrup 2010b). Reduced paw withdrawal thresholds does thus not necessarily implicate an increased aversion to the stimulus. Furthermore, much reduced brain activity evoked by electrical stimulation of skin corresponding to below-level dermatomes in similar (but anesthetized) SCI rats has been reported, which actually implicates a state of hypoesthesia, not hyperesthesia (Hofstetter 2003). Hence, BL-WRs are probably due to autonomic spinal hyperreflexia and one might even not be perceived/conscious at all. Also in human SCI patients below-level hypoesthesia is more the rule than the exception, even in patients with below-level central neuropathic pain (Celik 2012, Finnerup 2003c). Also, below-level pain in human SCI patients typically has a relative late onset, up to months, even years, after injury (Siddall 2003).

Therefore, we here test combination of existing paradigms in which sensitivity thresholds for below-level evoked thermal and mechanical stimuli are not assessed with BL-WRs, but with supra-spinally mediated Above-Level Escape Responses (AL-ERs) (Baastrup 2010b, Chaplan 1994, Detloff 2008, Vierck 1995), and see how they relate to the development of BL-WRs. AL-ER thresholds or latencies are defined as force (mechanical) or time (heat) required to elicit 1) escape or struggling behavior, or 2) vocalization. The possibility to behaviorally quantify below-level sensitivity not only allows for a better interpretation of (sensory-feedback-dependent) motor tests, but also allows evaluations of therapy efficacy on sensory function, including hypesthesia and hypersensitivity.

Furthermore, it is important to realize that *BL-WRs* have now been postulated to represent mechanisms related to the spastic syndrome (instead of central neuropathic pain) (Baastrup 2010b). This spastic syndrome does not occur as evident in most SCI rats (including SCI rats) as it does in humans patients, in whom the most evident feature of the spastic syndrome often is the increased muscle resistance/activity (i.e. spasticity). However, spasticity in its most common and narrow definition, is defined as a velocity-dependent increase in muscle activity after tonic passive stretch (stretch hyperreflexia). Thus, such spasticity (and other reflex-like features of the spastic syndrome, such as spasms or clonus) can be easily overlooked in rats, as increases in spastic syndrome related muscle activity only needs to occur, or becomes more evident, after being evoked (e.g. by stretch).

Therefore, in the present study we also investigate whether spasticity, defined as stretch/proprioceptive hyperreflexia (Marsala 2005), coincides with the nociceptive hyperreflexia seen with BL-WRs in SCI rats.

Animals, methods, and materials

Animals and surgeries

All animal studies were approved by the University of California, San Diego institutional animal care and use committee. Twenty-two 12 weeks old Female Sprague-Dawley rats were anesthetized with isoflurane (5% induction, 2% maintenance; room air), placed on a Stereotaxic frame (Stoelting, Cat# 51600 with Cat# 51695, Wood Dale, IL, USA), and maintained at a core temperature of $37\pm0.3^{\circ}\text{C}$ using a heating blanket. A Th9 laminectomy was performed using a dental drill. The MASCIS/NYU apparatus (WM Keck Center for Collaborative Neuroscience, Rutgers University, Piscataway, NJ, USA) was used for SCI. The laminectomy site was filled with mineral oil in which the tip of a small thermocouple (Physitemp, Cat# IT-14, Clifton, NJ, USA) was submerged and used to manually keep the spinal cord at $37\pm0.3^{\circ}\text{C}$ with warmth from surgical lights (Fiber-Lite, Cat# MI-150 & BGG1823M, Dolan-Jenner, Boxborough, MA, USA). Next, oil and lamps were removed and the rod dropped using a height of 6.25 (mild SCI, $n=7$), 12.5 (moderate SCI, $n=5$), or 25mm (severe SCI, $n=5$). Five sham operated animals were included.

Group designation was not known by the experimenters doing behavioral testing. SvG was involved in all behavioral testing, ML, and SN were involved in Basso, Beattie, Bresnahan (BBB) scoring.

Post-surgical care

Buprenorphine (0.05 mg/kg, s.c., Reckitt Benckiser, Richmond, VA, USA), 5 mL of Lactated ringer, and 10 mg/kg of Cefazolin (Novaplus/Sandoz, Holzkirchen, Germany) was given after surgery. Bladders were emptied manually twice daily. Sulfamethoxazole and Trimethoprim USP (200mg & 40mg per 250mL drinking water, Hi-Tech Pharmacal, Amityville, NY, USA) was given for at least 10-14 days or until autonomic bladder voiding occurred. Any ill-appearing animals received additional days of above-mentioned antibiotic treatments.

Open field locomotion testing

Locomotion recovery after SCI was monitored using a rating scale based on the BBB open field locomotor rating scale (0 to 21), as described before (Basso 1995, Basso 1996).

Sensory testing

Hind paw withdrawal response thresholds (dorsally applied von Frey filaments; see below, this section) and supraspinally mediated 'above-level'-escape response (AL-ER) thresholds were assessed. An AL-ER is defined as an escape-attempt from the investigator's loose, but fully supportive, grip (Fig.1) and has to include the elaborate use of their front paws or a vocalization. The applied stimuli were either a gradually increasing force to the hind paws (using the Analgesy-Meter; Cat# 37215, Ugo-Basile, Collegeville, PA, USA), or a constant heat stimulus (intensity 17, cut-off at 30 sec) to the hind paws (using an constant infrared heat source; Cat# 37360, Ugo-Basile, Collegeville,

Figure III-1: Schematic of Above-Level Escape Response testing upon below-level stimulation. Shown is the way the animals were held for the AL-ER testing upon hindpaw stimulation with a mechanical (black arrow, dorsal) or a thermal (grey arrow, plantar) stimulus. The animals were loosely held so that animals could escape from the investigator's grip. An Above-Level Escape Response refers to escape behavior elicited by structures innervated above the level of injury used. Here we used a thoracic injury; hence, AL-ERs were defined as the transition of a tranquil state to a state of fleeing/distress with respect to activation of the frontlimbs, and shoulder-/neck-/head-area, and/or vocalization. Per time point, the hindpaws were alternately tested with an interval of 1-2hours, up to a total of four times per paw for each stimulus.

PA, USA). The hindpaw tested was gently restrained by the investigator to prevent reflex-related paw withdrawal (**Fig.III-1**).

For the AL-ER tests, both hind paws were tested four times, alternately, for each test, with a testing interval of 1-2 hours and maximal four measurements per day. Maximum cut-off values for the stimuli or latency were at approximately two times that of response threshold of uninjured animals to prevent tissue damage. Prior to (7 days) and during the entire experimental period (5 days a week), the animals were extensively habituated to the experimenter in such a way that the animals could be held upright (loosely) during all sensory assessments. Habituation consisted of picking up the animal and holding/handling it twice daily for 3-5 minutes. During habituation the animals were presented to the test setup, as if they were tested, but without the application of mechanical or thermal stimuli (the testing devices were switched on/operated during habituation, to acclimatize the animals to any noise of

each device when operated).

To determine the hind paw withdrawal thresholds (von Frey monofilaments, up-down method (Chaplan 1994)) the animals were also loosely held upright, to circumvent the requirement of weight support (Detloff 2008).

Assessment of stretch induced muscle resistance

Peripheral muscle resistance was measured using a previously described spasticity-meter (Marsala 2005). Briefly, animals were placed in a restrainer and a hind paw was taped to a metal plate driven by a computer-controlled stepper motor. The resistance of the ankle to dorsiflexion was measured during stepper motor-driven ankle flexion (40°), and always in conjunction with the EMG of the ipsilateral m.gastrocnemius (described below, this section). Resistance was measured in awake animals using medium, fast and slow ankle rotation speeds (i.e. 40° in 0.5, 0.25 and 1 sec, respectively). Consecutively, the muscle resistance was measured in anesthetized animals (at medium speed), to identify the mechanical (vs. the neurogenic, isoflurane-sensitive) component of measured ankle resistance. Hence, active muscle resistance could be computed (resistance when awake minus resistance when anesthetized). Also, spastic components of muscle resistance could be detected, as spasticity is velocity-dependent. To record EMG activity, a pair of tungsten electrodes was inserted percutaneously into the gastrocnemius muscle 1 cm apart. EMG signals were bandpass filtered (100 Hz to 10 kHz) and recorded before, during, and after ankle dorsiflexion. Only EMG data from the fast ankle rotation measurements was used for the detection of rotation-induced EMG amplitude increases. Average EMG amplitudes were calculated for the two seconds before ankle rotation, and a period of 0.20 seconds in which the maximal ankle rotation was achieved. Each recorded value was the average of three repetitions.

Statistical analyses

Results were analyzed using ANOVA (one-way, or two-way group $\times$ time repeated measures, using a fixed-effect model), with a Bonferroni *post hoc* test for multiple comparisons (GraphPad Prism, La Jolla, CA, USA). Unequal variances were not observed. Results are analyzed as two-tailed and expressed with standard error of the mean (SEM), unless specified otherwise. A P-value of 0.05 was considered significant.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Results

Figure III-2: Spinal and supraspinal pain-like responses and spasticity after thoracic SCI. A) Shown are the typical open-field BBB locomotor scores of the sham, mild, moderate, and severe SCI groups. B) Using the up-down method to the dorsal aspect of the hindpaws we confirmed that below-level withdrawal responses decrease after SCI animals. Compared to sham-operated animals only more than mild SCI animals showed significantly decreased hindpaw withdrawal thresholds (Repeated Measures ANOVAs; $P < 0.001$). C-D) In contrast, pain-perception/AL-ER thresholds to below-level mechanical (C) and thermal (D) stimuli increased in animals with a more than mild SCI (Repeated Measures ANOVAs; $P < 0.001$). E) The occurrence of below-level paw withdrawal hyperreflexia did not coincide with stretch-evoked muscle activity, as no increases in (active) muscle resistance were noted in any SCI group up to the end of the study (i.e. six weeks post injury).

SCIs induced with the MASCIS/NYU apparatus using 6.25mm, 12.5mm, and 25mm impact/drop heights, resulted in significantly different injury severities, as assessed with the BBB-locomotor score (Variation between any two experimental groups; Bonferroni; $P \leq 0.04$; Fig.2A). Trends in BBB-scores are similar to the ones reported originally, indicating a similar extent of the injuries (Basso 1995, Basso 1996). Weight-support (i.e. BBB score ≥ 9) was absent in moderate SCI animals prior to the 3 weeks assessment and remained absent in most severe SCI animals.

Graded SCCI resulted in a decreased hind paw withdrawal threshold in the moderate and severely injured groups (i.e. 12.5 and 25mm weight drop groups), when compared to the sham-operated group (Repeated Measures ANOVA; $P < 0.0001$; Fig.2B). However, mild injury (6.25mm) did not give significant threshold changes in comparison to sham operated animals ($P = 0.21$). No significant differences were present between moderate and severe SCI.

Using supraspinally mediated above-level escape responses (AL-ERs), the below-level sensitivity thresholds were assessed. AL-ER thresholds for mechanical stimuli changed only for moderate and severe SCI, when compared to sham-operated animals (Repeated Measures ANOVA; $P < 0.001$; **Fig. III-2C**). Testing was somewhat difficult due to pronounced bilateral and complex hind limb motor activity (i.e. jumping/kicking-like behavior), but AL-ER thresholds after moderate SCI were noted to increase for thermal stimuli ($P < 0.001$; **Fig. III-2D**), yet it was impossible to confidently assess thermal AL-ER thresholds in the severe SCI rats. The complex motor response to stimuli was present even in the acute SCI phase, in which stepping was absent during open-field testing.

Analysis of active muscle resistance in the gastrocnemius muscle upon ankle rotations to assess stretch hyperreflexia, a measure of spasticity, showed no difference between experimental groups at any rotation speed (One-way ANOVAs; $P \geq 0.63$) nor time point measured (i.e. one, three, and six weeks after injury; $P = 0.94$, $P = 0.92$, and $P = 0.88$, respectively; **Fig. III-2E** shows active muscle resistance at six weeks). Also, ankle rotations m.gastrocnemius EMG amplitudes did not increase in any of the experimental groups, when compared to their resting stages (relative values; fastest ankle rotation speed: sham, mild, moderate, and severe SCI groups; Student's t-tests; $P = 0.75$, $P = 0.82$, $P = 0.48$, and $P = 0.25$, respectively).

Discussion

Here we show that thresholds for below-level sensitivity increase after more than mild SCI (assessed with AL-ER thresholds for both thermal and mechanical stimuli) during the first six weeks after SCI, which makes it a useful test for SCI therapy evaluations. In contrast, BL-WR thresholds decreased in those same animals, indicating a limited correlation of BL-WR with pain perception. Furthermore,

the AL-ER test revealed forceful bilateral hindpaw motor ability (kicking/jumping) upon modest sensory stimulation of a single hindpaw, even when SCI animals were still unable to support their bodyweight during locomotion. The latter is in line with observations that complex BL-WR can also be evoked in complete spinal cord transected animals, even within hours after injury (Schouenborg 1992).

Importantly, although AL-ERs are supraspinally mediated (strictly speaking they are above-level mediated), they still should be carefully interpreted as responses to pain, as AL-ERs reflect supraspinal responses which do not necessarily require cortical processing and could thus merely be brainstem mediated reflexes (Vierck 2006).

The observed nociception-evoked hyperreflexia is different from spasticity or rigidity, as it did not coincide with increased (stretch-evoked) muscle activity. Nonetheless, both the problems arising from the spastic syndrome in humans and the decreased BL-WR thresholds in SCI rats respond to baclofen (GABA_B agonist) treatment, while central neuropathic pain in humans hardly does, if at all (Teasell 2010). Nevertheless, the spastic syndrome consists of an array of only partly overlapping 'positive signs' of the Upper Motor Neuron syndrome; which includes not only spasticity, but also spasms, pathological reflexes, clonus, hypertonia, and dystonia (whereas its 'negative signs' relate to the loss of motor control). The hindpaw hyperreflexia as seen in the current study/model (i.e. complex hindpaw motor activity upon sensory stimulation in paraplegic rats) could still correspond well to the spastic syndrome, as observed in human SCI patients, as it shows similarities to, for instance, spasms or clonus. The latter has similarities with the withdrawal responses in terms of the rhythmicity and a fading ending, although clonus is classically elicited through stretch receptors.

When compared to humans (Jacobs 1995), it is remarkable that rats with moderate (or even severe) SCI show a high degree of motor function as well as complex below-level paw withdrawal behavior, but still have poor below-level perception. In addition, rat SCI result in much fewer cases of spasticity and less pronounced spinal shock phases (Akhtar 2008, Bennett 1999). Apparently, inter-species differences in the degree of spinal autonomy (supra-spinal involvement) for locomotion function do exist. This suggests a strong complex autonomic sensorimotor function of the rodent spinal cord, which will be mostly attributed to the relative large role of the Central Pattern Generator (CPG) in the locomotion of quadrupeds (see our other manuscript) (Barriere 2008, Magnuson 2005). In summary, nociception-evoked hindpaw hyperreflexia as observed in SCI rats still has, on the one hand, a debatable clinical relevance as it doesn't correlate with hypersensitivity and could be solely due to species specific properties of spinal function, but, on the other hand, resembles aspects of spasms and/or clonus, as seen in human SCI patients.

IV. Backwards locomotion on a rotating rod; a highly sensitive test to evaluate thoracic spinal cord contusion injury in rats?

Translation of the rat thoracic contusion model; part 2 – forward versus backwards locomotion testing

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Abstract

Locomotion analyses in rat spinal cord contusion injury (SCI) models are widely used for the evaluation of recovery of supraspinal locomotor control. However, much locomotor recovery recorded with commonly used tests are independent of below-level perception, highly mediated through autonomic propriospinal circuitry, and thus lack sensitivity for spinal cord integrity. Here we report a behavioral motor test which is more sensitive for spinal cord integrity and/or the disability in the chronic phase after injury: the backwards locomotion rotating rod. A standard rat rotating rod test was run in reverse while modified to accommodate SCI rats. The rod diameter was increased and a thin rubber lining was added. As a reference, we included commonly used motor tests: BBB locomotor scoring, Catwalk gait analysis, Motor Evoked Potentials, and the Single Frame Analysis, but also a forward rotating rod test and the 55° Inclined Ladder Test. Unlike commonly used motor test, the backwards locomotion rotating rod test does significantly discriminate both sham-operated vs. mild SCI animals, and mild vs. moderate SCI animals at three and six weeks post injury. Moderate SCI animals were virtually unable to make even the slightest backwards hind paw movement. The backwards locomotion ability in the chronic post injury phase correlate best with *acute* BBB locomotor scores. Our data shows that backwards locomotion is a highly sensitive test to assess spinal cord injury and its integrity after rat SCI. Backwards locomotion testing may enhance translatability of experimental results into the clinic.

Introduction

The application of animal models in spinal cord injury (SCI) research is complicated due to difficulties in sensitive and specific quantification of locomotor outcome, though behavioral outcomes are the most important argument for using animal models. Locomotor outcomes are often assumed to correlate well with SCI regeneration (long-tract integrity), but this is a feature which might be poorly present in widely used animals models. Behaviorally, in the chronic phase after SCI in rodents, i.e. only a few weeks after injury, most mildly injured SCI animals have spontaneously recovered to be indistinguishable from sham-operated animals, although only half of the spinal cord is spared (Basso 1996). Furthermore, moderate SCI animals generally show merely a few mild deficits, and even severely injured SCI animals (reported to have even <10% spared spinal cord tissue) regain weight supported stepping (Basso 1996). Also, recovery of below-level sensory perception seems barely, if at all, required for regaining basic quadruped locomotion (see the accompanying manuscript and Hofstetter et al. (Hofstetter 2003)). Hence, the question is: if current behavioral tests have difficulties to detect even such fairly large disruptions in the spinal cord, how reliable are these tests for (not) measuring therapeutic effects?

Agreed, the animal spinal cord (below the level of injury) is since long known to be able to implement/recover much basic motor functionality autonomously by strengthening preexisting propriospinal circuitries and, in particular the central pattern generator (see our other manuscript, Barriere et al, and Tillakaratne et al. (Barriere 2008, Tillakaratne 2010)). This notion is further supported by the fact that even after full spinal cord transection, but only when performed *after* a prior contusive SCI, some recovery in hindlimb function is still noted (Basso 1996). However, locomotion recovery through autonomic spinal cord circuitry can be expected to be of much less relevance in the human condition. This is due to the higher degree of instability of the basic posture during human bipedalism which would logically require more advanced (read: supraspinal) modulation. In this perspective it is not surprising that, in contrast to quadrupeds, I) adequate below-level sensory perception after SCI in humans has proved to be a good predictor for locomotor recovery and II) it takes a human relatively long to learn how to walk as a child. Hence, basic locomotor tests in SCI models not only seem to lack desirable sensitivity for SCI injury, but it also (or therefore) seems not to be sufficiently specific for the human SCI condition.

Subsequently, we focused on motor tests which (on theoretical grounds) might be more sensitive for spinal cord integrity through the requirement of supraspinal control (i.e. requiring more advanced sensorimotor modulation). Hence, the climbing test (55° Inclined Ladder Test) and the rotating rod test were included and tested for their sensitivity for mild and moderate thoracic spinal cord

contusion injury in rats, a model widely used and with reproducible results. It is known (from their use in other SCI models) that more severely injured animals barely, if at all, recover function as assessed with these test, which is suggestive for a relatively high requirement of supraspinal motor control (Hamm 1994, Semler 2011, Sheng 2004, Simard 2012). In order to further increase the requirement of supraspinal motor control on locomotion testing and decrease the impact of basic spinal autonomic locomotion circuitry, the backwards locomotion rotating rod test was developed. As a reference, severe SCI animals were included, Myogenic Motor Evoked Potentials (MMEPs) to transcranial electrical motor cortex stimulation were measured, and locomotion ability was scored with the widely used Basso, Beattie, Bresnahan (BBB)-locomotor scoring method (Basso 1995, Basso 1996). To objectify some of the key locomotor features of the BBB-score, the single frame analysis (Rump Height Index and Foot Stepping Angle) and Catwalk gait analysis (Regularity Index) were used (Koopmans 2005, Semler 2011).

Animals, methods, and material

Animals and surgeries

All animal studies were approved by the University of California, San Diego institutional animal care and use committee. Twenty-two 12 weeks old Female Sprague-Dawley rats were anesthetized with isoflurane (5% induction, 2% maintenance; room air), placed on a Stereotaxic frame (Stoelting, Cat# 51600 with Cat# 51695, Wood Dale, IL, USA), and maintained at a core temperature of $37\pm0.3^{\circ}\text{C}$ using a heating blanket. A Th9 laminectomy was performed using a dental drill. The MASCIS/NYU apparatus (WM Keck Center for Collaborative Neuroscience, Rutgers University, Piscataway, NJ, USA) was used for SCI. The laminectomy site was filled with mineral oil in which the tip of a small thermocouple (Physitemp, Cat# IT-14, Clifton, NJ, USA) was submerged and used to manually keep the spinal cord at $37\pm0.3^{\circ}\text{C}$ with warmth from surgical lights (Fiber-Lite, Cat# MI-150 & BGG1823M, Dolan-Jenner, Boxborough, MA, USA). Next, oil and lamps were removed and the rod dropped using a height of 6.25 (mild SCI, n=7), 12.5 (moderate SCI, n=5), or 25mm (severe SCI, n=5). Five sham operated animals were included.

Post-surgical care

Buprenorphine (0.05 mg/kg, s.c., Reckitt Benckiser, Richmond, VA, USA), 5 mL of Lactated ringer, and 10 mg/kg of Cefazolin (Novaplus/Sandoz, Holzkirchen, Germany) was given after surgery. Bladders were emptied manually twice daily. Sulfamethoxazole and Trimethoprim USP (200mg & 40mg per 250mL drinking water, Hi-Tech Pharmacal, Amityville, NY, USA) was given for at least 10-14 days or until autonomic bladder voiding occurred. Any ill-appearing animals received additional days of above-mentioned antibiotic treatments.

Open field locomotor testing

Locomotion recovery after SCI was monitored using a rating scale based on the Basso, Beattie, Bresnahan (BBB) open field locomotor rating scale (0 to 21), as described before (Basso 1995, Basso 1996).

Gait analysis

The CatWalk apparatus (7.1, Noldus, The Netherlands) was used to quantify gait parameters by semi-automated footprint analyses during runway crossings (Koopmans 2005). Animals were trained for smooth walkway crossing and animals had always access to their home cage and a treat (Certified Supreme Mini-Treats™, Cat# F05472-1, Frenchtown, NJ, USA) at the end of the run (done for catwalk, single frame, and ladder tests).

Single frame hind limb motion analysis

Bilateral video captures allowed analysis of the foot-stepping angle (FSA) and the rump-height index (RHI), as described previously (Semler 2011). The FSA is the angle at which the hind paw is placed on the ground. The RHI was defined as the highest point of the base of the tail during a run. VirtualDub 1.9.11 (<http://www.virtualdub.org>) and Screen Ruler V1.0.1a (<http://www.caveworks.net>) were used for analyses.

Integration of objective measures (Catwalk, FSA, and RHI) into BBB-locomotor score

BBB-scores allows some experimenter subjectivity, hence we tried to objectify some of the mid-range items of the BBB-score, which are regarded as key items in the recovery of SCI. First, animals were grouped in BBB-scores ≤ 7 , 8, 9, 10, or ≥ 11 , using the Foot Stepping Angle (to define plantar stepping) and Rump Height Index (to define weight support) from the Single Frame Analysis (Semler 2011). Secondly, frequency of frontlimb-hindlimb coordination was objectively assessed using the Catwalk gait analysis by counting the coordinated runs (out of three runs) (Koopmans 2005).

Inclined ladder test

The 55° Inclined Ladder Test was performed using an inclined 20-rung ladder, as previously described (Semler 2011). The correct placing of a hind paw and sustained position until its next forward move was counted over the 18 center rungs.

Myogenic Motor Evoked Potentials

Myogenic Motor Evoked Potentials (MMEPs) in the gastrocnemius muscle were measured after being elicited by transcranial electrical stimulation of the motor cortex using two percutaneously placed electrodes, as described before (van Gorp 2013).

Rotating rod and the backwards Locomotion test

A rotating rod apparatus (Rota-Rod, Stoelting, Cat# 52790, Wood Dale, IL, USA) was modified and used to assess motor performance (increased diameter from 3¼" to 5" and lined with 1.5mm thick rubber). Rats were placed on the elevated reversed rotating rod, which was started at 4 rpm, linearly accelerated to 40 rpm over 60 sec, with a cut-off of 150 seconds. Maximum falling latency out of three trials was recorded. Timing started after hindpaw movement was observed. Rats were given a minimum of 30 min. between trials. Two hand-held small transparent acrylic plates (400 × 50mm; see **Fig. IV-1**) and treats (as above) prevented rats from changing direction on the rotating rod and to motivate the animals for rotating rod testing.

Statistical analyses

Results were analyzed using ANOVA (one-way, or two-way group × time repeated measures, using a fixed-effect model), with a Bonferroni *post hoc* test for multiple comparisons (GraphPad Prism, La Jolla, CA, USA). Unequal variances were not observed. Results are analyzed as two-tailed and expressed with standard error of the mean (SEM), unless specified otherwise. A P-value of 0.05 was considered significant.

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

Figure IV-1: Backwards locomotion rotating rod. A rotating rod apparatus was slightly modified by increasing the rod diameter to 5" and lining it with 1.5mm thick rubber. Two hand-held small transparent acrylic plates (400 × 50mm) and treats prevented rats from changing direction on the rod.

Results

All SCI severities caused a significant acute (three days post injury) reduction in BBB scores, compared to sham operated animals (**Fig. IV-2A** solid lines; Bonferroni: $P < 0.001$). Next, a partial or complete recovery in BBB scores followed (i.e. up to six weeks post injury). Notably, differences between sham and mildly injured animals merely differed in the very acute phase (Bonferroni: $P < 0.001$), while differences between moderately and severely injured animals only became marginally notable after three weeks post injury (Bonferroni: $P < 0.05$). These BBB-scores are similar to the ones reported originally, using the same SCI model (Basso 1995, Basso 1996). There, most mild SCI animals also recovered to a BBB score of ≥ 20 , although a $>50\%$ white matter loss was reported at the level of injury.

Catwalk, FSA, and RHI barely improve sensitivity for SCI compared to the BBB-locomotor score

Implementing some objective measures to the BBB-locomotor scores gave only slight, non-significant, changes in original BBB-scores (compare solid and dotted lines in **Fig. IV-2A**; Repeated Measures ANOVA per injury severity: $P \geq 0.63$). Individually, frontlimb-hindlimb coordination, as assessed by number of fully coordinated walkway crossings during gait analysis (coordinated crossing: a regularity index of 100%) (Koopmans 2005), was not significantly affected in mild SCI animal when compared to sham-operated animals (**Fig. IV-2B**; Repeated Measures ANOVA: $P = 0.31$). Coordination in moderate and severe SCI animals differed significantly from each other at three and six weeks post injury (Bonferroni: $P < 0.05$). Also, after 3 weeks Rump Height Indices only show significant disabilities for the severe SCI animals, when comparing with sham animals (**Fig. IV-2C**; Bonferroni: $P \leq 0.001$). Last, Foot Stepping Angles were only significantly increased for severe SCI animals (at one and three weeks post injury; **Fig. IV-2D**; Bonferroni Post-Hoc: $P < 0.05$) and moderate SCI animals (at one week post injury; Bonferroni Post-Hoc: $P < 0.05$), when compared to either sham animals. Nonetheless, at the acute phase (one week post injury) Foot Stepping Angles, as well as Rump Height Indices, did show significant different disabilities between moderate and severe SCI animals (Bonferroni: $P < 0.05$), while BBB-scores did not.

Figure IV-2: Results from locomotion tests which depend little on supraspinal locomotion control. Note that sham-operated and mild SCI rats are indistinguishable after the acute phase post SCI. A) open-field locomotion scores as assessed with the BBB locomotor scale (solid lines), and the BBB locomotor scale which was objectified for coordination, plantar stepping, and weight-support (using the Catwalk, Foot Stepping Angles, and Rump Height Index, respectively; interrupted lines). Note that even severe SCI animals regain weight support (scores of ≥ 9). All four groups differed significantly from each other (Repeated Measures ANOVA: $P \leq 0.02$), but sham-operated and mild SCI only differed significantly at 3 days post injury (Bonferroni: $P \leq 0.001$). B) Displayed is the number of coordinated runs as assessed by the Catwalk gait analysis. Only moderate and severe SCI animals differed significantly from the other groups (Repeated Measures ANOVA: $P < 0.05$). C-D) Shown are the Rump Height Indices (C) and Foot Stepping Angles (D) as measured in the Single Frame Analysis test. Again, only moderate and severe SCI animals differed significantly from sham-operated animals (ANOVA: $P \leq 0.03$). *, **, and *** correspond to P-values of < 0.05 , < 0.01 , and < 0.001 , respectively.

The rotating rod, 55° Inclined Ladder Test, and Myogenic Motor Evoked Potentials did not improve sensitivity for SCI, but showed identical on/off patterns for supraspinal locomotor control

The patterns for chronic motor deficits (more than one week post injury) are identical as measured by the rotating rod (with forward locomotion), 55° Inclined Ladder Test, and Myogenic Motor Evoked Potentials. Again, no significant differences between sham and mild SCI were detected for the rotating rod test and MMEPs (**Fig. IV-3A&C**; ANOVA: $P \geq 0.41$). The 55° Inclined Ladder Test did show significant differences between sham-operated and mild SCI animals up to three week post injury (**Fig. IV-3D**; Bonferroni: $P < 0.05$). Even at six weeks post injury, the 55° Inclined Ladder climbing and MMEPs were significantly and fully reduced in both moderate and severe SCI animals, when compared to sham and mild SCI animals (Bonferroni: $P < 0.05$). Nonetheless, even severe SCI animals showed constant stepping-like hindpaw movements during ladder climbing test tests, although these movements remained largely dysfunctional. In the rotating rod test, any hindlimb movement soon contributed to improvement in outcome, which was also largely due to stomach dragging/frontlimb compensation.

The backwards locomotion test showed improved sensitivity of SCI-induced locomotor disability

Significant and chronic (measured up to six weeks post injury) decreases in locomotor function on the backwards locomotion test were measured for all SCI severities, which specifically includes mild SCI (when compared to sham-operated animals; **Fig. IV-3B**; Bonferroni Post-Hoc: $P \leq 0.01$). The ability to perform backwards locomotion was strikingly absent in animals with moderate and severe SCI, as virtually no movement in the hindlimbs was noted in these animals during testing. By far most moderate SCI animals were unable to make even slight hindlimb movements when attempting to walk backwards, even at six weeks post injury, which was in sharp contrast to their front limb movements.

Figure IV-3: Results from MMEP assessments, ladder climbing, and rotating rod tests which depend largely on supraspinal locomotion control. Note the lack of differences between sham-operated and mild SCI animals in all but the backwards locomotion rotating rod test in the chronic stage post injury. A) Displayed are drop-off latencies of the rotating rod testing with forward locomotion. Only differences between mild and moderate SCI animals, not sham and mild, or moderate and severe SCI animals, were significant (Repeated Measures ANOVA), for all three timepoints (Bonferroni Post-Hoc: $P < 0.01$). B) Displayed are drop-off latencies of the backwards locomotion rotating rod test. The backwards locomotion rotating rod test was the only test which showed significant differences between sham-operated, mild, and moderate SCI animals, at all time points (Bonferroni Post-Hoc: $P \leq 0.01$). C) Shown are the Myogenic Motor Evoked Potentials measured in the m. gastrocnemius after transcranial electric stimulation at six weeks post injury. Only between mild and moderate SCI occurred a significant drop in evoked potentials (One Way ANOVA: $P < 0.05$). D) Shown are the number of correct steps/paw placements while climbing the rungs of a 55° Inclined Ladder. Differences at six weeks post injury were only significant between mild and moderate SCI animals (Bonferroni: $P < 0.001$). *, **, and *** correspond to P-values of < 0.05 , < 0.01 , and < 0.001 , respectively, and are only displayed at the latest time point on which two consecutive injury severity groups showed significant differences.

Discussion

This study shows that the backwards locomotion test, unlike regular post-SCI hindpaw locomotor tests, is sensitive enough to allow reliable discrimination between both sham-operated vs. mild SCI animals, and mild vs. moderate SCI animals in the chronic post-SCI phase. Surprisingly, moderate SCI animals were virtually unable to perform even the slightest backwards hindpaw movement. Hence, backwards locomotion assessment is not only the first test sensitive enough to detect distinct and chronic differences in locomotor ability between sham, mild and moderate SCI animals, but also provides a wide continuous scale, from sham to (near) severe injury, which is likely to be of great use for translational therapy evaluations. Furthermore, abovementioned properties of the backwards locomotor test can be hypothesized to be due to the high dependency on supraspinally coordinated sensorimotor control as A) final neural networks of forward and backwards quadruped locomotion are believed to be similar (Musienko 2012), and B) among all performed locomotor tests, the backwards locomotion rotating rod test showed best correlation with 1) MMEPs, 2) acute (non-compensated by autonomic circuitry) scores of the BBB-locomotor score, and 3) deficits in sensory perception thresholds observed in the hindpaws of all animals (see the accompanying manuscript).

Below-level perception only becomes important for locomotor tests when they depend on supraspinal motor control, as sensory input first has to pass through the injury epicenter. Hence, sensory disturbances have been less relevant in locomotor tests like the BBB-locomotor score which depend more on autonomic propriospinal locomotor circuitries (Hochman 2012). These circuitries likely make that even numb rats can walk (Hofstetter 2003). This notion is nicely shown by the 55° Inclined Ladder Test, where mild SCI animals could normally detect and step on the rungs (largely unaffected hindpaw sensory thresholds; see the accompanying manuscript), while moderate SCI animals could not (altered sensory function), although the latter still showed pronounced (spinal autonomic) hindlimb motor activity during climbing. The latter is in line with reports showing vast rat spinal cord (forward) locomotor autonomy by propriospinal circuits (Rossignol 2011a, Rossignol 2011b, Tillakaratne 2010).

Although the extent of spinal autonomic locomotion in quadrupeds is interesting by itself, its value is believed to be much less in humans (Battistuzzo 2012, Wessels 2010). It then mostly remains an important hurdle for clinically translatable assessments of long tract regeneration in rats. Interestingly, MMEPs are typically reported not to correlate to basic locomotor testing (Loy 2002), but it does display a pattern more related to the backwards locomotion test and studies in human SCI patients showed high sensitivities of MMEPs for spinal integrity and functional outcome (Clarke 1994, Di Lazzaro 1999, Di Lazzaro 2004). Hence, the backwards locomotion test seems to correlate

closely with the integrity of long tracts which corresponds to those which are most relevant for function in humans. Further investigation will show which (combinations of) major descending (cortico-, rubro-, raphe-, reticulo-, vestibule-, and coeruleo-spinal) tracts rat backwards locomotor ability depends, and if (spontaneous) reactivity in any of these tracts corresponds with recovery.

In conclusion, we recommend backwards locomotion testing for better translatability of experimental SCI research.

V. Evaluation of olfactory ensheathing cell transplantation for spinal cord injury therapy in a rat hemisection model

Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord

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Abstract

Olfactory ensheathing cells (OEC) are a promising graftable cell population for improving functional outcomes after experimental spinal cord injury. However only few studies have focused on experimental models with large cavitations, which require bridging substrates to transfer and maintain the donor cells within the lesion site. Here, a state-of-the-art collagen-based multi-channelled three dimensional scaffold was used to deliver olfactory ensheathing cells to 2 mm long unilateral low-thoracic hemisection cavities. For a period of 10 weeks, allodynia of the hindpaws was monitored using the Von Frey hair filament test, while an extensive analysis of motor ability was performed with use of the CatWalk gait analysis system and the BBB locomotor scale. No substantial improvement or deterioration of motor functions was induced and there was no effect on lesion-induced allodynia. On the basis of these data, we conclude that relatively large spinal cord lesions with cavitation may present additional hurdles to the therapeutic effect of OEC. Future studies are needed to address the nature that such lesion cavities place on cell grafts.

Introduction

Spinal cord injury (SCI) has dramatic effects on the individuals concerned as well as on society. Debilitating consequences such as deficits in motor function and development of neuropathic pain are directly related to the disruption of spinal tissue and the cellular and molecular processes induced by trauma (Berger 2011, Hulsebosch 2009). Many different experimental approaches have been used to promote tissue repair or plasticity (e.g. regeneration of severed axon tracts) (Berger 2011). Delivery of axon-growth promoting cells into the injured spinal cord has proven to be a popular approach in experimental medicine, and clinical trials have been initiated. In particular, olfactory ensheathing cells (OEC) have been considered a highly promising population of cells for SCI repair because they support the regeneration of olfactory nerve fibers from the periphery into areas of the central nervous system (CNS) throughout the life-span of mammals (Raisman 2007).

OEC express many neurotrophic factors, and unlike many other types of cells can be applied as autografts, thereby by-passing side-effects of immunosuppression that would be required for allografts or xenografts (Munoz-Quiles 2009), while requiring minimal pre-grafting *in vitro* manipulations. A main advantage of grafting OEC into lesioned CNS is their ability to intermingle with- and migrate amongst CNS glia, such as astrocytes (Lakatos 2000). Moreover, OEC tend to form and maintain open channel-like structures that form a regenerative substrate, not only in their normal habitat (Li 2005), but also when transplanted into small electrolytic lesions of the of the corticospinal tract (Li 1998). Implanting OEC into partial or complete lesions of the rat spinal cord has been found to induce autonomic and voluntary motor improvements (Li 2003, Ramon-Cueto 2000).

However, when considering human SCI with the formation of large cystic cavitation, it is unlikely that grafting donor cells alone will be sufficient to promote repair. The bridging of large cystic cavities requires a physical implant which would act as a carrier for the donor cells and thus be a substrate capable of supporting nerve fiber regeneration. We have previously demonstrated the cytocompatibility of a degradable biomatrix, (based on polylactic acids) with OEC (Deumens 2004). However, implantation of such OEC-poly lactide matrices into cavities of the injured rat spinal cord did not lead to clear improvements in functional outcome (Deumens 2006b). A main limitation in the latter approach may have resided in the fact that OEC were cultured on the surface of the matrices before grafting, rather than throughout the matrices' internal 3D micro-architecture.

A major advance in tissue engineering for long distance axon regeneration within the lesioned CNS has been the development of a 3D collagen-based scaffold containing longitudinally orientated micro-channels that demonstrates excellent cytocompatibility with a range of neural cells including dorsal root ganglion neurons, SH-SY5Y neuroblastoma, human neural progenitor-derived astrocytes,

rat Schwann cells, rat astrocytes, and rat OEC (Bozkurt 2007, Bozkurt 2009, Fuhrmann 2010, Mollers 2009). In the present study, OEC-seeded or non-seeded collagen scaffolds were implanted into unilateral 2 mm resection thoracic spinal cord injuries of the adult rat. This model of SCI was selected because of its relevance to study both motor deficits and symptoms of neuropathic pain (Bennett 2000a, Christensen 1996). Lesioned animals that received no treatment served as controls. Motor outcome and allodynia were investigated over a post-surgical period of ten weeks. Motor performance was studied using the conventional BBB locomotor test and the CatWalk gait analysis system. Allodynia was assessed using the von Frey hair filament test.

Animals, methods, and materials

Female adult Lewis rats (Charles River, Germany) weighing 185-220 grams used for the present study were maintained in accordance with the guidelines of the German animal protection statute and experimental protocols were approved by the German governmental ethical committee. Every attempt was made to minimize the animal number as well as any pain or discomfort. Animals were housed under temperature-controlled conditions at 21 ± 1 °C, with a normal 12:12h light/dark cycle with *ad libitum* access to water. A diet restriction protocol (15g chow/rat/day) was used during a 2-week pre-operative period of CatWalk training (see below) and once per week (the day preceding post-operative CatWalk testing). During the rest of the study, food was available *ad libitum*.

The two weeks preceding surgery were used for daily training of animals on a 1.5m long CatWalk runway (Noldus Information Technology, The Netherlands). This training period resulted in rats making consecutive, uninterrupted runs with crossing times between 1.1 and 1.6 seconds. The methodology of CatWalk training is described extensively elsewhere (Deumens 2006a). During the week before surgery, animals were habituated to the von Frey set-up used to obtain paw withdrawal thresholds to mechanical stimulation. This set-up consisted of Perspex boxes positioned on a wire-mesh surface. Animals were allowed to get used to the apparatus for a minimum of 15 minutes before testing. The hindpaw withdrawal threshold (PWT) to mechanical stimulation was assessed using von Frey hair filaments of increasing thickness (0.41, 0.69, 1.20, 1.40, 2.00, 3.63, 5.50, 11.70, 15.14, and 28.84 g) and the up-down method (Chaplan 1994). Following three pre-operative sessions, baseline PWT values were obtained.

Adult GFP-transgenic Lewis rats were used to obtain OEC as described previously (Alexander 2002). Primary cultures of OEC, reaching near confluence by 10 days *in vitro* were enriched using magnetic cell sorting (MACS) and the low-affinity nerve growth factor (NGF) p75 receptor for separation. The OEC were seeded onto 2mm-long cylindrical collagen scaffolds with a diameter of 2mm (Matricel GmbH, Herzogenrath, Germany) at a number of 400,000 cells in a volume of 20 µL. OEC were

allowed to adhere to the scaffold for 30 min before adding growth-factor-containing medium. At 24h after seeding, the OEC-seeded scaffolds were used for transplantation. Control scaffolds were treated identically but without OEC.

Animals received a subcutaneous injection with Buprenorphine (Temgesic 0.1 mg/kg body weight; Schering-Plough, Utrecht, The Netherlands) 30-60 min before surgery. Anesthesia was induced by 5% isoflurane with air as carrier gas at a flow rate of 250 mL/min using a U-400 anaesthesia unit (Agntho's, Lidingö, Sweden) with an open mask system. When the corneal blink reflex and paw withdrawal reflexes were absent, maintenance of anesthesia was set at 2% isoflurane. An ophthalmic ointment was applied to the eyes to prevent drying during surgery. Then, the back of the animal was shaved and disinfected before making a skin incision. A laminectomy of the T10-11 vertebral level was performed, exposing the T13 spinal cord level. The dura was opened gently and four suture stitches (9/0 sutures of monofilament polyamide; 9/0 Ethilon[®], Ethicon Inc., Somerville) were positioned at the corners of the opened dural membrane. A right-sided hemisection, about 2.0 mm long, was created using a no.10 scalpel blade and microscissors. Care was taken to prevent damage to the major dorsal blood vessels or vascular branches. Completeness of hemisection was verified microscopically following aspiration of the lesion site. Animals were divided into three groups at random: (1) receiving no implant (n=10), (2) receiving an empty collagen scaffold (n=10), and (3) receiving an OEC-seeded collagen scaffold (n=10). Dural sutures were used to stabilize the implant after which muscle and skin were closed with 4/0 single suture stitches (4/0 Prolene[®], Ethicon Inc., Somerville). Three animals died during surgery due to respiratory depression and four animals showed inappropriate deficits in both hind paws instead of unilateral deficits (no weight support up until four weeks post-surgery) probably due to local bleeding and/or edema. As a consequence, the numbers of animals were 9 control animals without graft, 7 animals receiving empty collagen scaffolds, and 7 animals receiving OEC-seeded collagen scaffold.

During the first week after surgery, BBB scores were obtained at post-operative day (dpo) 1, 3, 5 and 7, and thereafter on a weekly basis. Although both hindpaws were scored in this test, deficits were exclusively detected in the hind paw ipsilateral to the lesion. Weight support was regarded a prerequisite for reliable testing with the von Frey filaments and this was observed in the hindpaws of all animals from 21 dpo. PWT were obtained at four weeks and at 10 weeks post-surgery. Post-operative CatWalk data were acquired at these same time points (before obtaining PWT to mechanical stimuli).

Behavioral data were analysed using the SPSS 15.0 software. A general linear model (GLM) with repeated measures (for time) used to analyze overall time, group, and time x group interaction

effects (Dunnett's *post-hoc* correction with untreated animals as control reference). In case of an overall time difference, an Analysis of Variance (ANOVA) was used to analyze effects at individual time points (Dunnett's *post-hoc* correction with pre-operative baseline values as control reference). Data are indicated as mean $\pm$ standard error of the mean (SEM). A *p*-value of 0.05 was considered as the level of statistical significance.

Results

Unilateral hemisection of the low-thoracic spinal cord in rat resulted in onset of hypersensitivity to mechanical stimulation of both hind paws, as reported previously (Christensen 1996). Untreated animals showed a decrease in PWT to mechanical stimulation of the ipsilateral hind paw of about 40% and 75% at 4 and 10 weeks after injury, respectively (**Fig. V-1A**). The decrease in PWT of the contralateral hindpaw was delayed compared to the ipsilateral hind paw (**Fig. V-1B**). Statistical testing showed an overall time difference in ipsilateral PWT ($F_{2,20}=36.6$; $p<0.001$) and contralateral PWT ($F_{1,20}=23.5$; $p<0.001$). However, no significant group differences were found, neither for ipsilateral nor for contralateral PWT.

Robust locomotor deficits could be detected using the BBB scale. BBB scores decreased immediately after surgery and a gradual and spontaneous recovery was observed over a period of about two weeks after which a plateau phase was reached. Scores dropped to around 8.5 at five days and around 14.5 at four and ten weeks after injury. BBB scores did not differ for the three different animal groups. At the end point of the investigation (10 weeks after SCI), BBB scores were 13.7 ± 0.6 , 15.3 ± 0.8 , and 14.5 ± 0.7 for untreated animals, scaffold-treated animals, and OEC-scaffold-treated animals, respectively. *Post-mortem* analysis confirmed the reproducibility of the lesion in this investigation with no statistically significant differences in lesion size for the three different animal groups (data not shown).

In order to objectively determine more delicate changes in motor function due to the spinal cord lesion with- or without treatment, CatWalk gait analysis was performed. Importantly, the crossing times required for the CatWalk runs did not differ for time or treatment. This fact is of vital importance to the interpretation of the data, because speed of gait has a strong impact on most gait parameters. We found that unilateral low-thoracic spinal cord injury in the rat affects some, but not all parameters recorded by gait analysis. The stride length (i.e. the distance between two subsequent placements of a single paw) was for example unaffected for both hind paws (data not shown). Also, the regularity index, which is a measure of coordination, remained relatively stable after injury (**Fig. V-1C**). The most prominent motor deficits detected by the CatWalk gait analysis turned out to be the base-of-support (BOS) of the hind paws (i.e. the distance between both hind

paws) (**Fig. V-1D**). Indeed, the animals tended to place their ipsilateral hind paw much more laterally after SCI. This was reflected by an increase in the BOS from about 1.5 cm at pre-injury baseline to about 3.0 cm after injury (**Fig. V-1D**). Statistical analysis showed a strong time effect ($F_{2,20}=82.7$; $p<0.001$), but no group effect. Also the ratio of the print area of the ipsilateral over the contralateral hind paw was significantly reduced following SCI (Time effect: $F_{2,20}=45.8$; $p<0.001$) (**Fig. V-1E**), but without treatment effects. The ratio of paw print intensity (ipsi/contra) was slightly reduced by the SCI (**Fig. V-1F**). However, this decrease did not always reach statistical significance. No group/treatment differences were found for this measure either.

Figure V-1: Effects of OEC-collagen repair strategy of unilateral low-thoracic spinal cord hemisection. A,B: the 50%-paw withdrawal threshold (PWT) is shown at baseline (BL), 4 weeks and 10 weeks after injury for the ipsilateral (A) as well as contralateral (B) hind paw of animals without treatment (open histograms), treated with empty scaffolds (striped histograms), and treated with OEC-scaffolds (dotted histograms). **C-F:** CatWalk parameters are shown for the different animal groups at baseline (BL), 4 weeks and 10 weeks after injury; note that the regularity index is calculated taking all paw into consideration while the base-of-support, print area and intensity are calculated in relation to the hind paws (ratio ipsi/contra for print area and intensity).

Discussion

Transplanted OEC have, for some time, been regarded as having substantial therapeutic potential in SCI and have even been subject to clinical trials (Mackay-Sim 2008). Implanting OEC seems to be of major therapeutic benefits including their supportive effect on the regeneration of many different descending fiber systems (Deumens 2005) and their re-myelination potential. Earlier studies by the group of Raisman had shown that injections of OEC into highly defined, small, electrolytic lesions of the rat corticospinal tract induced substantial recovery of motor function, possibly due to the regeneration of severed corticospinal fibers (Li 1998). The functional benefits of implanting suspensions of OEC have not only been limited to small spinal cord lesions. When implanted into complete thoracic spinal cord transection injuries, donor OEC were found to enhanced weight support and even locomotor function (Ramon-Cueto 2000). Although the completeness of the spinal lesion increases the challenge to spinal cord repair, it does not necessarily represent a good working model for cystic cavitation, which is an important aspect of human SCI. Large spinal cord cavitations require bridging strategies in which donor cell (e.g. OEC) transfer needs an implantable biomaterial and preferably present an orientated substrate capable of promoting efficient and directional axon regeneration. Raisman and co-workers reported on a method of adjusting implanted OEC to include their own dense extracellular matrix, but did not test this method in a model of larger cavitation (Li 2003). An alternative strategy adopted by other research groups has been to use bioengineered scaffolds, from synthetic or biological polymers, to act as cell carriers and guidance frameworks for axon regeneration across SCI lesion cavities. It is still very early for clear statements on the therapeutic potential of such approaches and much more research in this area is needed. We previously found that immediate or delayed implantation of OEC on polylactide-based matrices into thoracic dorsal hemisection cavities did not improve motor function, nor were any regenerative effects of substance observed (Deumens 2006b). An obvious factor influencing the therapeutic effect of this type of tissue engineering intervention strategy is the choice of the material. Polylactic acid may be sub-optimal as it may acidify the implant environment once it breaks down. In recent years, we have focused our attention on the use of a novel, state-of-the-art collagen-based biomaterial, which is highly porous and contains multiple longitudinally orientated microchannels. *In vitro* studies have indicated that this three-dimensional scaffold can be seeded with both neuronal and non-neuronal cells, which readily adhere to and grow within the scaffold (Bozkurt 2007, Bozkurt 2009, Fuhrmann 2010, Mollers 2009). Importantly, OEC were found to migrate and proliferate within the scaffold (Mollers 2009). In the present investigation, we implanted OEC-collagen scaffolds in the cavities created following unilaterally hemisectioning of the low-thoracic spinal cord of adult rats. A major advantage of donor OEC over many other types of graftable cells is their ability to interact

with host astrocytes. Indeed, other cell types, including Schwann cells, form physical as well as molecular boundaries when confronted with astrocytes (Lakatos 2000).

The main aim of our investigation was to study the effect of a tissue engineering strategy on motor and sensory parameters after experimental SCI. Motor deficits and allodynia present two major symptoms which negatively affect the quality of life of an individual living with SCI. Experimental work has shown that treatment approaches which affect plasticity in neural systems may have opposing consequences on pain and motor function (Deumens 2008, Hofstetter 2005). Increased plasticity in severed fiber tracts as well as local spinal cord neuronal circuitries is generally thought to benefit motor functions. Increased plasticity in nociceptive circuits of the spinal cord on the other hand might underlie neuropathic pain (Berger 2011). As such it is of utmost importance to consider both of these two outcomes in preclinical investigations. The use of the CatWalk gait analysis system in the present study allowed a detailed investigation of gait parameters during locomotion of the lesioned animal. Most strikingly, the base-of-support of the hind limbs was increased 2-fold in this injury model. Implantation of OEC-seeded or empty collagen scaffolds into the lesion cavity exerted no demonstrable effect on BOS or any other motor or sensory parameters of the present investigation. Our findings may reflect (1) the difficulty in obtaining functional benefits by approaches to repair cavities in the injured spinal cord, (2) that the concentration of donor cells may not have been sufficient for such a lesions, (3) that trophic support has to be added to the grafting procedure, and/or (4) that some degree of distortion of the scaffold may have occurred or that the chosen lesion model may simply not have been the best for demonstrating implant-induced effects. Despite these rather disappointing preliminary results, we are still hopeful for the future. It seems essential to determine what limits the therapeutic effects of OEC grafting in spinal cord lesion cavities. Obviously, inflammatory mediators may restrict grafted OEC within the inflammatory loci (Su 2009), which may an OEC-supported long-distance regeneration of severed fibers. Concomitant immune modulatory approaches might then be required to optimize the therapeutic effects of this type of cell grafting therapy. Interestingly, immune modulatory therapies such as administration of the TNF-decoy receptor etanercept, effectively reduces allodynia after experimental SCI (Marchand 2009).

In conclusion, the present investigation showed that implantation of a collagen-based scaffold, seeded with OEC or unseeded, did neither improve nor deteriorate motor outcome and allodynia following thoracic spinal cord hemisection in the rat. The most reasonable interpretation of such data is that this particular combination of donor cells and scaffold is not the best for this particular lesion model associated with clinically relevant lesion cavities. As cavitation is closely related to

human SCI, we feel that experimental repair approaches need to ultimately be tested for their efficacy in models containing cavities. Lack of therapeutic effect, then needs to be investigated more thoroughly to improve repair approaches, thereby getting closer to clinical treatments of the severe condition SCI represents.

VI. Evaluation of human neural stem cell transplantation for spinal cord injury therapy in a rat lumbar injury model

Amelioration of motor/sensory dysfunction and spasticity in a rat model of acute lumbar spinal cord injury by human neural stem cell transplantation.

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Stem Cell Research & Therapy

Abstract

Intraspinal grafting of human neural stem cells represents a promising approach to promote recovery of function after spinal trauma. Such a treatment may serve to: 1) provide trophic support to improve survival of host neurons, 2) improve structural integrity of spinal parenchyma by reducing syringomyelia and scarring in trauma-injured regions, and 3) provide neuronal populations to potentially form relays with host axons, segmental interneurons, and/or α -motoneurons. Here we characterized the effect of intraspinal grafting of clinical grade human fetal spinal cord-derived neural stem cells (HSSC) on recovery of neurological function in a rat model of acute lumbar (L3) compression injury. Three-month-old female Sprague-Dawley rats received L3 spinal compression injury. Three days post-injury, animals were randomized and received intraspinal injections of either HSSC, media-only, or no injections. All animals were immunosuppressed with tacrolimus, mycophenolate mofetil, and methylprednisolone acetate from the day of cell grafting and survived for 8 weeks. Motor and sensory dysfunction was periodically assessed using open field locomotion scoring, thermal/tactile pain/escape thresholds and myogenic motor evoked potentials. The presence of spasticity was measured by gastrocnemius muscle EMG response during computer-controlled ankle rotation. At end-point, gait (Catwalk), ladder climbing, and single frame analyses were also assessed. Syrinx size, spinal cord dimensions, and extent of scarring were measured by MRI. Differentiation and integration of grafted cells in the host tissue were validated with immunofluorescence staining using human-specific antibodies. Intraspinal grafting of HSSC led to a progressive and significant improvement in lower extremity paw placement, amelioration of spasticity, and normalization in thermal and tactile pain/escape thresholds at 8 weeks post-grafting. MRI volume reconstruction and immunofluorescence analysis of grafted cell survival showed near complete injury-cavity-filling by grafted cells and development of putative GABA-ergic synapses between grafted and host neurons. Peri-acute intraspinal grafting of HSSC can represent an effective therapy which ameliorates motor and sensory deficits after traumatic spinal cord injury.

Introduction

Extensive experimental and clinical data show that the mechanisms leading to a clinically-defined loss of neurological function after spinal trauma can in general be considered in two categories. First is the pathology and corresponding functional loss resulting from a direct mechanical injury of axons at the injury epicenter, and second is a progressive appearance of secondary changes (local edema, hematoma, excitotoxicity and ischemia) which can evolve over hours to weeks after the initial impact (for review see Hagg & Oudega (Hagg 2006)). Consistent with our current knowledge of the mechanism which leads to the development of secondary post-injury cascade, the current experimental and clinical treatment strategies primarily focus on: 1) improvement of local metabolism and blood flow (e.g., through decompression therapy and hypothermia) (Jones 2012, Oudega 2012), and 2) modulation of local inflammatory response (e.g., with methylprednisolone) (Ankeny 2009, Bracken 2012, David 2011, Fleming 2006).

A separate group of experimental treatment modalities are aimed at improving the local neurotrophic activity at and around the injury epicenter with the primary goal of increasing the survival of partially injured axons and/or neurons. In this category of experimentation, besides the use of locally delivered trophic factors (such as BDNF-, GDNF-, and FGF-peptides or growth factors-gene-encoding vectors) (Lu 2008), regionally grafted fetal or embryonic stem cell-derived neuronal precursors are frequently used (Llado 2004, Sahni 2010). In addition, the use of neuronal precursors for spinal grafting and development of functional synapses between grafted neurons and the neurons of the host can serve to create a functional “relay” system throughout the injured spinal cord region. In these studies, for example, it has been demonstrated that acute grafting of fetal rat spinal cord-derived neuronal and glial-restricted precursors into a site of cervical dorsal column lesion leads to formation of functional ascending spinal relays *in vivo* as validated electrophysiologically and histologically in the dorsal column nucleus (Bonner 2011). Similarly, using electrophysiologically-defined phrenic nerve activity and qualitative histology, functional restoration of the spinal phrenic nucleus circuitry was demonstrated after acute grafting of fetal rat spinal cord-derived neural precursors into the injury epicenter in rats (White 2010). Embryonic rat cerebral cortex-derived neural precursors, grafted acutely or at 4 weeks post-injury into the epicenter of a spinal kainic acid-induced lesion, showed good engraftment up to 12 weeks after grafting (Magnuson 2001). Consistent with histologically and electrophysiologically validated maturation and functional incorporation of grafted neuronal cells in previously trauma-injured or chemically-lesioned spinal cord, a beneficial behavioral effect was also noted in several experimental studies. Acute spinal grafting of mouse neural precursors (isolated from fetal neural tube tissue) into the epicenter of L2 spinal cord compression injury (10 g for 5 min), improved hindlimb motor and

sensory function in mice (Boido 2011). Similarly, acutely grafted human embryonic stem cell-derived oligodendrocyte progenitors and/or motoneuron progenitors into a complete thoracic transection model resulted in partial restoration of hindlimb locomotor function and motor evoked potentials in rats (Erceg 2010). Delayed (7 days post injury) grafting of neurally pre-differentiated mouse embryonic stem cells into quisqualic acid-lesioned lumbar spinal cord led to a reduction in spontaneous and thermally-, mechanically-, or chemically (paw formalin injection)-evoked pain-like behavior in mice (Hendricks 2006).

Recently, well-defined protocols were developed which permit the isolation and long-term stable expansion of (non-immortalized) human fetal brain or spinal cord tissue-derived neural stem cells (Carpenter 1999, Ferrari 2010, Svendsen 1998, Uchida 2000, Vescovi 1999, Yan 2006). Using these protocols, continuing neurogenic potential, as evidenced by neuronal differentiation and the ability of differentiated neurons to generate action potentials *in vitro*, was documented at even high (>20) passage numbers (Guo 2010, Vescovi 1999). Some of these lines were successfully used for: 1) generation of GMP-grade clonally-derived cell lines, 2) extensive pre-clinical evaluation using a variety of neurodegenerative small and large animal models, and 3) subsequently used successfully in Phase I human clinical trials (Jacobs June 2011, Neuralstem Inc. - Emory University 2000, StemCells Inc. 2000 - cited: 2012 Aug 10).

In our previous studies, we have extensively characterized the *in vivo* treatment effect after spinal grafting of cGMP-grade human fetal spinal cord-derived stem cells (NSI-566RSCs line) using a spinal ischemia model in rats and transgenic rat model of ALS (SOD1^{G93A}). In those studies, we have shown that: 1) grafting of NSI-566RSCs into lumbar spinal cord of adult Sprague-Dawley (SD) rats with previous spinal ischemic injury is associated with a progressive improvement of ambulatory function which correlates with long-term grafted cell survival and extensive neuronal differentiation (Cizkova 2007), and 2) bilateral lumbar grafting of NSI-566RSCs in pre-symptomatic SOD1^{G93A} rats provides a transient functional benefit and suppression of α -motoneuron degeneration, i.e., a protective effect which was absent in media-injected animals (Hefferan 2012). Using the same cell line, we have also demonstrated the optimal dosing regimen and safety after grafting into the lumbar spinal cord of immunosuppressed minipigs (Cizkova 2007). The dosing design defined in this pre-clinical minipig study was then subsequently used in a recently completed Phase I human clinical trial in ALS patients receiving lumbar and cervical grafts of NSI-566RSCs (Boulis 2011, Neuralstem Inc. - Emory University 2000). In a more recent study using an immunodeficient rat model of complete spinal cord Th3 transection, it was shown that NSI-566RSCs or rat embryonic neural precursor cells, embedded in a fibrin matrix with trophic factors and grafted one week after injury, were able to form functional

relays. The formation of functional relays was validated behaviorally (BBB locomotor score), electrophysiologically (spinal cord evoked potentials), and histologically (host on graft and graft on host synapses) (Lu 2012).

The goal of our present study was to characterize the effect of NSI-566RSCs grafted spinally in a clinically relevant L3 spinal compression model in continuously immunosuppressed adult SD rats. The presence of treatment effect was assessed by analysis of 1) motor and sensory function, 2) myogenic motor evoked potentials (MEPs), 3) spasticity response during computer-controlled ankle rotation, and iv) qualitative analysis of grafted cell survival and maturation.

Material and Methods

Animals and surgeries

All animal studies were approved by the University of California, San Diego Institutional Animal Care and Use Committee. The study design is outlined in Fig. 1. Twelve-week-old Female Sprague-Dawley rats were anesthetized with isoflurane (5% induction, 1.5-2% maintenance; in room air) and placed into a Lab Standard Stereotaxic frame (Stoelting, Cat# 51600, Wood Dale, IL, USA). The animal was elevated 2 cm by placing it on a homeothermic heating blanket (set at 37°C with feedback from the rectal thermometer, Harvard Apparatus, Cat# 507214, Holliston, MA, USA) which sits on a plastic rectangular block. The animal was then placed in Spine Adaptors (Stoelting, Cat# 51695, Wood Dale, IL, USA) and a wide Th13 laminectomy was performed using an air-powered dental drill and binocular microscope (exposing the dorsal surface of spinal segment L3). An acrylic rod ($\varnothing$ 2.9 mm, length 15 cm; 35 g) was then slowly lowered on the exposed L3 segment until it slightly touched the spinal cord but without inducing any compression. The laminectomy site was then filled with mineral oil in which the tip of a small thermocouple (Physitemp, Cat# IT-14, Clifton, NJ, USA) was submerged and touched the dura. The light from the two fiber optic light pipes of the surgical light (Fiber-Lite, Cat# MI-150 & BGG1823M, Dolan-Jenner, Boxborough, MA, USA) were focused on the surgical site (and directly illuminating the temperature probe). Next, the light intensity was manually regulated so that the spinal cord/mineral oil was warmed to 37°C and remained at $37 \pm 0.3^\circ\text{C}$. If necessary, a 100W infrared lamp was used to gradually adjust and maintain the animal's core temperature at 37°C (rectal). When both temperatures (i.e., paraspinal and rectal) were at $37 \pm 0.3^\circ\text{C}$ for at least 5 min, the rod was slowly lowered until its weight fully rested, perpendicularly, onto the spinal cord. The rod was kept in place for 15 min, while both temperatures were maintained at $37 \pm 0.3^\circ\text{C}$. After spinal compression, the rod and mineral oil was removed and the wound sutured in anatomical layers.

Post-surgical care

Buprenorphine (0.05 mg/kg, s.c., Reckitt Benckiser, Richmond, VA, USA), 5 mL of lactated ringer's, 10 mg/kg of Cefazolin (Novaplus/Sandoz, Holzkirchen, Germany), and standard triple antibiotic ointment to cover the incision site (Bacitracin, Neomycin, Polymyxin B) was given after every surgery. Bladders were manually emptied twice daily (if full). Sulfamethoxazole and Trimethoprim USP oral suspension (200 mg & 40 mg per 250 mL drinking water, Hi-Tech Pharmacal, Amityville, NY, USA) was given for at least 10-14 days after SCI or until autonomic bladder voiding occurred and for 1-2 days after any other surgery (sham or grafting). Food was provided by placing it at the bottom of cage and water bottles with an elongated drinking tube were used, until regular overhead supplies could be reached by the animal. Animals diagnosed with bacterial infections throughout the study were treated with Sulfamethoxazole (as above), 10 mg/kg/day of Cefazolin, and lactated ringer's 5 mL/0.5 day.

Cell derivation and preparation

The cells, named "NSI-566RSC", were produced by Neuralstem Inc. (Rockville, MD, USA), as described before (Hefferan 2011). Briefly, human spinal cord neural precursors (HSSC) were prepared from the cervical-upper thoracic region obtained from a single eight week fetus. Meninges and dorsal root ganglia were removed and dissociated into a single cell suspension by mechanical trituration in serum-free, modified N2 media (human plasma apo-transferrin, recombinant human insulin, glucose, progesterone, putrescine, and sodium selenite in DMEM/F12). For growth of the HSSC, 10 ng/ml basic fibroblast growth factor (bFGF) was added to the modified N2 media and expanded serially as a monolayer culture on poly-D-lysine and fibronectin (Johe 1996). Approximately 6.1×10^6 total cells were obtained upon the initial dissociation of the spinal cord tissue. The growth medium was changed every other day. The first passage was conducted 16 days after plating. At this point, the culture was composed mostly of post-mitotic neurons and mitotic HSSC. Mainly the mitotic cells were harvested through brief treatment with trypsin and subsequent use of soybean trypsin inhibitor. The cells were harvested at approximately 75% confluence, which occurred every 5-6 days (20 passages). At various passages, the cells were frozen in the growth medium plus 10% dimethyl sulfoxide at $5-10 \times 10^6-10$ cells/ml. The frozen cells were stored in liquid nitrogen. Upon thawing, the overall viability and recovery was typically 80-95%. A cell bank of passage 16 cells was prepared and used for this study.

For the production of GFP-labeled NSI-566RSC, a Lentiviral vector was constructed containing the human Ubiquitin C promoter driving expression of enhanced green fluorescent protein. Viral particles produced by infected 293FT cells were collected after overnight incubation, then

concentrated by centrifugation and stored frozen. Neural stem cell cultures were infected by overnight incubation in growth medium supplemented with viral supernatant. Infected stem cells were washed with PBS and cultured as described above. After multiple passages, >90% of cells were EGFP positive (assessed after immunohistochemical staining). A cell bank of passage 17 cells was prepared and used for this study.

One day prior to each grafting day, one cryopreserved vial of the previously prepared cells was thawed, washed, concentrated in hibernation buffer, and shipped from the cell preparation site (Neuralstem, Inc., Rockville, MD, USA) to the surgery site (University of California, San Diego, CA, USA) at 2-8°C by overnight delivery. Upon receipt the following day, the cells were used directly for implantation without further manipulation. Before and after implantation, the viability of cells was measured with trypan blue (0.4%; Sigma). Typically, a >85% viability rate was recorded.

Inclusion and exclusion criteria, randomization and blinding

Three days following SCI and prior to grafting, animals were randomly divided into three groups: the vehicle-injected group, non-injected group, or the HSSC-injected group. SCI animals with an open-field locomotion score of ≤ 1 and appearing healthy enough were included. Animals found moribund or automutilating at any point during the study were excluded and euthanized. A total of 42 animals were employed and divided into 6 experimental groups, as follows:

Group A (n=14): SCI animals-NSI-566RSC-grafted,

Group B (n=10): SCI animals-vehicle-injected,

Group C (n=8): SCI animals-non-injected,

Group D (n=6): sham operated (laminectomy only),

Group E (n=6): naïve animals (no surgical manipulation)

Group F (n=2): SCI athymic animals-ubiquitin.eGFP⁺ NSI-566RSCs-grafted.

One animal was excluded in Group A because of automutilation of the hind paw; two animals were excluded in Group C, one because of automutilation of the hind paw and 1 because of bacterial infection. Six animals had been replaced before dosing/randomization, 5 due to inadequate injuries and 1 because of bacterial infection.

Figure VI-1: Schematic diagram of experimental design. A: To induce spinal cord injury, a 35 g circular rod was placed on the exposed L3 spinal segment and spinal cord compressed in dorso-ventral direction for 15 min. B: Three days after injury, animals were randomly assigned to experimental groups and received spinal graft of HSC or media only. A total of 12 injections were performed targeting the injury epicenter and adjacent areas (Spinal Injection Map). C: After spinal injections, animals survived for 2 months while being continuously immunosuppressed and periodically tested for recovery of motor/sensory functions, changes in motor evoked potentials (MEPs) and gastrocnemius muscle spasticity response evoked by computer-controlled ankle rotation. D: At 2 months after treatment, animals were perfusion fixed with 4% paraformaldehyde and spinal cord MRI-imaged *in situ* before histological processing. E: After MRI imaging, spinal cords were dissected from the spinal column and spinal blocks prepared for plastic embedding (injury epicenter region) or cryostat sectioning and used for immunofluorescence staining (the regions just above and below the injury epicenter).

Grafting procedure

For the intraparenchymal injections, the animals were placed in the stereotactic frame as described above. The L3 spinal cord (i.e., the dura mater) was then re-exposed at the previous laminectomy site. Injections were performed using a 33 gauge beveled needle and 100 μ L Nanofil syringe (World Precision Instruments, Cat# NF33BV & Nanofil-100, Sarasota, FL, USA) connected to a microinjection unit (Kopf Instruments, Cat# 5000 & 5001, Tujunga, CA, USA). The duration of each injection was ≥ 45 sec followed by a ≥ 30 sec pause before slow needle withdrawal. The center of the injection was targeted intermediate of the ventral and dorsal horn and close to the lateral funiculus (distance from the dorsal surface of the spinal cord at L3 level: 0.80 mm). Twelve injections (20,000 cells/ μ L) were done; four injections (0.5 μ L each, 0.8-1.0 mm apart, rostrocaudally) at each lateral boundary of the injury (8 in total), plus two (bilateral) injections (0.5 μ L each) 1.5 mm caudal from the previous, most caudal injections, and two injections at the core of the epicenter (1 μ L at each side of the dorsal vein, bilaterally; see diagram in **Fig. VI-1**). After the injections, the incision was cleaned with penicillin-streptomycin solution and sutured in two layers.

Immunosuppression

Two days after injury (i.e., 1 day before grafting), a methylprednisolone acetate (Depo-Medrol, 10 mg/kg, i.m.) was given, which was repeated thereafter 3 times with 1 mg/kg/week i.m. Starting directly after grafting, all animals received 3 mg/kg/BID s.c. of Tacrolimus (Prograf/FK506, Astellas, Deerfield, IL, USA) until the end of the study. For post-transplant days 0-10, the animals also received 30 mg/kg/day s.c. of Mycophenolate mofetil (CellCept, Genentech, CA, USA). Immunosuppression was also given to the non-grafted Sprague-Dawley animals (i.e., the naïve, sham operated, and all SCI-control animals).

Open field locomotion testing

Locomotion recovery after spinal cord contusion injury was monitored using a modified Basso, Beattie, Bresnahan (BBB) open field locomotion rating scale (Basso 1995). The BBB score was modified to reflect the distinct locomotor recovery stages observed after L3 SCI. The modified score entailed 8 well-defined degrees of locomotor recovery: 0-1: are identical to the BBB-score, 2: is cumulative score of 2 and 3 of the BBB score, 3: is cumulative score of 4, 5 and 6 of the BBB score, 4: is cumulative score of 7 and 8 of the BBB score, 5: reflects weight support with poor paw clearance, 6: is broadened and/or shortened stepping, and 7: is normal walking. In the present study, the locomotor score was obtained before grafting and weekly after injury until the end of the study (i.e., 8.5 weeks post-injury). In addition to a modified BBB score, a regular full 21 scale BBB score was periodically assessed.

Gait analysis

The CatWalk apparatus (CatWalk 7.1, Noldus Technology, The Netherlands) was used to quantify gait parameters during walkway crossings (e.g., paw positioning, base of support, stride length, front limb vs. hind limb coordination) by footprint analysis (Deumens 2006b). Animals had to walk down a horizontal glass walkway ($109 \times 15 \times 0.6$ cm, L×W×H), of which the glass is illuminated along the long edge. At the end of the walkway, animals had access to their home cage and were given a treat upon arrival (Certified Supreme Mini-Treats™, Cat# F05472-1, Frenchtown, NJ, USA). The light only enters the (side of the) glass and reflects merely internally (when the glass is bordered by air). As an animal walks on the glass walkway, light reflects off of the animal's paws, producing a series of bright footprints when viewed through the glass, from below the walkway. The illuminated footprints were then recorded by a video camera with a wide-angle objective that was located underneath the elevated glass walkway. In order to get an optimal contrast between the paws and the surroundings, the test was performed in a room that was totally darkened. The animals were trained for smooth walkway crossing on the 5 days prior to the video acquisitions. To obtain accurate and meaningful data, the following criteria concerning walkway crossings needed to be met: (1) the animal needed to walk uninterrupted across the walkway, at a constant pace, and (2) a minimum of three such crossings per animal were required. Animals without bilateral paw clearance could not be analyzed (n=4 control-SCI animals, and 3 HSSC-treated animals). Digital data analysis consisted of assigning labels (left-fore, left-hind, right-fore, or right-hind) to the animal's paw prints in a recorded walkway crossing, using dedicated CatWalk software. Next, the software calculated gait parameters. Data from the three proper crossings was averaged for statistical analysis.

Inclined ladder test

The inclined ladder test was performed as described before (Apostolova 2006, Semler 2011). Using an inclined ladder (55°) with twenty 120 mm wide rungs (diameter: 1/4"), spaced at equal intervals (60 mm), and having 150 mm-high side walls was used. The rats were trained for this test so that smooth runs were recorded. At the end of the ladder, the animals had access to their home cage and received a treat (as above). The rats were placed at the bottom, and in front, of the ladder. The bottom of the ladder was placed on a 20 cm elevated platform. Climbing was video recorded from a position below the ladder, so that the ventral aspect of the animal is recorded. All animals were able to climb up the ladder. The correct placing of a hind paw and sustained position until its next forward move was counted over 18 rungs (placement on first and last rung not counted).

Single frame hind limb motion analysis

Two parameters were measured in bilateral video captures of animals crossing a runway: the foot-stepping angle (FSA) and the rump-height index (RHI), as described before (Apostolova 2006, Semler 2011). The FSA is the angle at which the hind paw is placed on the ground just after the swing phase. The angle is defined by a line parallel to the dorsal surface of the paw and a horizontal line behind the paw. Four to six measurements were made for each hind limb (a total of 8 to 12 step cycles). The RHI was defined as the highest point of the base of the tail during the (recorded part of the) run. The values for the left and right paw of each animal were averaged. The elevated runway bar was made of a wooden plate/beam (1500 × 150 × 20 mm, L×W×H). The animals were trained to smoothly walk the beam. Once more, at the end of the beam the animals had access to their home cage and received a treat (as above). The videos (i.e., the selected frames) were selected and analyzed using the video tool VirtualDub 1.9.11 (Written by Avery Lee, <http://www.virtualdub.org>) and the on-screen measurement tool Screen Ruler V1.0.1a (<http://www.caveworks.net>).

Myogenic motor evoked potentials

Animals were anesthetized with ketamine (80 mg/kg i.p., Ketaset, Fort Dodge Animal Health, Overland Park, KS, USA). Myogenic Motor Evoked Potentials (MEPs) were elicited by transcranial electrical stimulation (with a pulse duration of 1 ms at 7 mA using a DS3 constant current isolated stimulator (Digitimer LTD., Welwyn Garden City, UK)) of the motor cortex using two percutaneously placed 30G stainless steel stimulation electrodes. Responses were recorded from the gastrocnemius muscle using 30G platinum transcutaneous needle electrodes (distance between recording electrodes ~1 cm; Grass Technologies, Astro-Med, Inc., West Warwick, RI, USA). Recording electrodes were connected to an active headstage (3110W Headstage, Warner Instruments LLS) and signal amplified using DP-311 differential amplifier (Warner Instruments LLS). Amplified signal was acquired by the PowerLab 8/30 data acquisition system (AD Instruments, Inc., Colorado Springs, CO) at sampling frequency of 20 kHz, digitized and stored in PC for analysis. MEPs were measured until three to five highest (stable) recorded potentials were similar. Those traces were averaged per animal and multiplied by one thousand (μ V; all values >1). Next, for data normalization, a logarithmical transformation was applied for further analysis (amplitudes of MEP traces tended to vary much more in animals with higher MEPs amplitudes).

Measurement of muscle spasticity

At 1.5 weeks and 2 months post-injury, the presence of muscle spasticity in the lower extremities was measured using a previously described system (Marsala 2005). Briefly, animals were placed in a restrainer and a hindpaw was taped to a rotational metal plate driven by a computer-controlled

stepping motor. The metal plate is interconnected loosely to the “bridging” digital force transducer (LCL454G, 0–454 g range; Omega, Stamford, CT, USA). The resistance of the ankle to dorsiflexion was measured during stepping motor-driven ankle dorsiflexion (40°; MDrive 34 with onboard electronics; microstep resolution to 256 microsteps/full step; Intelligent Motion Systems, Marlborough, CT, USA) at 3 different ankle-rotational velocities (40, 60 or 80°/sec). The EMG signal was recorded from the ipsilateral gastrocnemius muscle during the same time frame. To record EMG activity, a pair of tungsten electrodes was inserted percutaneously into the gastrocnemius muscle 1 cm apart. EMG signals were bandpass filtered (100 Hz to 10 kHz) and recorded before, during, and after ankle dorsiflexion. EMG responses were recorded with an alternating current-coupled differential amplifier (model DB4; World Precision Instruments, Sarasota, FL, USA). EMG was recorded concurrently with ankle resistance measurements, both with a sample rate of 1 kHz. Both muscle resistance and EMG data were collected directly to the computer using custom software (Spasticity version 2.01; Ellipse, Kosice, Slovak Republic). Each recorded value was the average of three repetitions. The presence of spasticity response was identified as an increased ankle resistance and concurrent increase in recorded EMG activity during computer-controlled ankle dorsiflexion. To measure the contribution of “mechanical” component in measured resistance (i.e., caused by ankle ankylosis in chronically paraplegic animals), animals were anesthetized with isoflurane at the end of each recording session and the relative contribution of neurogenic (i.e., isoflurane-sensitive) and mechanical (i.e., isoflurane non-sensitive) component identified. The magnitude of antispasticity effect after any manipulation was then expressed as the maximum possible anti-spasticity effect measured under isoflurane anesthesia minus the value of the mechanical component.

Sensory testing

Recovery of sensory function was assessed through quantification of supraspinal “above-level” escape response (AL-ER; i.e., an escape or escape-attempt with incorporation of the forelimbs) thresholds to 1) a gradually increasing force to the hind paws (using the Analgesy-Meter, no disc weights added; Cat# 37215, Ugo-Basile, Collegeville, PA, USA), and 2) AL-ER latencies to a constant heat stimulus (intensity 17, cut-off at 30 sec) to the hind paws (using an constant infrared heat source; Cat# 37360, Ugo-Basile, Collegeville, PA, USA). The hind paw tested was gently restrained by the investigator to prevent withdrawal. For the heat perception test the apparatus was switched on ≥ 15 min prior to testing, to allow it to warm up.

For the AL-ER tests, both hind paws were tested four times, alternately, for each test, with a testing interval of ≥ 1 hour. No more than four measurements per day were performed, rendering two testing days per test. Maximum cut-off values for the stimuli or latency were at approximately two

times that of response threshold of uninjured animals, to prevent tissue damage. Prior to (one week) and during the experimental period, the animals are extensively habituated to the experimenter so that the animals can be held upright (loosely) during all sensory assessments. Habituation consists of picking the animal up and holding/handling it twice daily for ≥ 3 minutes. Subsequently, in absence of a stimulus, animals did only rarely show escape behavior when held for the time it would take to reach cut-off values. We measured the AL-ER thresholds/latencies before injury (baseline) and every 2nd week after injury. The final measurement was done at eight weeks post-injury. Two or less (out of the total of eight, bilateral) measurements could manually be assigned as outliers and be excluded per time point (done while blinded for time point, animal, and treatment group). In addition, individual scores were log transformed before analysis and we calculated the Maximal Possible Effect, using these log scores, as previously suggested (Mills 2012). Hence, we used the standard formula to calculate the Maximal Possible Effect, and assuming a logarithmic relation between stimulus intensity and perceived intensity:

$$100 \times \frac{\log(x_{final}) - \log(\bar{x}_{final \text{ of SCCI control animals}})}{\log(\bar{x}_{baseline \text{ of SCCI animals}}) - \log(\bar{x}_{final \text{ of SCCI control animals}})}$$

Here, x_y is the average AL-ER threshold of an individual animal at time point y (either for a thermal or mechanical stimulus).

Magnetic resonance imaging

Eight weeks after cell grafting, rats were deeply anesthetized with 2 mg pentobarbital and 0.25 mg phenytoin (0.5 mL of Beuthanasia-D, Intervet/Schering-Plough Animal Health Corp., Union, NJ, USA) and transcardially perfused with 200 ml of heparinized saline followed by 250 ml of 4% paraformaldehyde (PFA) in PBS. A 3 cm piece of the vertebral column (Th8-L1) was placed in a tight small latex container filled with 4% PFA to prevent the formation of air bubble/tissue interface artifacts. Samples were scanned using Magnetic Resonance Imaging (MRI). Images were acquired using a 7T Bruker (Bruker Biospin Billerica, MA) horizontal bore small animal magnet, and a 2.5 cm imaging volume transmit/receive coil. A 3D turboRARE sequence was used with the following imaging parameters: Echo time/Repetition time 45/1500 ms, Flip angle 180 degrees, field of view $16 \times 16 \times 16$ mm, matrix $256 \times 256 \times 70$ with a resulting voxel size of $62 \times 62 \times 229$ microns. The imaging time was 84 min per sample.

Volume reconstructions and calculations were done using Amira software (Visage Imaging GmbH, Berlin, Germany).

Axon counting in plastic semi-thin sections

After MRI imaging, spinal cords were dissected from the spine, transverse (1.5-mm-thick) spinal cord block cut from the injury epicenter, was then removed from the spinal cord and prepared for plastic embedding as previously described (Navarro). Briefly, dissected tissue blocks were treated with 0.1% osmium tetroxide in 0.1 M non-saline phosphate buffer (pH 7.4) for 12 h, followed by adequate rinsing in non-saline phosphate buffer. This was followed by progressive alcohol dehydration according to standard procedures up to 100% ethanol, with the addition of further dehydration in a 1:1 solution of ethanol/propylene oxide, and lastly in 100% propylene oxide. Dehydrated blocks were then prepared for resin infiltration by incubation in a 1:1 solution of resin/propylene oxide on a rotator in a fume hood overnight. The resin solution used consisted of: Eponate 12, Araldite 502, dodecenyl succinic anhydride, and 2,4,6-tri [dimethylamino-methyl] phenol (DMP-30; Ted Pella, Inc., Redding, CA), mixed in ratios of 10:10:25:1, respectively. The blocks were then transferred to 100% resin for subsequent overnight infiltration on a rotator. Finally, the tissue blocks were embedded using fresh resin in multi-chamber silicone rubber molds made from a Silastic® E RVT Silicone Rubber Kit (Dow Corning Corp., Midland Township, MI). The molds with embedded sections were placed in an oven at 60°C for 1 day to facilitate resin polymerization. Semi-thin (1 μ m) transverse sections were then cut using a microtome (Leica Supercut RM 2065) with a 8-mm diamond knife (Histo Diamond Knife, Cat# LM 7045, DiATOME, Hatfield, PA, USA). The sections were mounted on slides with distilled water and allowed to dry on a slide warmer. Prior to staining, the slides were incubated at 60°C in an oven for 10-15 min, and then contrast-stained with 4% para-phenylene-diamine (PPD).

Mosaic images were taken of two sections per animal at 20X using a Zeiss Imager.M2 fitted with a Zeiss MRm camera (Carl Zeiss Microscopy, Thornwood, NY, USA), a BioPrecision2 stage (Cat# 96S100, Ludl Electronic Products, Hawthorne, NY, USA), and Stereo Investigator software (MBF Biosciences, Williston, VT, USA). Complete mosaic images were loaded into ImageJ 1.45s. Axonal quantification involved manual definition of pixel threshold (0-255, grayscale; using the Triangle method). Next, ImageJ's Analyze Particles option was used to find particles with a size of 0.20-250 μ m² and a circularity of 0.5 to 1.0 (which corresponded to axons). All acquisition and analysis values were held consistent throughout the study. Final measurements acquired were the minimal diameter (Feret's) of each particle (and particle counts). Particles with a minimum diameter >10 μ m were excluded. Employment of this parameter allowed for further axonal analysis, in which axons were divided into empirically-derived caliber sizes of small, medium, and large axons (0.3–1.0 μ m, 1.0–2.5 μ m, and 2.5–10 μ m, respectively). Data was acquired per spinal region (i.e., dorsal, ventral, and lateral funiculi).

Immunofluorescence staining

After removing the 1.5 mm block from the spinal cord at the injury epicenter, the remaining caudal and rostral parts of the spinal cord (± 1 cm each) were placed in 30% sucrose for cryoprotection for a minimum of 5-7 days. Transverse spinal cord sections were then prepared from the L6 segment. The segment(s) in between the L6 and the injury epicenter and the one rostral to the injury epicenter were sectioned coronally and used for identification of grafted human cells. All sections were cut on a cryostat and stored free-floating in PBS with thimerosal (0.05 wt%).

Sections were stained overnight at 4°C with primary human-specific (h) or non-specific antibodies in PBS with 0.2% Triton X-100: mouse anti-Nuclear Mitotic Apparatus (hNUMA, 1:100; Millipore, Billerica, MA, USA), mouse anti-Neuron Specific Enolase (hNSE, 1:500; Vector Labs, Burlingame, CA, USA), mouse anti-Synaptophysin (hSyn, 1:2,000; Millipore), rabbit anti-Glial Fibrillary Acidic Protein (hGFAP, 1:500; Origene, Rockville, MD, USA), mouse anti-Neuronal Nuclei (NeuN, 1:1,000; Millipore), chicken anti-Green Fluorescent Protein (GFP, 1:1,000; Aves Labs,), rabbit anti-Anti-Glutamate Decarboxylase 65 & 67 (GAD65&67; 1:300; Millipore), mouse anti-GFAP (Cy3-labeled; 1:500; Sigma-Aldrich; St. Louis, MO, USA), rabbit anti-Ki67 antibody (mitotic marker, 1:100; Abcam), and rat anti human axonal neurofilament antibody (hHO14; 1:100; gift from Dr. Virginia Lee; University of Pennsylvania). Mouse anti-Growth Associated Protein 43 (GAP43, 1:16,000; Millipore), rabbit anti-Calcitonin Gene-Related Peptide (CGRP, 1:1,000; Biotrend, Destin, FL, USA), and rabbit anti-Ionized calcium Binding Adaptor molecule 1 (Iba1, 1:1,000; Wako, Richmond, VA, USA), were used on the L6 transverse sections. Following washing in PBS 3-5 min, sections were incubated with fluorescent-conjugated secondary donkey antibodies (Alexa® Fluor 488 & 647; 1:500; Jackson Immuno Research, West Grove, PA, USA; & Alexa® Fluor 555, 1:500; Invitrogen). Sections were then mounted on slides, dried at room temperature, and covered with Prolong anti-fade kit (Invitrogen Corp., Carlsbad, CA, USA).

Confocal images (1024 × 1024 pixels) were captured with a Fluoview FV1000 microscope (Olympus, Center Valley, PA, USA) with a 20X or 40X objective, optical section spacing of 0.5 μ m, and pulse speed of 20 μ sec/pixel. Other images were taken using the Zeiss Imager.M2 setup as described above, using a 10, 20 or 63X magnification. CGRP, GAP43, and Iba1 stainings on L6 transverse sections were quantified using densitometry measurements of the main dorsal horn region (Laminae I through IV; area as marked in **Fig. VI-7B**). ImageJ software was used for quantification by using the Background Subtraction function.

Statistical analyses

Behavioral data were analyzed using ANOVA (one-way, or two-way group $\times$ time repeated measures, using a fixed-effect model, and a Bonferroni *post hoc* test for multiple comparisons). A *P* value of 0.05 was considered significant. Unequal variances were explored prior to using ANOVA analyses using the Bartlett's test, but were not identified. *Post hoc* tests were only calculated if overall group differences were found. Results are expressed as means with the standard error of the mean (SEM). To analyze differences between the two groups (e.g., vehicle injected vs. non-injected SCI animals), we used Student's *t*-tests (unequal variances were explored with the *F*-test, but not found) or repeated measures ANOVA. Naïve and sham operated animals were grouped (and named "non-injured") in all outcomes besides the sensory tests. All statistical analyses were done using GraphPad Prism (La Jolla, CA, USA), SPSS statistics 17 (for K-Means clustering; IBM, Armonk, NY, USA), or STATA 12 (for precise post-hoc test *P*-value calculations; StataCorp LP, College Station, TX, USA) and performed two-tailed.

Results

General animal health and survival of animals during long-term immunosuppression

From the total of 35 SCI Sprague-Dawley rats employed in this study, 32 survived until planned sacrifice while continuously immunosuppressed; 14 NSI-566RSC-injected (1 excluded; because of automutilation of hind paw on day two post-injury), 10 vehicle-injected, 8 non-injected (2 excluded; 1 because of automutilation of hind paw on day 7 post-injury & 1 because of excessive BW loss on post-injury day 18 (likely related to immunosuppression-related toxicity)). In four surviving animals, lower extremity ulcers developed but were effectively treated with local standard triple antibiotic ointment (Bacitracin, Neomycin, and Polymyxin B) and cohesive bandages. In most animals, the Crede's maneuver needed to be performed for 3-5 days after spinal trauma (exceptions: 3 animals in NSI-566RSC-injected group and 2 animals in non-injected SCI-control group, of which one died due to health issues; see above). No additional worsening (i.e., a lowering in open-field locomotor scores at one day post-grafting, compared to pre-grafting values) was noted in intraspinal media- or cell-injected animals.

Spinal injection procedure did not alter neurological outcome in previously L3-contused rats

In order to define the effect of spinal injection itself in modulating the functional recovery profile (i.e., potential worsening in neurological outcome) in L3-injured animals, we have first compared the effect of spinal media injection only with spinal injury animals which received no injections (10 vehicle-injected and 8 non-injected SCI animals). No significant differences were found between

these two groups in any of neurological or electrophysiological outcome measures used in this study (repeated measures ANOVA for open field locomotor scores; Student's *t*-test for others). Based on these data, which showed no significant differences between both control groups, these two groups were then pooled into one control group and used for subsequent comparison with HSSC-grafted animals.

Assessment of motor function

Gait analysis was conducted at 8 weeks after grafting (or corresponding time point in controls) using the Catwalk apparatus (Koopmans 2005). The following parameters were analyzed: 1) runway crossing time, 2) rostro-caudal hindpaw positioning, 3) hind paws base of support, 4) regularity index/coordination, 5) stride length, and 6) phase dispersions.

Rostro-caudal hindpaw positioning (RCHPP): In control non-injured animals, the RCHPP was 0 ± 1.7 mm (i.e., the animals are able to achieve a near complete overlap in the hindpaw positioning relative to the last ipsilateral frontpaw print; full rostro-caudal overlap is represented by a value of "0"). Rats receiving spinal HSSC grafts showed significantly better RCHPP, when compared to control SCI animals (-9.0 ± 1.9 vs. -18.2 ± 3.1 mm, respectively, **Fig. VI-2A**; Bonferroni: $P=0.04$). Examples of the paw positioning are shown in **Fig. VI-2B** for a non-injured control, SCI control, and a HSSC-treated SCI animal (**Fig. VI-2-B1, -B2, and -B3**, respectively).

No significant differences were detected in other Catwalk parameters (runway crossing time, hind paws base of support, regularity index/coordination, stride length, phase dispersions), myogenic motor evoked potentials, or behavioral motor tests (open field locomotor score (modified BBB score, and regular BBB scores), single-frame motion analysis or ladder climbing test) (**Fig. VI-8A-D**).

To identify the presence of spasticity (i.e., potentiation in muscle stretch-evoked EMG activity) in animals after SCI, a computer-controlled ankle-rotational force was applied on the right or left paw in fully awake restrained animals and resulting change in EMG activity in the gastrocnemius muscle and correlative ankle resistance was measured (Marsala 2005).

Independent of SCI group (control or HSSC-injected), two quantitatively different EMG patterns and corresponding resistance response (EMG/RES) patterns were recorded in spinally-injured animals. First, if compared to control non-injured animals, little or no change in EMG/RES response was seen at 1.5 weeks after SCI. Second, SCI induced an increased spasticity response in a portion of the animals at 1.5 weeks after injury. A K-Means clustering method was used to group all 44 (SCI and non-injured) animals into 2 groups based on magnitude of resistance to ankle rotation at 1.5 weeks post-injury (or equivalent time point in non-injured animals).

Figure VI-2: Improvement in paw positioning and muscle spasticity. A: The gait analysis of hind paw positioning at 2 months after treatment. In comparison A significant improvement was seen in HSSC-grafted animals. B1-B3: Example of the sequence of paw step images in naïve (B1) SCI-control (B2) and SCI-HSSC-treated animals (B3). Note a near complete paw footprint overlap between the front and the hind paws in naïve animals (B1) but a substantial dissociation in the paw footprint overlap in SCI controls (B2). An improvement in paw footprint placement in SCI-HSSC-treated animals can be seen (B3). C: Analysis showed significant suppression of spasticity response (expressed as a muscle resistance ratio: values at 2 months vs. 7 days post injury in “HIGH spasticity” HSSC-treated animals if compared to “HIGH spasticity” controls. D: To identify the presence of muscle spasticity in fully awake animals, the hind-paw ankle is rotated 40° at a velocity of 80°/sec. The spasticity response is identified by exacerbated EMG activity measured in the gastrocnemius muscle and corresponding increase in muscle resistance. In control SCI animals with developed spasticity (i.e., “high spasticity”/HIGH group), no change in spasticity response if compared to 7 days post-vehicle injection was seen at 2 months (compare D1 to D3). In contrast, a clear decrease in spasticity response was seen in SCI-HSSC-treated animals at 2 months after cell injections (compare D4 to D6). To identify the presence of mechanical resistance, animals are anesthetized with isoflurane at the end of the recording session and the contribution of mechanical resistance (i.e., isoflurane non-sensitive) is calculated. (D2, D5: data expressed as mean±SEM; one-way ANOVAs).

Seven animals of each SCI group (i.e., control or HSSC-injected) were found to be clustered in the high “spasticity” group (HIGH), which had a 31.7 ± 3.9 g increase in measured muscle resistance during ankle rotation, compared to the low “spasticity” group (LOW) showing 8.9 ± 1.5 g resistance (Student’s *t*-test: $P < 0.0001$). No difference in the incidence of this high “spasticity” response was noted between SCI control vs. cell-treated groups (incidence: χ^2 : $P = 0.53$; extend: Student’s *t*-test: $P = 0.24$). No naïve or sham operated animals were found to be clustered into the HIGH group. Resistance to ankle rotation measured 8 weeks after treatment (and expressed as relative change from 1.5 weeks post-injury values) showed a significant decrease in the HSSC-injected HIGH resistance group when compared to HIGH resistance animals from the control SCI group (**Fig. VI-2C**; i.e., decline of 24.8 ± 6.4 g in HSSC-injected animals and 4.8 ± 6.3 in control SCI animals; Bonferroni: $P = 0.048$).

Fig. VI-2D shows an example of raw data depicting a post injury EMG response (red channel) and corresponding increase in muscle resistance (black channel) during ankle rotation in a SCI-control (**Fig. VI-2D1-3**) and a HSSC-injected animal (**D4-6**) at 7 days after treatment and at the end of the 8-week survival. Clear suppression of potentiated EMG response and muscle resistance can be seen in HSSC-treated animals (compare **D4** to **D6**). To identify and dissociate neurogenic (i.e., isoflurane-sensitive) versus mechanical (i.e., isoflurane non-sensitive) components, muscle resistance was re-measured after isoflurane anesthesia and the relative contribution of mechanical component calculated. The induction of isoflurane anesthesia near completely blocked the ankle rotation-evoked EMG response and resulting increase in muscle resistance (**D2, D5**).

Assessment of sensory functions

Analysis of mechanical and thermal sensory function was performed by comparing hindpaw thresholds improvements of evoked above-level/supra-spinal withdrawal responses (i.e., an escape response in which the frontlimbs and/or vocalizations are used) between experimental groups over several time points. Groups consisted of naïve control, sham operated control, SCI-control, or SCI-HSSC-injected animals. Response thresholds were measured before injury and every second week thereafter. No differences were measured between naïve and sham-operated animals at any time point in response thresholds to both mechanical and thermal stimuli (repeated measures ANOVA).

Prior to injury, no differences in mechanical thresholds to trigger escape responses were measured between all four experimental groups (on average 92 ± 2 g). After SCI, the thresholds increased significantly in both SCI-control and SCI-HSSC-injected animals compared to control non-injured groups, at all time points (Bonferroni; $P < 0.001$). From 4 weeks post injury, SCI-HSSC-injected animals displayed a trend towards progressive improvement in response thresholds if compared to SCI

controls (at eight weeks: 177 ± 10 g and 216 ± 10 g, respectively; **Fig. VI-3A**; repeated measures ANOVA: $P=0.14$). This resulted in a significant higher percentage of the Maximal Possible Effect for improvement of mechanical stimulus perception in SCI-HSSC-injected animals compared to SCI-control animals (**Fig. VI-3C**; Student's t -test: $P=0.03$).

Prior to SCI, measurement of thermal (infrared) stimulus-evoked paw withdrawal threshold showed no significant differences between all experimental groups (17.3 ± 0.3 sec; one-way ANOVA). At two weeks post SCI, significant increase in paw withdrawal latencies in both the control SCI group and in SCI animals receiving spinal HSSC grafts were measured, when compared to control non-injured (sham operated and naive) groups (26.1 ± 0.7 sec in SCI-control animals and 26.5 ± 0.7 sec in HSSC-grafted animals vs. 18.3 ± 0.2 sec in control non-injured animals; **Fig. VI-3B**; Bonferroni: $P<0.001$).

From four weeks after treatment a trend towards a progressive normalization in response threshold was seen in HSSC-treated animals if compared to SCI controls (at eight weeks: 24.0 ± 0.9 sec in SCI-control and 21.4 ± 0.9 sec in HSSC-injected animals, respectively; repeated measures ANOVA: $P=0.09$). This resulted in a significantly higher percentage of the Maximal Possible Effect for the improvement of thermal stimuli in SCI-HSSC-injected animals compared to SCI-control animals (**Fig. VI-3C**; Student's t -test: $P=0.02$).

Figure VI-3: Amelioration of hypoesthesia in SCI-HSSC-grafted animals. A & B: Baseline and biweekly assessments of perceptive thresholds for (A) mechanical and (B) thermal stimuli, applied below the level of injury, showed a trend towards progressive recovery in SCI-HSSC-grafted animals. C: When expressed as percentages of the Maximal Possible Effect for mechanical and thermal perceptive thresholds improvements, SCI-HSSC-treated animals showed significant improvements in sensory function for both mechanical and thermal components. (A-C: data expressed as mean $\pm$ SEM; A-B: repeated measures ANOVAs; C: Student t -tests).

Postmortem spinal cord MRI showed cavity-filling effect by grafted cells in HSSC-injected animals

For lesion volume analyses, a 2 cm long portion of the fixed spinal column was dissected out, kept in 4% paraformaldehyde, and imaged using a 7 TESLA MRI magnet. The primary goal of this analysis was to generate quantitative data on the cavity-filling effect by grafted cells and to assess the extent of rostro-caudal cavitation in vehicle-injected vs. HSSC-injected animals using quantitative volume analysis (**Fig. VI-4**). In vehicle-injected animals, the presence of fluid-filled cavities was readily identified as the presence of homogenous white areas and scarring as black areas (**Fig. VI-4B1**; compare with non-injured: **Fig. VI-4C**). In contrast, in animals receiving cell injection, the cavity was partially or completely filled with grafted cells as evidenced by the presence of low density tissue masses (**Fig. VI-4A1**). The identity/presence of grafted cells in the “low density tissue masses” was further validated by analysis of semi-thin plastic sections taken from the same region (compare **Fig. VI-4A2** which depicts the presence of cell grafts vs. extensive cavity in **Fig. VI-4B2**). **Fig. VI-4A** shows a 3D reconstruction image of a cell injected animal (areas identified as grafted cells are labeled green). **Fig. VI-4B** shows a SCI-control (media-injected) animal with cavity labeled in light-green-yellow.

Quantification of the cavity volume showed a significantly larger injury-induced cavity in SCI-control animals than in HSSC-injected animals ($3\pm0.4\text{ mm}^3$ vs. $0.6\pm0.2\text{ mm}^3$, respectively; **Fig. VI-4D**; Student's *t*-test: $P<0.0001$). Similarly, the scar volume seen in SCI-control animals was larger than in cell-injected animals ($3.3\pm0.3\text{ mm}^3$ vs. $1.9\pm0.3\text{ mm}^3$, respectively; Student's *t*-test: $P<0.001$).

To assess the potential excessive grafted cell proliferation and resulting spinal cord tissue expansion, we next compared the total volume (i.e., the volume of the remaining spinal cord, scar, cavity, and/or grafted cells) of cell-grafted spinal cord segments with corresponding segments of control animals. The measured volumes were: $71.8\pm3.2\text{ mm}^3$ in non-injured control animals, $54.6\pm2.8\text{ mm}^3$ in SCI-control animals, and $59.0\pm2.2\text{ mm}^3$ in SCI-HSSC-injected animals (Student's *t*-test: $P=0.27$; SCI-control vs. SCI-HSSC-injected animals).

Figure VI-4A-D: Effective cavity-filling effect by transplanted cells in SCI HSSC-injected animals. At the end of the 2-month post-treatment survival, animals were perfusion fixed with 4% paraformaldehyde, spinal column dissected and MRI-imaged in situ before spinal cord dissection for further histological processing. **A**, **B**: 3-D MRI images of spinal cord segments in animals with previous traumatic injury and treated with spinal HSSC (**A**) or media (**B**) injections. Note the near complete injected-cells cavity-filling effect in HSSC-treated animals. **A1**, **A2**, **B1**, **B2**: To validate the presence of grafted cells or cavitation at the epicenter of injury, the same region was histologically processed, semi-thin plastic sections prepared and compared to corresponding MRI image (compare **A1** to **A2** and **B1** to **B2**). **C**: 2-D MRI image taken from naïve-non-injured animal. **D**: Quantification of the cavity and scar volume from serial MRI images showed a significantly decreased cavity and scar volumes in SCI-HSSC-injected animals if compared to media-injected SCI controls. (**D**: data expressed as mean±SEM; Student *t*-tests), (Scale Bars: **A**, **B**: 5 mm; **A1**, **A2**, **B1**, **B2**, **C**: 3 mm).

Survival, maturation and integration of grafted HSSC

To identify the presence of human cells in the rodent spinal cord tissue, two different immunostaining/analytical methods were used. First, GFP-tagged grafted cells were identified by the presence of GFP autofluorescence/immunoreactivity and then co-stained with neuronal and non-neuronal markers. Second, a set of human-specific antibodies was first used to validate the presence of human cells and then combined with other human-non-specific neuronal or non-neuronal antibodies.

Staining with anti-GFP, -NeuN (neuronal marker) and -human-specific synaptophysin antibody showed a near complete repopulation of compression-induced lesion cavity by grafted GFP+ cells (**Fig. VI-5A**-yellow dotted area). A comparable spinal injury-cavity filling by grafted cells was seen after grafting with GFP or non-labeled HSSC as evidenced by the presence of dense hNUMA-immunoreactive grafts (**Fig. VI-5A** inserts). Analysis of axo-dendritic sprouting from grafted GFP+ cells showed extensive rostro-caudal neurite sprouting particularly well-developed in the lateral white matter (**Fig. VI-5B**). In addition, numerous GFP+ axons branching from innervated lateral funiculi and projecting towards α -motoneurons and interneurons was identified (**Fig. VI-5B**; insert). Triple staining with NeuN, hSYN and GFP antibody showed a high density of hSYN punctata in GFP+ innervated regions (**Fig. 5C**-yellow arrows) as well in the vicinity of endogenous NeuN+ neurons. Staining with hNUMA, hNSE and DCX antibody revealed that the majority of hNUMA+ grafted cells were DCX or DCX/hNSE immunoreactive (**Fig. VI-5A**-insert; **Fig. VI-5D**). Probing for glial phenotype in grafted cells by double staining with hNUMA and hGFAP or hNUMA and Olig2 antibody revealed well-developed groups of hGFAP+ astrocytes. These GFAP+ cell populations were primarily found in the white matter or at the periphery of individual DCX/hNSE+ grafts (**Fig. VI-5E**). Less than 2% of hNUMA+ cells showed Olig2 immunoreactivity (**Fig. VI-5F**; yellow arrows). To assess the presence of mitotically active grafted cells, sections were double-stained with hNUMA and Ki67 antibody. An estimated 0.5-1% of hNUMA+ cells were Ki67 positive. These double hNUMA/Ki67+ cells were regularly distributed throughout the grafted regions but no cluster(s)-like formations of hNUMA/Ki67+ cells were seen in any animal (**Fig. VI-5G**; yellow arrows).

Confocal analysis of spinal cord sections triple-stained with hSYN, GFP and NeuN antibodies showed numerous hSYN punctata colocalizing with GFP+ processes. Several hSYN punctata were found to reside in the vicinity of interneuronal and/or α -motoneuronal membranes (**Fig. VI-6A**; inserts; white arrows). Probing for the presence of GAD65/67+ terminals derived from grafted neurons by using triple stained GAD(65/67)/GFP/NeuN sections and confocal microscopy showed the presence of GFP/GAD65/67+ terminals in the vicinity of α -motoneuronal membranes (**Fig. VI-6B**; white arrows).

Figure VI-5: Survival, differentiation and outgrowth. A: Grafted cells filling near completely the lesion cavity at 8 weeks after grafting (yellow dotted area; inserts). B: Detail from “A”: a dense GFP+ neurite network in the lateral funiculus (LF) and with axons projecting towards α -motoneurons and interneurons in the gray matter (insert). C: In areas with a dense GFP+ axodendritic network, hSYN immunoreactivity associated with GFP+ processes can be seen (yellow arrows). D: The majority of grafted cells showed development of the neuronal hNSE/DCX+ phenotype. E, F: A subpopulation of grafted hNUMA+ cells showed astrocyte (hGFAP+) and oligodendrocyte (Olig 2) phenotype (F; yellow arrows). G: Using mitotic marker Ki67, regularly distributed hNUMA/Ki67+ grafted cells were identified (yellow arrows). (Scale Bars: A: 1.5mm (inserts: 200 μ m); B: 600 μ m (insert: 75 μ m); C: 60 μ m; D: 20 μ m (insert: 5 μ m); E-G: 10 μ m).

Figure VI-6: Development of putative GABA-ergic synaptic contact between HSSC and the host neurons. A: Confocal analysis of hSN/GFP/NeuN-stained sections shows numerous hSN punctata associated with GFP+ processes derived from grafted cells. Some of the hSN/GFP+ terminals were found to be in the vicinity of the host interneurons or α -motoneurons (A; inserts; white arrows). B: Triple staining with GAD65+67/GFP/NeuN antibody showed numerous double-stained GAD65+67/GFP+ terminals residing on or in the close vicinity of lumbar α -motoneurons (white arrows). (Scale Bars: A: 150 μ m (inserts: 30 μ m); B: 20 μ m).

Figure VI-7: Figure 7A-C: Significant decrease in the dorsal horn CGRP immunoreactivity caudal to the injury epicenter in SCI-HSSC-treated vs. SCI-control animals. A-C: CGRP- (A), GAP-43- (B), and Iba1- (C) immunoreactivity in the dorsal horns (DH) caudal of the injury epicenter at 2 months after L3 SCI. The region of interest (ROI) was defined as outlined in B and C (left panels, red dotted line). A: The quantitative densitometry analysis of CGRP-immunostained images in the dorsal horns of SCI-HSSC-treated animals (A2) showed significantly decreased CGRP expression when compared to SCI-control animals (A1). B, C: The dorsal horn GAP-43 or IB1 immunoreactivity was not significantly different between experimental groups. (A-C: data expressed as mean $\pm$ SEM; student *t*-tests). (Scale Bars: A - C: 500 μ m).

Normalization of CGRP expression in L6 dorsal horns in SCI-HSSC-treated animals

To analyze changes in the spinal expression of putative central pain neuromodulators/indicators, including CGRP, GAP43 and Iba-1 (Hains 2006, Oндarza 2003), we next stained transverse L6 sections (i.e., below-injury-level region) with respective antibodies at 8 weeks after treatment (**Fig.7 A, B, C**). Densitometry analysis showed that CGRP immunoreactivity was significantly reduced in SCI-HSSC-treated animals (**Fig. 7A2**) when compared to SCI-controls (**Fig. 7A1**; Student's *t*-test: $P=0.04$). We did not find significant group differences in IHC staining intensities of either GAP43 or Iba1 (**Fig. 7B, C**; Student's *t*-test: $P=0.58$ and $P=0.24$, respectively).

Quantitative assessment of axonal survival in the epicenter of injury using semi-thin plastic sections

For quantitative analysis of axonal survival, a transverse spinal cord block taken from the injury

epicenter was used. Using osmium/p-phenylenediamine-stained semi-thin (1- μ m-thick) plastic sections, the total number of axons (divided in 3 subgroups based on the axonal caliber; 0.3–1.0, 1.0–2.5, and 2.5–10 μ m in diameter) was then counted using ImageJ software.

Figure VI-8: Effect of spinal HSSC grafting on locomotor function (BBB), foot stepping angle, ladder climbing test and motor evoked potentials. A: Weekly measurement of the BBB scores modified for the L3 injuries (left y-axis) and regular BBB scores (right y-axis) showed progressive recovery in both HSSC-grafted and control SCI animals. While there was a trend toward better motor performance in HSSC-grafted-animals, this effect was not significant for both scoring systems. B: Single Frame Analysis showed a tendency towards regaining normal foot stepping angles between the paw and floor (measured at stance-phase initiation; see insert/drawing in B) in SCI-HSSC-treated animals. However, the angles were not significantly improved if compared to SCI controls. C: Using the ladder climbing test, we found a significant decrease in number of correct steps in SCI animals if compared to naïve controls. No significant difference was seen between SCI-control and SCI-HSSC-treated animals if analyzed at 2 months after treatments. D: Motor Evoked Potentials recorded at baseline (i.e., before injury) and at 8 weeks post injury showed a significant decrease only for the SCI-control animals. No significant difference between HSSC-grafted and control SCI animals was detected.

Figure VI-9: Axonal survival in the injury epicenter. A: Schematic diagram of the axon counting design used in our current study. Axons were counted in plastic osmium-stained sections in the dorsal, lateral and ventral funiculi using ImageJ software. An example of the detection threshold to identify individual axons in a selected field is shown in A2 and A3. B: Transverse plastic section depicting a bilaterally distributed graft (red dashed line) and completely filling the cavity created by previous spinal compression. Note that the fusion of the graft with the host tissue is so advanced that the border between the previous injury-evoked cavity and the graft is difficult to delineate (red asterisks). C: An example of transverse spinal cord section taken from an animal receiving media injection. An extensive cavity occupying near completely the region of previous gray matter can be seen. D: Quantification of axons in SCI-control and SCI-HSSC-treated animals showed no significant differences if analyzed in dorsal, lateral or ventral funiculi or if sub-divided into axons of different caliber (S=small=0.3–1 μ m; M=medium=1–2.5 μ m; L=large=2.5–10 μ m). (Scale Bars: A-C: 500 μ m).

Systematic quantification of the total number of myelinated axons counted bilaterally in a control naïve animal showed 281,352 axons (**Fig. VI-9A**). Thirty-three percent was represented by small caliber axons, 57% by medium caliber axons and 10% by large caliber axons. In SCI-control animals, the total number of axons was on average $55,137 \pm 5,168$ and was $55,340 \pm 5,650$ in HSSC-injected animals (Student's *t*-test: $P=0.98$; **Fig. VI-9B-D**). Intergroup statistical analysis of the axons at specific diameter (i.e., 0.3–1.0, 1.0–2.5, and 2.5–10 μm) showed *P*-values of 0.88, 0.84, and 0.51 (Student's *t*-tests) between SCI-control and SCI-HSSC-grafted animals, respectively. Intergroup statistical analysis of the axons at specific funiculi (i.e., dorsal, lateral, and ventral funiculi) showed *P*-values of 0.73, 0.82, and 0.72 (Student's *t*-tests) between SCI-control and SCI-HSSC-grafted animals, respectively (**Fig. VI-9D**). Additional intergroup analyses of axon survival categorized by both size and location/funiculus did not show significant differences between SCI-control and SCI-HSSC-grafted animals ($P>0.36$; Student's *t*-tests) (**Fig. VI-9D**).

Discussion

In the present study, we investigated the treatment effect of spinally grafted GMP-grade human fetal spinal cord-derived neural stem cells (HSSC) in a L3 spinal compression injury (SCI) model in rats. HSSC were grafted into and around the epicenter of the contusion-injured L3 spinal segment at 3 days after spinal trauma in continuously immunosuppressed Sprague-Dawley rats. In comparison to control SCI animals with no treatment or receiving intraspinal injections of media only, the intraspinal grafting of HSSC led to a progressive and significant improvement in: 1) gait/paw placement, 2) muscle stretch-induced spasticity, and 3) mechanical and thermal sensitivity. These behavioral benefits were associated with robust graft survival and near complete injury-cavity-filling effect with grafted cells and corresponding lack of syringomyelia otherwise seen in control SCI cell-non-treated animals. In addition, the development of putative GABA-ergic synapses between grafted neurons and interneurons and/or α -motoneurons of the host were identified. These data demonstrate that intraspinal grafting of HSSC into an injured spinal cord segment in the acute phase of injury represents a safe and effective treatment modality. This cell-replacement therapy was effective in providing qualitatively- and quantitatively-defined functional benefits and also led to significant and long-term improvement in the structural integrity of previously trauma-injured spinal cord segments.

Rationale for early spinal cell-replacement therapy after spinal trauma

Both experimental and clinical data show that the spinal pathological processes following acute spinal injury are in part characterized by continuing axonal/neuronal degeneration, which can then continue for months to years after injury (Bramlett 2007, Ek 2010, Falcì 1997, Hagg 2006, Wirth 2001). It is believed that such an ongoing axonal degeneration is in part the result of the lack of local trophic support associated with loss of neurons/glia cells at and around the injury epicenter. Thus, the use of treatment strategies which can replace or supplement the loss of the local neurotrophic activity and are initiated during this acute period should thus lead to a measurable treatment effect. Previous studies have demonstrated that neural stem cells of mouse, rat or human origin are a rich source of extracellularly released trophic factors (such as NGF, BDNF, GDNF, EGF, IGF-1, and VEGF) in *in vitro* cultured cells and that these cell populations retain a high level of neurotrophin expression after *in vivo* grafting in naïve animals and in a variety of neurodegenerative models including spinal injury and transgenic amyotrophic lateral sclerosis (ALS) models (Fagerlund 2011, Hawryluk 2012, Lu 2003, Xu 2006, Yan 2004, Yan 2007). In addition, using long-term post-grafting survival periods, it was shown that *in vivo* grafting of neural precursors with neurogenic potential into either the spinal cord at 9 days post spinal cord injury, the brain at 3 days post ischemic insult, or the CNS (brain or spinal cord) of adult or developing rats, there is the development of functionally and morphologically-defined synaptic contacts between grafted neurons and the neurons of the host (Cummings 2006, Lepore 2006, Rota Nodari 2010).

Based on these characteristics of neural precursors (NPCs), the use of NPCs for acute spinal cord grafting after trauma serves three purposes. First, it serves to provide local trophic support in the areas of previous injury (provided that grafted cells are able to home and survive long-term once grafted into the injured spinal cord milieu) and to minimize or halt the process of progressive axonal/neuronal degeneration. Second, it serves to provide a cavity-filling effect by replacing previously injured-degenerated necrotic tissue and thus prevent the long-term (or progressive) formation of rostro-caudal cavitations (i.e., syringomyelia) (Wirth 2001). Third, by the development of synaptic contact with the host axons/neurons above and below the injury level it can potentially lead to formation of a functional relay through the injury site.

Effect of spinal grafting of HSSC on the recovery of motor function and muscle spasticity

In our current study, a combination of several motor performance tests were employed including open field modified BBB scoring, Catwalk gait analysis, inclined ladder climbing, single frame hind limb motion analysis, and myogenic motor evoked potentials to identify the degree of motor function recovery after cell grafting. The changes in muscle spasticity in lower extremities (i.e., below the level of injury) were also measured using a computer-controlled ankle rotational system (Marsala 2005). The catwalk gait analysis showed significantly improved paw placement in HSSC-injected SCI animals when compared to control SCI animals. In addition, a significant suppression of otherwise exacerbated muscle spasticity response measured during ankle rotation was seen in cell-treated animals.

A previous study has shown that spinal grafting of adult rat spinal cord-derived NPCs at 7 days post thoracic contusion injury (MASCIS/NYU weight drop from 12.5 mm) resulted in significant improvement in BBB/open field and grid-walk scores over 9 weeks after grafting in rats (Hofstetter 2005). Similarly, spinal grafting of mouse NPCs immediately after L2 spinal segment compression in mice resulted in a significant improvement in posture and hindlimb motor response to hindlimb extension with no significant improvement in the grid-walk or grip test (Boido 2011). Another study showed significant improvement in BBB-score over 8 weeks after spinal grafting of rat embryonic tissue-derived spinal cord-derived NPCs and Glia Restricted Precursors (GRPs) at 9 days after thoracic contusion injury (MASCIS/NYU weight drop from 25 mm) in rats (Mitsui 2005). In the same study, no significant improvement in the grid-walk was seen.

Mouse embryonic stem cell-derived NPCs grafted spinally at 9 days after thoracic contusion injury (MASCIS/NYU weight drop from 25 mm) were associated with a significant BBB-score improvement in rats measured up to a month after cell grafting (McDonald 1999). More recently, a significantly improved functional recovery (as assessed by BBB-scoring and spinal cord evoked potentials) after spinal grafting of acutely dissociated rat embryonic spinal cord tissue at 2 weeks post injury or after spinal injections of HSSC (the same cell line as used in our current study) at 1 week post injury was reported in a Th3 complete transection model in rats (Lu 2012). In the same study, to further facilitate the cell engraftment in the region of complete spinal cord transection, the cells were first embedded into the fibrin matrix enriched with a cocktail of neurotrophins and growth factors, which was then placed into a 2 mm long tissue gap created by the previous Th3 complete transection. Similarly, we have previously demonstrated that spinal grafting of HSSCs (same cell line as used in the current study) 21 days after induction of spinal ischemic injury in Sprague-Dawley rats resulted in a significant recovery of motor function and amelioration of spasticity when assessed up to 12 weeks

post-grafting (Cizkova 2007). In another study, human fetal NPCs (cultured as neurospheres or enriched for oligodendrocyte precursors) grafted spinally at 7 days post thoracic contusion injury in rats (MASCIS/NYU weight drop from 25 mm) showed a temporary significant improvement during the initial 5 weeks post cell grafting, but this effect was no longer present at longer (up to two months) post-treatment intervals (i.e., a faster recovery was seen in HSSC-treated animals) (Alexanian 2011).

Several other studies showed no motor improvement (as assessed by BBB or grid-walk) after spinal grafting of mice NPCs, or rat GRPs or GRP-derived astrocytes (GDAs, induced by CNTF *in vitro* pre-treatment), when grafted 8 days after thoracic contusion injury in rats (MASCIS/NYU weight drop from 25 mm) or immediately after cervical dorsolateral funiculus transection in rats, respectively (Davies 2006, Davies 2008, Macias 2006). However, immediate spinal grafting of GDAs, generated after Bone Morphogenetic Protein-4 (BMP-4) *in vitro* pre-treatment in a rat cervical dorsolateral funiculus transection model, did result in significantly less foot-faults on the grid-walk test (Davies 2006, Davies 2008). No motor improvement (as assessed by BBB-scoring) was seen after spinal grafting of human GRPs or BMP-4-induced GDAs at 9 days after thoracic contusion injury in athymic rats (MASCIS/NYU weight drop from 25 mm) (Jin 2011).

These results indicate that cell replacement therapies which employ cell lines with both gliogenic and neurogenic potential can be beneficial in promoting motor/sensory recovery after SCI. In addition, these studies and our current study demonstrate that a clear therapeutic effect can be achieved once cells are grafted during the early post-injury period (i.e., 3-7 days after spinal trauma).

Effect of spinal grafting of HSSC on the recovery of sensory function

In our study, we assessed the sensory function below the level of injury (hind paws) by measuring the mechanical and thermal thresholds for supraspinally mediated escape behavior. We found significant improvement of both SCI-induced mechanical and thermal hypoesthesia. It is important to note that the sensory thresholds did not yet plateau at the end of the 2-month survival period. We speculate that an additional quantitative and qualitative improvement in the sensory function would likely be seen should a longer post-grafting interval be studied. In addition to sensory tests, quantitative analysis of spinal parenchymal markers indicative of developing (spinal) hypersensitivity (i.e., CGRP/GAP43, an indicator of aberrant sprouting of primary sensory neurons (Macias 2006, Ondarza 2003), and Iba1 staining, a marker of microglia activation (Hains 2006)) were studied and showed a significant decrease in CGRP staining intensities in HSSC-treated animals if compared to SCI controls. This suggests that the recovery/decrease in sensory thresholds observed in our study is not a result of aberrant sprouting or microglia activation.

Consistent with the observations from our study, previous studies from other laboratories have demonstrated similar findings. Spinal grafting of human fetal neural cells (A2B5+ NG2+) 7 days after thoracic contusion injury (MASCIS/NYU weight drop from 25 mm) partially, but significantly, improved below-level thermal hypoesthesia at 7 weeks post-grafting (Alexanian 2011). Spinal grafting of adult rat spinal cord neurogenin-2-expressing cells at 7 days post rat thoracic contusion injury (MASCIS/NYU weight drop from 12.5 mm) improved below-level hyposensitivity (assessed by fMRI) (Hofstetter 2005). Similarly, spinal grafting of human GDAs at 9 days after thoracic contusion injury in athymic rats (MASCIS/NYU weight drop from 25 mm) resulted in a trend towards improvement of a SCI-induced increase of frontpaw (above-level) withdrawal thresholds to thermal stimuli (Jin 2011).

The comparison of the effect of spinal NPC grafting on sensory function (measured thresholds for escape behavior) used in our study with other studies reporting on (SCI- or therapy-induced) decreased paw withdrawal thresholds is to some extent speculative, because the relation between paw withdrawal thresholds and supraspinal escape behavior is still not well-defined (Baastrup 2010a). With that in mind, previous studies have demonstrated that GRPs or GRP-derived astrocytes (GDAs, acquired through CNTF treatment), which were spinally grafted in rats immediately after high cervical dorsolateral funiculus transection, resulted in decreased forepaw mechanical and thermal withdrawal thresholds (i.e., below the level of injury) (Davies 2008). GDA induction from GRPs with BMP-4 prevented this hypersensitivity and the related occurrence of aberrant CGRP sprouting in the cervical spinal dorsal horn (Macias 2006). Spinal grafting of human BMP-4-induced GDAs at 9 days after thoracic contusion injury in athymic rats (MASCIS/NYU weight drop from 25 mm) resulted in significant improvements of a SCI-induced decrease of hindpaw (below-level) withdrawal thresholds to mechanical stimuli (Jin 2011).

Similarly, spinal grafting of human fetal NPCs 7 days post thoracic contusion injury (MASCIS/NYU weight drop from 25 mm) resulted in a minor improvement of SCI-induced lowered below-level paw withdrawal reflexes, when grafted cells were A2B5+/NG2+ NPCs (not NCAM+/A2B5+ or NCAM+/A2B5-) (Alexanian 2011). Yet another study showed restoration of SCI-induced lowered tail withdrawal latencies to a heat stimulus as assessed up to 8 weeks after spinal grafting of rat embryonic spinal cord-derived NRPs and GRPs at 9 days after thoracic contusion injury in rats (MASCIS/NYU weight drop from 25 mm) (Mitsui 2005). This indicates that a partial glial differentiation in grafted cells observed in the current study might be one of the mechanisms responsible for amelioration of aberrant CGRP sprouting and resulting decrease in spinal sensitization.

Differentiation of grafted cells and mechanism of HSSC-mediated therapeutic action

In our current study, near pure population of nestin⁺ human fetal spinal stem cells were grafted intraspinally at 3 days after contusion-induced spinal cord injury. Analysis of the graft survival at 2 months after grafting showed a dense population of grafted hNUMA⁺ cells in grafted previously trauma-injured regions. In addition, numerous hNUMA⁺ cells which migrated out of the graft in distances ranging between 2-3 mm were also seen. Using human-specific antibodies against NSE and synaptophysin (markers of mature neurons), we have also shown that a majority of grafted cells developed into a neuronal phenotype. Many human specific synaptophysin⁺ boutons were found to reside in the vicinity of host neurons.

Quantitative analysis of the host axon survival in the injury epicenter showed no significant sparing effect in HSSC-grafted SCI animals vs. medium-injected or untreated SCI animals. These data suggest that 1) the majority if not all axons which succumb to pathological processes resulting from secondary changes post injury such as edema, ischemia were already lost or irreversibly damaged at 3 days after trauma (i.e., the time point when the cells were grafted), or 2) regional cell grafting is not therapeutically effective in providing acute neuroprotection.

Analysis of the neurotransmitter phenotype in grafted cells showed the development of putative inhibitory GABA-ergic synapses with host neurons. These data show that the restoration of the local functional inhibitory circuitry by grafted cells can in part lead to the observed functional improvements. Similarly, in our previous study using the same cell line, we have demonstrated the development of putative GABA-ergic synaptic contacts between grafted neurons and persisting α -motoneurons of the host in a rat spinal ischemia model. In the same animals, a significant amelioration of spasticity was measured (Cizkova 2007). In a recent study using EM analysis, we have confirmed the development of synaptic contacts with the host neurons at 9 months after intraspinal grafting of HSSC in normal non-injured immunodeficient rats (Hefferan 2012). Similarly, in a more recent study, the development of functional contacts and restoration of axon potential conductivity across the region of complete Th3 spinal transection by grafted HSSC was seen (Lu 2012).

In addition to restoration of the local motor circuitry, significant amelioration of otherwise increased spinal CGRP expression seen in non-treated SCI animals was measured in SCI animals receiving spinal injections of HSSC. Consistent with this observation, previous studies have demonstrated that improvement of local spinal GABA-ergic tone, as achieved by subcutaneous inoculation of a replication-incompetent herpes simplex virus (HSV) encoding GAD67 gene in a Th13 spinal cord hemisection model, led to a similar decrease in otherwise increased CGRP expression (Liu 2004). Second, previous studies have shown that spinally grafted HSSC show the expression of several

trophic factors (GDNF, BDNF, and VEGF) at 2 months after grafting in SOD+ rats (Xu 2006). We speculate that the release of these tropic factors can potentiate the sprouting of persisting axons of the host below and above the injury and accelerate the development of new synaptic contacts particularly at longer post-grafting intervals.

Finally, we have demonstrated a near complete injury-cavity filling effect by the grafted cells at 2 months after grafting when the cells were grafted at 3 days after injury. This was in contrast to media-injected animals which showed consistent and extensive rostro-caudal spinal cord cavitation. These data suggest that early post injury cell grafting is desirable as it can effectively block the formation of the spinal cavity and its expansion and related long-term secondary spinal cord degeneration. A comparable cavity-filling effect and prevention in the progression of syringomyelia has been shown after spinal grafting of human embryonic or fetal SSCs in human patients with progressive post-traumatic syringomyelia (Biyani 1994, Falci 1997, Wirth 2001).

It is important to note that the cavity-filling effect demonstrated in our current study was achieved without the use of any supporting matrices or additional topical growth factor(s) delivery. In our preliminary study, we have determined that while the density of grafted cells is relatively low to fill the cavity-forming region, the grafted cells continue to proliferate after grafting to the point when a cavity is near completely filled with grafted cells [unpublished data]. The cell proliferation is inhibited once the cavity is filled and after that the cells differentiate normally. That the cells do not develop into pre-neoplastic or neoplastic cells has been assessed in a 9-month tumorigenicity study with nude rats whose Th9 spinal cord segment was first injured by contusion (manuscript in preparation). Similarly, using the same cell line as used in our current study, we have previously reported a comparable low level of mitotic activity in grafted cells at 6 weeks to 9 months after grafting in naïve immunodeficient rats or immunosuppressed minipigs (Usvald 2010).

In our current study, we demonstrate a functionally-defined treatment effect after spinal grafting of human GMP-grade fetal spinal stem cells in immunosuppressed SD rats with previous L3 compression injury. This treatment effect was expressed as a significant improvement in motor and sensory function (gait/paw placement, stretch-induced muscle spasticity, and, mechanical and thermal sensitivity). In addition, an effective filling of trauma-induced spinal cavity with grafted cells was seen in HSSC-treated animals at 2 months after grafting. Jointly, these data demonstrate that the use of this clinical grade NSI-566RSC cell line with already established favorable clinical safety profile represent a potential cell candidate for cell replacement therapy in patients with previous spinal traumatic injury.

VII. Tumorigenesis safety study of human fetal neural precursor cells in SCI rats

Preclinical safety assessment of contemporary experimental stem cell therapies for clinical trial authorization; an instructive example with neural stem cells

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Abstract

A main concern for any novel therapy for clinical applications is its safety. The same holds true for stem cell based therapies. In particular the potential tumorigenic property of many contemporary experimental stem cell therapies requires distinct preclinical safety assessments. As no standard guidelines or any stem cell safety studies have yet been published, it is still a matter of debate on how to perform such assessments. In this study we describe the preclinical assessment of the safety of a promising human fetal spinal cord derived neural stem cell therapy as part of a phase 1 clinical trial application. The design of the ensuing safety study is the result of a dialogue between researchers in this field, and the national drug authority (the Food and Drug Administration). This study and the concomitant efficacy/therapeutic studies resulted in the approval of the currently ongoing clinical trial in patients with amyotrophic lateral sclerosis. Therefore, to provide a reference and guide for future preclinical stem cell safety studies, we here report on the main considerations and outcomes of our preclinical safety study.

Introduction

An essential prerequisite of a successful transfer of any experimental therapy into clinical practice is a tolerable safety profile; i.e. its toxicity. Typically, a toxicity study is conducted after the initial proof of efficacy (disease modification) is established and follows a relatively well defined guideline of a supervising agency, like the Food and Drug Administration (FDA)-guidelines-based experimental design (Food and Drug Administration 1997). An Investigational New Drug application for clinical trials (IND) to the FDA requires study reports which assess potential toxicities in their target tissues of animal species that are relevant for the clinical development plan of the drug (U.S. National Archives and Records Administration 2012b).

Essentially, this is not different for cell-based therapies, but a key item of toxicity potentially associated with any stem/progenitor-cell-based therapy is tumorigenicity; i.e. the propensity of grafted donor cells to form tumors, either by itself or by inducing host cells. Either can occur in the targeted tissue, but also, due to cell migration, in non-targeted tissues. Generally, the risk of tumorigenesis is higher when allogeneic cell-based therapies have long-term, or even permanent, survival and integration, have differentiation and proliferation capacity, have a higher chance/need for a systemic route of administration, or are not irreversibly implantable.

Unfortunately, practical guidelines to assess tumorigenic potential of any group of IND candidate stem cell population in an animal model are not defined. One test of tumorigenicity is provided in an FDA guidance document (Food Drug Administration 1993). In this test, a bolus cell suspension consisting of at least 10^7 cells are injected subcutaneously in at least ten athymic (immunodeficient) mice and assessed up to a month. However, for development of a therapeutic cell-based product, an additional tumorigenicity study would be required in which the cells are injected into the clinically intended target tissue with a cell dose at the very least consistent with planned human dose. Obviously, although tumorigenesis is an important outcome when assessing the safety profile of a cell-based therapy, the other 'classical' signs of toxicity should not be neglected and require extensive health observations.

Here, we report on the safety assessment of NSI-566RSC, a human fetal spinal cord-derived neural stem cell population (Yan 2007), developed for spinal neurodegenerative diseases and spinal cord injury for final use in human trials. NSI-566RSCs are developed using reproducible and well-defined protocol which permit a long-term expansion of human spinal stem cells isolated from cervical spinal cord of human first trimester fetuses. NSI-566RSCs are now well-established and fully characterized *in vitro* (Guo 2010, Lunn 2010). The cells are produced under Good Manufacturing Practice (GMP) conditions (U.S. National Archives and Records Administration 2012a, U.S. National Archives and

Records Administration 2012d). Efficacy studies to define the therapeutic use of this particular cell line after spinal grafting have been reported to i) extend life span and regional Alpha-motorneuron protection in SOD+^(G93A) transgenic rats (Hefferan 2012, Xu 2006), ii) improve motor function and ameliorate spasticity in spinal ischemia-injured Sprague-Dawley (SD) rats (Cizkova 2007), iii) improve motor function after 2mm long full spinal transection in SD rats (Lu 2012), and, iv) improve hind limb function, and mechanical and thermal-stimulus evoked sensory function, and no engraftment procedure related deterioration in L3 spinal cord contused SD rats [unpublished observation]. With the availability of a stable, safe to engraft, and effective product, all logical prerequisites for a preclinical safety assessment are met.

This safety study was designed in conjunction with FDA reviewers (Office of Gene and Cell Therapy/CBER/FDA) for guidance on how to design the safety study to include suitable tumorigenicity assessment. Following are the main considerations to assess the toxicity of (neural) stem cell-based therapy, with special focus on tumorigenesis, as a frame of reference.

As a first prerequisite, the study must be performed in a Good Laboratory Practice (GLP)-compliant manner (including external auditing) and in a contract research facility (U.S. National Archives and Records Administration 2012c). Any safety study is designed specifically to have a high sensitivity for potential systemic and local toxicities. The goal is to show the safety of a final stable product (e.g. GMP-compliant) in a concentration which is evidently higher than will be used in human patients. Formulating such a maximal tolerated dose also involves anticipating the normal proliferation of the cell-based therapy. In addition, factors possibly enhancing the inherent proliferating potential (or tumorigenicity) of the grafted cells in/on the targeted tissue should be taken into account (e.g. spinal cord disease increased mitotic potential of NSI-566RSCs). Preliminary dose finding studies are thus advisable. Next, standard toxicology endpoints are studied, including mortality, clinical observations, and (histo-)pathology of targeted and non-targeted tissues, with a special focus on tumorigenesis through examination of all suspected lesions and masses by an external certified pathologist. The use of athymic (RNU⁻/RNU⁻) animals is first choice since they possess genetic immunodeficiency (no T-cells), which is required for perfect cell survival while the rats remain relatively resistant to simple/opportunistic infections due to an intact innate immune system. In agreement with FDA reviewers, this study needed to hold at least a final 30 treated animals and 30 appropriate control animals with a survival of 6 months, with periodic toxicity assessments, to be considered adequately conclusive.

In conclusion, the goal of the present study is to characterize the safety profile of NSI-566RSCs after spinal grafting in spinal cord contused athymic rats, as defined by i) toxicity, specifically entailing ii)

tumorigenesis, and iii) any additional health observations. In addition, although strictly not part of a toxicity assessment, it was decided best to collect additional supporting safety data by characterizing NSI-566RSC after long-term and high dose spinal cell grafting in terms of iv) safety for neurological function (motor and sensory), and v) its acceptance by/integration in host tissue.

The data from this study and the abovementioned efficacy and safety studies were used for IND application and successfully led to IND approval of NSI-566RSC. NSI-566RSC is currently in ongoing Phase I clinical trials in amyotrophic lateral sclerosis patients (Glass 2012, Neuralstem Inc. - Emory University 2000).

Animals, materials, and methods

The study design and animal usage were reviewed and approved by the Institutional Animal Care and Use Committee. Animal welfare for this study was in compliance with the U.S. Department of Agriculture's (USDA) Animal Welfare Act (CFR 9 - Parts 1, 2, and 3) (Department of Agriculture's Code of Federal Regulations 9 2009), and the Guide for the Care and Use of Laboratory Animals (National Research Council 1996).

Animals

Rats, Athymic Nude (rnu-/rnu-) - Hsd: RH-Foxn1rnu (Harlan, Madison, WI, USA) were used. A total of 90 male rats were enrolled, of which 8 were used for long descending anterograde tract labeling (**Fig. VII-1**). Animal ages at injury ranged from 10 to 13 weeks, and body weights varied between 309 to 345 grams.

Animal housing

Animals were single-housed in cages with irradiated bedding. Acclimation times prior to the experiment varied, but were at least 8 days. Environmental controls were set to maintain temperatures from 64° to 79°F (18° to 26°C), with fluorescent lighting on a 12 hour/12 hour on/off inverted cycle. Animals were fed irradiated Harlan Teklad Global 18% Protein Rodent Diet T.2918.15 (Harlan Laboratories, Placentia, CA) ad libitum. In addition, animals that were selected for addition behavioral testing (see below) also received Fruit Crunchies™ (BioServ product # S05798-1) and HydroGel™ (ClearH2O®, ME 04101). Animals were provided with purified water (Mountain Dairy, Riverside, CA, USA) ad libitum.

Injury Surgery

The day prior to surgeries the back of the animals was shaved. On SCI surgery days, each animal received lactated ringer's (5 mL, s.c.). Animals were anesthetized with isoflurane (5% for induction, ~2% for maintenance, in air). The surgery site was wiped with alcohol and chlorhexidine diacetate solution (2-4%). Rat body temperature was maintained by a water blanket heating system. The skin over the vertebral column was opened, paravertebral muscles were dissected away, and the animal was mounted onto a Stereotaxic frame (Stoelting Lab Standard Stereotaxic - Single, Cat# 51600 Lab Standard) with Spine Adaptors (Stoelting, Cat# 51695 Rat Spinal Adaptor). A thoracic spinal segment (Th10) was then exposed using a dental drill. A moderate injury was made by letting the rod of a MASCIS (NYU) Impactor dropped from a height of 12.5 mm onto the exposed surface of the spinal cord. Next, the impactor was removed immediately, the animal was detached from the frame, the surgical site was irrigated with sterile saline, layers were closed with absorbable Vicryl suture, and Bacitracin/Neomycin/Polymyxin (triple antibiotic) ointment was applied to the incision site. Cefazolin (10mg/kg, s.c.) was also given at the day of surgery.

Post-surgical Care

All animals received Sulfamethoxazole and Trimethoprim oral suspension, (USP 200 mg/40 mg per 5 mL) in the water (5 mL per 250 mL) for 1-2 days before, up to 30 days after initial contusion, and as needed in case of infections. Animals were checked for bladder retention at least two times daily for the duration of the study. If full, bladders were emptied by manual expression. Consecutively, the surgical area was checked. Ketoprofen (4.0 mg/kg, s.c.) was given approximately one hour prior to surgery and approximately every 24 hours for up to 48 hours. Animals found ill were given up to 5 mL (s.c., ≤ 2 daily) Lactated Ringer's solution until improvement was observed. Hexa-Caine Spray and/or Bacitracin/Neomycin/Polymyxin triple antibiotic ointment was used to treat scabs or wounds.

Cells

The cells, named "NSI-566RSC", were produced by Neuralstem Inc. (Rockville, MD, USA), as described before (Hefferan 2011). The grafted cells were a clinical lot (Lot No. CRL471925-3, passage 12), a human neural stem cell line derived from fetal spinal cord of 8 gestational weeks. They were suspended in hibernation buffer at 10^4 cells/ μ L. Each batch (and vehicle) was prepared at Neuralstem Inc (Rockville, MD, USA) one day before surgery and shipped by overnight service in temperature monitored containers.

Figure VII-1: Study outline. Animals received a low thoracic moderate spinal cord injury with a MASCIC/NYU impactor (10 grams dropped from 12.5mm high). They randomly received either vehicle or NSI-566RSCs at one week post-injury through multiple intraspinal/-parenchymal injections. All health observations and behavioral assays we performed while blinded for treatment. Eight animals were found moribund or dead before one month post-grafting and had been replaced. Twelve animals were found moribund or dead after the one month post-grafting timepoint and underwent unscheduled necropsy. Remaining animals survived for nearly ten months after grafting.

Dosing surgery

One week following SCI surgeries, prior to grafting, animals were allocated to groups by stratified randomization based on body weights. Animals were assigned to either the vehicle group (n=45) or the cell-graft group (n=45). There were no exclusion criteria for dosing surgery other than appearing healthy enough. Moribund animals or animals found dead were replaced (up to 30 days post injury).

The day prior to grafting the animals were anesthetized to remove any remaining skin sutures and to shave the back of the animals. At the beginning of a grafting day (7 or 8 days after SCI), NSI-566RSC's viability was determined. The spinal cord was re-exposed. The dorsal aspect of the vertebra immediately caudal to the existing laminectomy was removed using the dental drill. Special care was taken to keep the dura intact. For the current safety assessment, a maximum feasible cell dose was estimated by several preliminary dose-range finding studies using subcutaneous and intraspinal delivery. The cell suspension (Group C) or vehicle/hibernation buffer-only (Group V) was injected as follows:

- 1) 20 injections peripheral from the injury epicenter (1 μ L each; 2 μ L/min) were made bilaterally into about 2 segments above and/or below the injury epicenter at 1mm intervals.
- 2) 5 injections (5 μ L each; 2 μ L/min) were injected around the borders of injury epicenter. Hence, 25 injections were made in each spinal cord, resulting in a total of 45 μ L (cells suspended at 10^4 cells/ μ L, resulting in a total of $\sim 4.5 \times 10^5$ cells).

The required volume of dosing for completing all injections of one animal was drawn into a 100 μ L Nanofil syringe (World Precision Instruments) with a 33-gauge needle (World Precision Instruments, Cat# NF33BV). The syringe was mounted onto a manually controlled syringe holder/injector (David Kopf, Model 5000+5001) attached to the stereotaxic frame. The needle tip was lowered to a depth of 1-1.5 mm from the pial surface at the Dorsal Root Entry Zone (DREZ) into the spinal parenchyma, and the injection was made. After a thirty second pause, the needle was gently drawn out of the spinal cord. The syringe/needle ensemble was cleaned by repeatedly rinsing with and immersion in 70% isopropanol/water solution for a minimum of 15 minutes before and after each animal.

Post injury Health observations, Measurements, and Specimens

General Health Observations (GHOs) were performed on the dosing day, pre-dose, post-dose, and weekly thereafter. GHOs included weight, general appearance, stool appearance, toxicity symptoms and other appearing additional health issues (Table 1). In addition, tumor presence was assessed by full body palpation. Any palpable cell mass was measured using a caliper. All animals were observed daily to ensure no loss of animals for necropsy.

All behavioral testing was performed under red light or dark conditions.

Open field locomotor rating

Locomotor recovery after spinal cord contusion injury was monitored using the Basso, Beattie, and Bresnahan (BBB) open field locomotor rating scale (Basso 1995). In the present study, the BBB score was obtained weekly until eight weeks post-injury, biweekly up until week 20, and every four weeks thereafter. Each examination was conducted by two experienced examiners and during five minutes. The animals selected for BBB testing comprised of the last cohorts of 20 animals in both therapeutic groups (n=40 total).

Additional behavioral testing was conducted on animals showing weight support during the last BBB assessments. Behavioral testing was conducted approximately 1.5 months prior to sacrifice. For training/testing, animals were transferred to the testing room in their original housing and allowed to habituate to the room for 30 minutes, during which white noise was generated.

Activity assessment in an open field

One week prior to the beginning of the test trials, animals were adapted to the apparatus/arenas (76 × 152 × 50 cm; W×L×H) for five minutes on each day. After habituation, the animals were placed in the center of a clean arena and tracked by a video tracking system under dark conditions (night/active portion of their diurnal rhythm), over a period of three hours. The distance traveled and a number of various movement variables was assessed (EthoVision, Noldus Technology, The Netherlands) (Noldus 2001). Three separate observation periods were evaluated. Each observation period was separated by at least 24 hours. During testing, animals had access to Hydrogel and food pellets.

Beam walk

Rats were trained to traverse elevated (1m) narrow beams (3, 2.5, and 2 × 200 inches; W×L) towards a darkened goal box (10 × 10 × 10 inches). Only motor impaired animals would produce foot faults (slips from beam), which number than represent a motor deficit. Bright light and white noise were produced near the start to promote beam crossing. Rats were allowed to fall from the beam (onto a container with padding). For training, each rat was placed in goal box for two minutes and afterwards placed on the balance beam for 1 minute per trial. The rat was considered trained when it was able to remain on the beam for three consecutive trials. Mean scores out of two trials for each

beam were used (i.e. right and left hind paw foot faults, and falls). The animal was allowed to take ~5 min to complete a crossing. In between runs an animal was placed in their home cage for ≥ 15 sec. If an animal was able to cross a beam for at least two thirds, it was also tested on the narrower beam (i.e. 2.5 or 2 inch).

Catwalk Gait Analysis

The CatWalk apparatus (CatWalk 7.1, Noldus Technology, The Netherlands) was used to quantify gait parameters by footprint analysis during walkway crossings (Hamers 2006). Animals walk down a horizontal glass walkway ($109 \times 15 \times 0.6$ cm; L×W×H), of which the glass is illuminated along the long edge. The light only enters the (side of the) glass and reflects merely internally (when the glass is bordered by air). As an animal crosses the walkway, light reflects off of the animal's paws, producing a series of bright footprints when viewed through the glass, which are recorded by a video camera. Hence, testing was performed in a darkened room. Walkway crossing was stimulated by rewarding the animal with little Fruit Crunchies™, placed at the end of the walkway. In conjunction, animals were further motivated by food restriction prior to training/testing. Hence, food was removed from their cages approximately 18 hours before testing (and returned after training/testing). In addition, the following criteria concerning walkway crossings needed to be met: (1) the animal needed to walk uninterrupted across the walkway, at a constant pace, and (2) a minimum of three such crossings per animal were required. Data from three proper crossings was averaged for statistical analysis.

Sensory function assessment (mechanical and thermal)

In this test, pain thresholds for a supraspinal response (vocalization or escape behavior) to a below-level evoked mechanical or thermal stimulus were assessed. The animals were habituated to the set-up, test room, and investigator for one week (no responses were elicited), twice daily for five minutes, prior to testing. The investigator held the animal in an upright position, fixed the tested hindpaw, and then the hindpaw was stimulated. When a response was elicited, the stimulus was stopped immediately and the maximal pressure elicited was recorded. Both hind paws were tested for four times while alternating between paws, with at least a 1 hour interval between trials. The first scores of each paw were removed.

The mechanical stimulus was created using a rigid tip mounted on a pressure transducer (IITC life sciences Electronic von Frey Anesthesiometer, Cat# 2393, Woodland Hills, CA), which was operated by a second investigator (SvG). The stimulus was applied on the dorsal side of a hind paw.

Compressing the paw of the animal with the tip on the pressure transducer at the distal metatarsal or metacarpal area in a gradual incremental fashion (paw rests on table surface).

For eliciting the thermal stimulus an infrared beam apparatus (Ugo Basile, Cat# 37360, Collegeville, PA, USA) was used. For testing, the investigator held the animal's plantar side of a hind paw over the infrared light beam mounted in the apparatus.

Necropsy

The post-grafting survival period varied between 272-274 days (~10 months). Animals were sacrificed by 2 mg pentobarbital and 0.25mg phenytoin (0.5mL of Beuthanasia-D, Intervet/Schering-Plough Animal Health Corp., Union, NJ, USA) followed by transcardial perfusion of saline, trailed by 4% paraformaldehyde (phosphate buffered). The central nervous system was dissected out and preserved in 4% paraformaldehyde. The following tissues were also taken out and placed in 10% formalin:

Skeletal muscle (thigh), skin, adrenals, aorta, rectum, cecum, colon, duodenum, esophagus, epididymides, eyes with optic nerves, femur, Harderian gland, heart, ileum, jejunum, stomach, kidneys, spleen, liver, lung with bronchi, mesenteric lymph node, mammary gland, pancreas, thymus, thyroid with parathyroid glands, trachea, pituitary, prostate, mandibular salivary glands, tongue, sciatic nerve, testes, seminal vesicles, and bladder.

Animals found clinically ill and moribund were euthanized, per protocol. On these animals, and all animals found dead, a gross necropsy was conducted (i.e. external surfaces and orifices, musculoskeletal system, cranial cavity (and brain), neck with associated tissues, and thoracic, abdominal and pelvic cavities (incl. associated organs and tissues).

At necropsy, any tissue with discoloration, lesion, necrosis, distension, malformation, and/or suspected tumor/mass were sent for external histopathological evaluations by a board-certified veterinary pathologist (J.E. Sagartz DVM PhD DACVP, Seventh Wave Laboratories LLC, Chesterfield, MO), as were the histological sections of the central nervous system (see below). Evaluation was performed in a manner blinded to treatment nature. Severity grades for pathology diagnoses were based on a 4 point scale as follows: Grade1= minimal, Grade 2 = moderate, Grade 3 = marked and Grade 4 = severe. After completion of pathology analysis, the pathologist was unblinded and the final conclusion was made.

Central nervous system histology

Each cord was photographed against a ruler (cm) along with its identification number (**Fig. VII-2**). The cords were bisected into approximately equal rostral and caudal pieces and embedded horizontally into a gelatin block (6.5 × 4.5 × 2.5 cm L×W×H). Spinal cords from 16 - 20 animals were embedded in 4 horizontal layers in a block 1 (**Fig. VII- 8**). The rostral pieces were placed in the first and third layers and the caudal pieces were in the second and fourth layers, with the ventral side facing down. The layers were separated by ±0.4 cm of gelatine. The cords (columns) were separated by ±0.2 cm from each other. After solidification of the gelatin block, it was placed in 30% sucrose in 4% formaldehyde for 48 hours. Then, it was frozen in cold 2-methylbutane using dry ice, cryostat sectioned horizontally (40µm thick), and mounted with intervals of 12 sections. Whole brains were also embedded into gelatin blocks, cryostat sectioned coronally at 40 µm thickness. All stained slides were sent to a Board-certified veterinary pathologist for evaluation (J.E. Sagartz DVM PhD DACVP, Seventh Wave Laboratories LLC, Chesterfield, MO). For histopathological stainings every 24th section was used.

The spinal cords used to assess the neuronal integration (see below) were not gelatin embedded, but were frozen directly (after 48h in 30% sucrose) using isopentane (-80°C), mounted in OCT compound (Tissue-Tek®) and cryostat sectioned at 30 µm in the horizontal, sagittal, or transverse plane, and collected for free-floating immunofluorescence staining.

Stainings used entailed: hematoxylin & eosin (H&E), human neurofilament antibody (h.HO14; rat 1:100; human specific axonal marker; gift from Dr. Virginia Lee, University of Pennsylvania, PA, USA), Glial Fibrillary Acidic Protein antibody (h.GFAP; rabbit 1:500; human specific astroglial marker; Origene, Rockville, MD, USA), and Green Fluorescent Protein antibody (GFP; chicken 1:2000; Aves, Tigard, OR, USA), Neuron-Specific Enolase antibody (h.NSE; mouse 1:500; human specific neurite marker; Vector Labs, Burlingame, CA, USA) Synaptophysin antibody (h.Syn; mouse 1:500; human specific synaptic marker; Millipore, Billerica, MA, USA), Human Nucleus antibody (h.HuMA; mouse 1:200; Millipore, Billerica, MA, USA), Glial Fibrillary Acidic Protein antibody (GFAP; mouse 1:500; Sigma-Aldrich; St. Louis, MO, USA), Oligodendrocyte lineage transcription factor 2 antibody (Olig2; rabbit 1:1000; Abcam ab81093; Cambridge, MA, USA), Ki67 antibody (proliferation marker; Abcam ab16667; 1:100; rabbit), and DAPI (In Prolong®; Life Technologies, Carlsbad, CA, USA). Immunostainings were finished using diaminobenzidine (DAB) staining or with fluorescent-conjugated secondary donkey antibodies (Alexa® Fluor 488 & 647; Jackson Immuno Research, West Grove, PA, USA; & Alexa® Fluor 555; Invitrogen; 1:500). The Ki67 immunostaining was also counterstained with 0.1% Neutral Red and finished with DAB.

Evaluation of histological integration of grafted cells

A small group of SCI animals (n=8; 4 with cell treatment), was used only to assess neuronal integration. Injury and treatment was identical up to almost two months prior to perfusion. Then animals underwent general anesthesia during which they received four brain injections of recombinant Adeno-Associated Virus (AAV) engineered to express Green Fluorescent Protein under the human synaptophysin promoter (AAV.synGFP; neuronal specific expression) into both motor cortices and both nuclei rubri. Stereotaxic coordinates used for the injections were: motor cortex: bregma -2.0mm, lateral 3.0mm, depth 1.5 mm, red nucleus: bregma -5.8, lateral 0.8mm, depth 7.1 mm. Each injection consisted of 5 μ L of the virus suspension at 10^{13} particles/mL and was injected over 5 min period using 32G stainless steel needle. The purpose of the virus injections was to label the host corticospinal and rubrospinal (motor) fibers in the spinal cord.

Statistical analysis

Results were analyzed using ANOVA (one-way, or two-way group $\times$ time repeated measures, and using a fixed-effect model), with a Bonferroni *post hoc* test for multiple comparisons. If the unequal variances were observed (Bartlett's test), the Kruskal-Wallis test with Dunn's *post hoc* comparisons were used. Results are expressed as means with the standard deviations of the mean (SD), unless specified otherwise. To analyze differences between the two groups, we used student's t-tests or Mann-Whitney test (Non parametric), or a repeated measures ANOVA (when appropriate). All statistical analyses were performed two-tailed, and a P-value of 0.05 was considered significant. Between-group variations are reported as cell-injected vs. vehicle-injected.

Results

In this study 41 animals were injected with NSI-566RSCs and 41 received vehicle injections, of which 4 had been replaced before dosing and 4 within the first month after dosing, with equal occurrence among both groups (**Fig. VII-1**). Eight additional animals were used for descending tract labeling (vehicle: n=4, NSI-566RSCs: n=4). A total of twelve (six animals from each group) out of all 82 animals were found ill, moribund, or dead during the course of the study and underwent unscheduled necropsy. The remaining 78 animals underwent scheduled necropsy. Cell viability was recorded to exceed 70% prior to all grafting procedures.

External pathology report of targeted tissue

The external report on Central Nervous System histopathology was made by a board-certified veterinary pathologist (J.E. Sagartz DVM PhD DACVP, Seventh Wave Laboratories LLC, Chesterfield, MO) and based on observations made in sections of all 82 animals (without labeled descending motor tracts), for each staining.

Figure VII-2: Representative examples of the gross-morphology of vehicle- and NSI-566RSC-injected spinal cords after a ~10 month *in vivo* period. Note the difference in volume-loss at the lesion site between vehicle- and NSI-566RSC-injected spinal cords.

Macroscopically, no tumors were noted. Traumatic tissue destruction (i.e. loss of actual spinal cord volume) that graded mild to severe was recorded in the anterior caudal spinal cord segment (lesion site) of 32/40 vehicle control animals and 11/41 NSI-566RSC injected animals. Tissue transparency, as an indication of subacute degeneration, was observed in gray or white matter at the injury/injection site of 5/40 vehicle controls and 30/41 test rats (**Fig. VII-2**). The severity of

the degeneration was classified as mild to moderate, being primarily moderate in the majority of affected rats from both NSI-566RSC-engrafted (90%) and vehicle-injected (80%) animals.

Microscopically, grafted NSI-566RSCs were generally present in a dispersed, random pattern with no recognizable organization. Occasionally, discrete foci of h.NuMA immunoreactive cell clusters were observed which were present at or near injection sites as well as in the central canal at and adjacent to the lesion site. The amount of Ki67 immunostained (mitotic) cells at the lesion site of vehicle control rats was always at low levels, whereas in the remaining spinal cord no Ki67 immunoreactive proliferating cells were visible. Of the NSI-566RSC-injected animals 52.5% had low levels of Ki67 immunoreactive proliferating cells (0 to 5 cells/200× field), 15% showed 5-20, 25% showed 20-40, and only 7.5% showed more than 40 cells per 200× field. Cell proliferation in these spinal cords was classified as orderly.

Ninety-five percent (39/41) of the NSI-566RSC grafted rats showed an abundance of h.NuMA immunoreactive cells at the site of injury/injections, mostly filling the cavities/syringes entirely. Two animals showed low numbers of grafted cells in their spinal cords. While the majority of grafted (h.NuMA immunoreactive, human nuclei) cells were found both anterior and posterior in the caudal cord segments, a few grafted cells were also observed in the rostral cervical segment, the segment furthest from the injury/injection site.

Both NSI-566RSC-engrafted and vehicle-injected animals showed spinal cord lesion sites with increases in cellularity (cell density), based on H&E stained sections, however, more so in grafted animals. The latter was deemed primarily due to the abundant presence of grafted, h.NuMA immunoreactive, cells. The increase in cellularity in vehicle injected animals was mainly due to the presence of GFAP immunoreactive astrocytes, probably related to the development of the astroglial scar.

There were no h.NuMA immunoreactive cells present in the brains of rats. And there were no intervention-related lesions in the brains of either vehicle injected or cell grafted rats. Hence, in conclusion, no major (histo-)pathological changes noted after NSI-566RSC spinal engraftment in spinal cord injured nude rats at almost 10 months.

Full body necropsy and external histopathology report of NSI-566RSC-engraftment-related tumor formation

Each animal underwent full body necropsy after sacrifice or when found dead. Any lesions/masses were isolated and offered for external histopathological analysis (as above).

Fourteen animals of the vehicle group and five of the NSI-566RSC grafted group showed lesions and/or suspected masses (i.e. swollen, discolored, inflamed, and/or shrunk organs/tissue). All of which were examined by the external pathologist. Two tumors were identified. Both tumors occurred in animals from the cell injected group:

Figure VII-3: Body weights of athymic rats at baseline and during the course of the study (vehicle vs. NSI-566RSC treated). No significant difference in bodyweights was noted. Note that NSI-566RSC-engrafted animals tended to have slightly higher bodyweights.

One had a trichoepithelioma of the skin of the left hind leg (a benign tumor with differentiation toward hair follicle epithelium).

The other had a multicentric malignant histiocytic sarcoma affecting multiple non-neuronal organs (abdominal and thoracic); a tumor commonly observed in aging rats.

All other lesions were also judged unrelated to NSI-566RSC engraftment.

In conclusion, no NSI-566RSC-engraftment-related tumors were identified.

NSI-566RSC-engraftment-related toxicity

Signs of toxicity were scored throughout the study in both the NSI-566RSC-engrafted and vehicle-injected group (**Table VII-1**). Symptom or toxicity ratings (number of symptoms) between NSI-566RSC-grafted and vehicle-injected groups showed no differences ($z=-.69$, Mann-Whitney U: $P=.49$). A staggered gait was the most frequent observation, which was observed in all vehicle and cell-engrafted animals after th.SCCI. Comparisons of general appearance ratings (based on the number of different symptoms expressed) between both experimental groups revealed no differences between groups ($z=-.90$, Mann-Whitney U: $P=.37$). Also stool appearance ratings (based on the number of different characteristics expressed) showed no differences between groups ($z=-1.02$, Mann-Whitney U: $P=0.31$).

Additional *in vivo* health observations

In addition to toxicity-related items, all changes in health status were monitored for both NSI-566RSC-engrafted animals and vehicle-injected animals throughout the study. Final weights were 478 ± 65 grams for the cell-injected animals and 458 ± 59 grams for the vehicle injected animals (**Fig. VII-3**; Two Way ANOVA: $F_{1,68}=1.114$, $P=.30$).

The most frequent clinical observation noted was scratching of the abdomen, which often also resulted in scabbed wounds. This was observed in the cell-injected group ($n=24/41$) as well as the vehicle-injected group ($n=20/41$; Pearson's χ^2 : $P=.38$). *In vivo* palpable masses were noted in three animals in the vehicle group and six animals in the NSI-566RSC-engrafted group. No differences were seen between groups in speed of symptom onset (measured in weeks; $z=-.44$, Mann-Whitney U: $P=.66$). Also, no differences were seen between both experimental groups when frequency of tumor expression was compared (Pearson's χ^2 : $P=.48$). Also, no differences in duration or frequency of manually bladder voiding requirements were present between groups.

Frequency of clinical observations	Vehicle	Cells
GENERAL APPEARANCE OBSERVATIONS		96.2
Appears normal; bright, alert, and responsive	37	37
Appears mildly depressed; quiet, slow to respond to stimulus	1	1
Appears markedly depressed; lethargic, non-responsive	3	4
Appears moribund; recumbent, unable to respond to stimulus	1	2
Found dead	1	1
STOOL APPEARANCE		96.2
Stool normal	41	39
Stool soft	0	2
Diarrhea, no blood or mucus	0	0
Diarrhea with blood or mucus	0	0
SYMPTOMS OF TOXICITY		96.2
No symptoms	41	40
Mechanical repetition of movements (e.g., circling)	0	0
Excessive grooming	0	0
Self biting	1	1
Excessive restlessness and irritability	0	0
Lethargy	4	6
Piloerection	0	0
Difficulty breathing	0	1
Tremors, twitches, and/or spasms	1	1
Staggering gait	41	41
Prostrate; unable to stand or walk	1	2
Convulsions	0	0
Death	1	1
OTHER HEALTH FINDINGS		96.2
Tumor/mass palpable	3	6
Scratching	24	20
Swollen paw(s)	8	6
Unscheduled Necropsy	6	6

Table VII-1: Main health observations after SCI in athymic/RNU^{-/-} rats throughout the course of the study (i.e. ~10 months). Data shows no differences in frequency of any health observation between treatment groups (vehicle- vs. NSI-566RS-injected; $z=-.69$, Mann-Whitney U: $P=.49$).

The following events were found at least once (slightly different events omitted for readability):

Tumor palpable, scratch/scab/sore, dehydration, lactated ringer given, cold to touch, favors a limb, bandage wrap applied, bloody/red urine, Crede maneuver, limb(s) dragging, eye discoloration, hunched, lacrimation, redness, swelling, hardness, pus(-tules), Cefazolin given, tooth discoloration, highly defensive, bent tail, tail tip discoloration, and missing tail tip

In summary, no differences in frequencies of toxicity symptoms and/or health status were noted between the NSI-566RSC-engrafted group and vehicle-injected group

To assess the safety of NSI-566RSC on neurological function, behavioral assessments of neurological function were made.

Locomotor function

The Basso, Beattie and Bresnahan (BBB) locomotor scoring results are shown in **figure VII-4A**. Using the BBB scores locomotor function was evaluated up to nine months after th.SCCI. No significant differences in BBB scores were seen between NSI-566RSC-engrafted and vehicle-injected animals for BBB scores (Repeated Measures ANOVA: $F_{135}=.61$, $P=.44$).

Sixteen animals of both the NSI-566RSC-engrafted and vehicle-injected group (32 in total) which showed weight support during BBB testing at the end of the trial, were subsequently used for additional behavioral sensory and motor assessments (see below). The additional behavioral sensory and motor assessments are no main outcomes for the safety study and were performed to provide supportive safety data. As such, these tests were performed in a fashion not strictly compliant with Good Laboratory Practice guidelines, for financial reasons.

Sensory function

At the end of the study, animals were tested for below-level pain thresholds for mechanical and thermal stimuli using both the right and left hind paws. Using escape-response thresholds for mechanical stimuli, no differences were seen between both experimental groups and tested paws (**Fig. VII-4B**; Two way ANOVAs: $P \geq .31$; with $F_{1,35}=1.06$ and $F_{1,35}=.61$, respectively). Also, no differences were seen in the escape-response latencies for thermal stimuli between NSI-566RSC-engrafted and vehicle-injected animals or between left and right hindpaws (**Fig. VII-4C**; Two way ANOVAs: $P \geq .052$; with $F_{1,35}=4.03$ and $F_{1,35}=.13$, respectively). The effect size of the trend towards between-group difference for thermal stimuli was small (partial $\eta^2=.10$).

Activity assessment

Also at the end of the study, the activity of the animals was evaluated over several trials in an open field (three hours each; one to three trials per animal). Analyses were conducted to compare differences across a number of motor activity variables between cell-grafted and vehicle-injected animals. No significant differences were noted for total distance moved ($P=.98$; **Fig. VII-4D**), frequency of mobility ($P=.46$; **Fig. VII-4E**), total duration of movement ($P=.07$; **Fig. VII-4F**), and mean velocity ($P=.54$).

Figure VII-4: Behavioral results show no functional differences after SCI between groups (vehicle- vs. NSI-566RSC-injected). A) Locomotor (BBB) scores of both treatment groups during the entire course of the study. A score of 0 was given if there was no spontaneous movement, and a score of 21 indicates normal locomotion. BBB scores generally increasing with time until week 17 post-graft, then dropping slightly until week 35 post-graft. No significant differences were seen between groups for BBB scores. B-C) Near the end of the study (8-9 months post-grafting), thirty-two of the animals were tested for thermal and mechanical pain thresholds. No differences in mechanical thresholds were seen (Figure B) between groups or between tested paws. Also, no differences in thermal sensory function were seen (Figure C) between groups or between tested paws. Note that while the treatment effect appears to be approaching significance, the effect size was small (partial $\eta^2 < .10$). D-F) Also near the end of the study (8-9 months post grafting) the latter animals were tested for motor activity. No differences were noted for total distance moved (total distance traveled by the subject's center; Figure D), frequency of mobility (total number of samples involving any movement of the subject's center-point; Figure E), and duration of movement (total amount of time involving movement; Figure 2F). Means $\pm$ SEMs.

Gait assessment by Catwalk

Thirty out of the thirty-two animals were successfully trained (run duration: 2.5 ± 0.2 sec; 2 animals did not perform smooth complete runs and were excluded) and were recorded on the Catwalk at the end of the study (**Fig. VII-5A-C**). Cell-grafted ($n=14$) and vehicle-injected ($n=16$) animals were compared to investigate the effects of engraftment on different aspects of gait using the CatWalk method for gait-analysis. Eleven variables were selected for between group (NSI-566RSC vs. vehicle injected) comparisons and showed no significant differences for run duration ($P=.55$), regularity index ($P=.56$; **Fig. VII-5A**), hindpaw stride length ($P=.99$; **Fig. VII-5B**), hindpaw Base of Support ($P=.88$; **Fig. VII-5C**), hindpaw stance duration ($P=.20$), and hindpaw swing duration ($P=.85$).

Sensorimotor/Beam walk function

Animals were tested sensorimotor integration based on their ability to traverse a beam (3", 2.5", or 2" wide) without foot-faults. There were no statistical differences (Two way ANOVAs) between the NSI-566RSC and vehicle injected groups for number of falls ($F_{1,12}=.18$, $P=.68$), left slips ($F_{1,12}=.03$, $P=.87$), and right slips ($F_{1,12}=1.21$, $P=.29$); as seen in **figure VII-5D-F**, respectively.

Therefore, neither the BBB-locomotor test, sensory assessment, gait analysis, or sensorimotor/beam walk test showed functional differences between NSI-566RSC-engrafted and vehicle-injected animals. In summary, no decline in neurological function was noted after high dose spinal NSI-566RSC engraftment in spinal cord injured nude rats for up to ~10 months.

Figure VII-5: Behavioral results show no functional differences at 8-9 months after SCI between treatment groups (vehicle vs. NSI-566RSC treated; initiated one week post injury). A-C) Thirty out of thirty-two animals (16 of the vehicle treated animals, 14 of the treated animals) successfully completed the training and were recorded on the Catwalk for analysis of gait performance. Of special relevance in SCI are: regularity index (Figure A), hind paw stride length (Figure B), hind paw Base Of Support (mm, Figure C). There were no significant differences between treatment groups. D-F) Thirty-two animals were tested on the beam walk. All animals received two separate trials on the first (and widest, 3") beam. If the animals were not able to traverse at least two thirds of the distance across the beam, they were not tested on the next (narrower, 2.5" or 2") size beam. There was no statistical difference in falls or paw slips between the cell and vehicle injected groups. Means $\pm$ SEMs.

Histological integration of grafted cells

To analyse integration/compatibility with host tissue and neural differentiation of NSI-566RSCs, further immunostainings were performed.

Seventy-eight percent (32/41) of the NSI-566RSC-engrafted rats showed human-specific axonal neurofilament protein (h.HO14) immunoreactive axonal fibers (**Fig. VII-6A**) that projected laterally and orthogonally near the injury site (**Fig. VII-6B**), and both rostrally and caudally away from the contusion site (**Fig. VII-6C**). Also, at the lesion site, h.HO14 immunoreactive cells were found with better delineated and thicker axons (**Fig. VII-6B**), when compared to those at places more distant from the lesion site (**Fig. VII-6C**), therefore appearing to be in a more mature state. Nine NSI-566RSC-grafted animals did not have adequate spinal cord sections for extensive evaluation, but h.HO14 immunoreactive cells were nonetheless observed in the cord. The average length of the h.HO14+ axons from five representative cords was 7.4cm (SD±.32), covering ±67% of the entire cord studied (~11cm). Hence, neuronal differentiation and neurite outgrowth show good compatibility of NSI-566RSCs with host tissue.

Figure VII-6: A human axon (h.HO14) staining of a nearly ten months *in vivo* surviving cell graft in an injured spinal cord from a athymic rat, showing graft survival, migration, and neuronal differentiation. A) Spinal cord injury epicenter and grafting site overview obtained from a mosaic montage of 2.5× images. B) Lateral and orthogonal spreads of bundles of human stem cells. Arrows depict HO14 bundles. Note the thick diameter of h.HO14+ fibers. C) The most rostral end of human stem cell fibers is shown. Arrows depict HO14+ fiber endings. Note the thinner diameter and rostro-caudal direction of the axonal endings, and compare to (more matured) axons in the contusion epicenter (B). Scale bar: 1mm (A), 150µm (B, C).

Figure VII-7: Immunostained spinal cords from spinal cord injured athymic rats, showing host GFP labeled descending motor tracts and graft survival, migration, and neural differentiation. A) Mosaic montages of GFP labeled host descending motor tracts (rubro- and corticospinal tracts; in green) and human nuclei (in red), showing widespread graft survival after ~10 months in the four animals with engrafted NSI-566RSCs. Note that host motor tracts traverse the graft. B) Human specific synaptophysin, indicating neuronal differentiation and synapse formations by NSI-566RSCs throughout the engrafted area, including areas of surviving host tissue. C) Human neurons stained by anti-Neuron Specific Enolase (in red), showing neuronal differentiation of the grafted NSI-566RSCs and formation of neurites, in relatively intact host tissue (i.e. white matter). D, E) Confocal images showing host GFP labeled descending motor tracts (in green) and human synapses stained with human specific anti-synaptophysin (in blue) and human axons (HO14; in red: panel D), indicating synapse formation between host and graft cells. F) Human specific GFAP (astrocytic marker; in red) expression in a transverse section of a spinal cord of a cell engrafted animal near the injury epicenter, indicating widespread astrocytic maturation of the NSI-566RSCs. Stained human nuclei (h.NuMA; in green) colocalizes with the human GFAP staining, showing human specificity of this GFAP staining. Scale bars: 4mm (A, B), 200 μ m (C), 50 μ m (D, E), 0.5mm (F)

Next to the 82 animals of the main study, eight additional animals had their long descending motor axons labeled (four received NSI-566RSCs, four received vehicle only; **Fig. VII-1**). This was done to show integration of grafted NSI-566RSC in the host tissue on a more detailed fashion. These animals were also used to further characterize the neural differentiation of the NSI-566RSCs nearly 10 months after grafting.

NSI-566RSCs were again generally found to be located in a dispersed, random pattern with no recognizable organization and almost entirely filling up the injury cavities (h.NuMA/human nuclei in red; areas with cavitations are marked in yellow dotted line; sections from vehicle injected animals are marked with an asterisk; **Fig. VII-7A**).

Grafted NSI-566RSCs were found to widely express human-specific synaptophysin (h.Syn in blue; **Fig. VII-7B**). At one and a half month prior to sacrifice, cortico- and rubrospinal axons (in the posterior funiculi and the posterior side of the lateral funiculi of rat spinal cords, respectively) were labeled anterogradely with Green Fluorescent Protein (GFP in green; **Fig. VII-7A**). Human Neuron-Specific Enolase immunoreactive neurites were observed in between host long descending motor axons (h.NSE/human neurites in red, host axons in green; **Fig. VII-7C**). Also, confocal imaging showed human-specific synaptophysin immunoreactive synapses bordering on long descending host motor-tract axons and/or human-specific neurofilament antibody immunoreactive axons (h.Syn in blue; HO14 in red; **Fig. VII-7D&E**). Last, human-specific GFAP immunoreactive astroglia cells (in red; with verified specific colocalization with h.NuMA immunoreactivity, in green; **Fig. VII-7F**) were widely present near the grafted lesion site in spinal cord of NSI-566RSC-grafted animals, but no Olig2-immunoreactive cells were found to colocalize with h.NuMA-immunoreactive cells.

In conclusion, our immunohistochemical analyses, albeit based on a limited number of animals, indicates that i) *in vivo* differentiation of grafted NSI-566RSCs into neuronal and astroglial lineages occurred, ii) there was a permissive environment for growth of neurites and formation of synapses from grafted NSI-566RSCs in the host spinal cord, and iii) synapses are formed from NSI-566RSCs which were found bordering on host descending spinal axons.

Discussion

This study showed that over nearly ten months after high dose spinal NSI-566RSC-engraftment in spinal cord injured immunodeficient rats: i) no signs of NSI-566RSC-engraftment-related toxicity were noted, and specifically, ii) no definitive evidence of tumor growth related to NSI-566RSC-engraftment was found within the spinal cords, brains, nor any other organs, and iii) no adverse

health observations occurred which could be related to the NSI-566RSC-engraftment. All major health issues that occurred during the study were all related to the use of immunodeficient rats, the injury and/or surgery, as well as observations seen with aging rats. Moreover, iv) no decline in long-term neurologic function was observed after high dose spinal NSI-566RSC-engraftment, and v) histological assessment showed that spinally grafted NSI-566RSCs integrated in the host tissue as they were a) present in substantial numbers at the lesion site and at sites more distant from the lesion site, b) differentiated in neuronal and astroglial cells, c) extended into the host spinal cords, and d) formed synapses with host cells, including synapses on long descending axons from the Cortico- and Rubrospinal Tracts.

Figure VII-8: Embedding strategy for preparation of external histopathological assessment of spinal cords. A) Full spinal cords (~11cm) were divided in pieces of equal length. The pieces were then mounted above each other in gelatin together with 17 other, similarly divided, cords. B) The location of the injury in the spinal cord and its relative position to the separation cut used for the gelatin embedding, as shown in Figure A.

Some major considerations for the design of a safety study for cell-based therapies have not yet been discussed. First, as there is no predefined cell dose for safety assessments, an optimal cell dose needs to be determined. Although it is recommended to use high doses of any cell-based therapy for safety assessments, it is important to note that if tumors and/or an increased morbidity are then found, the whole study might have to be repeated with a lower dose group. Too high concentrations of growth inducing cells, could lead to confounding morbidity/mortality in the toxicity study, not in the least due to host tissue unable to accommodate more engrafted cells (i.e. not due to therapeutic properties, but physical and/or metabolic effects) (Mannello 2011). Timing of cell-grafting was chosen at one week post injury to prevent pronounced metabolic competition between host tissue and grafted cells, which otherwise would occur in the acute phase post injury. While normal, most stem cells tend to proliferate after engraftment, like NSI-566RSCs, effectively increasing their initial cell dose, which can also cause physical problems. Moreover, injections of human neural stem cells into contusion rat spinal cords led to increased proliferation of the cells, beyond the level observed in intact tissue, as observed in our preliminary studies [unpublished data]. Inclusion of an intermediate-dose and a normal-dose group can be a safety net for toxicity studies. However, in our case, spinal lesioning and (delayed) intraspinal grafting of cells are laborious surgeries, which can quickly result in limitations to the amount of animals in a single study. The cost can also be prohibitive, not in the least since safety studies need to be carried out in a GLP-compliant contract research organization's facility over a considerable time period. Therefore, it is advisable to conduct preliminary studies (in target tissue and subcutaneously) to estimate the maximal feasible dose, during which a reasonable confidence is gained for the appropriate cell dose for the safety assessment.

Secondly, the animal model requires further considerations. Logically, the use of athymic animals, necessary for safety assessments in cell-based therapies, limits the availability of concurrent disease modeling. In our case, although the IND application of NSI-566RSCs was for a trial in ALS-patients, the spinal cord injury model is useful for the safety assessment for this application by offering spinal tissue with varying severities of spinal tissue disease/injury. Tumorigenicity was assessed in injured tissue near the epicenter up to fairly uninjured, more remote tissue, and thus provides a broad and sensitive assay to test for tumorigenicity. Having many unscheduled deaths due to disease modeling is also impractical and unethical, which is another reason for not using ALS models. It is also why in our study a moderate spinal cord injury model was chosen. In such a way the injury would barely lead to increased mortality and animals would soon recover spontaneous bladder functions and adequate locomotion (within several weeks) (Basso 1996). As a rule of thumb, a study design should not anticipate >25% mortality, even in case of natural mortality. In this perspective a 9-10 months

survival in 2-3 months old athymic animals remained feasible, as longevity of athymic rat has been reported to be 9-18 months (Festing 1982, Schuurman 1995). Rats were chosen because athymic animals are available, dosage can be higher than in mice, nude mice are more susceptible to infections than nude rats (van Loveren 1988), and most efficacy data of NSI-566RSCs was established in rats. Only male rats were used in this study, but there is no basis to suspect tumorigenicity of hNSCs is sex-dependent. In our experience, male rats tend to have mildly higher tendencies towards morbidity after SCI, thus possibly yielding higher/better toxicity sensitivity.

Regarding the therapeutic efficacy of NSI-566RSCs in this study, it should be emphasized that due to the design of the study, entailing mainly the large loading dose of NSI-566RSCs and amount of injections needed for the toxicity assessment, any therapeutic efficacy of the NSI-566RSC-engraftment was not expected. Nevertheless, NSI-566RSCs showed good histological integration and both neuronal and glial differentiation. Furthermore, albeit the gross morphology of the spinal cords around the lesion sites was qualitatively similar in spinal cords of rats that received injury plus vehicle-only as compared to those that received contusion injury plus NSI-566RSCs, the lesions tended to be more severe in control rats (i.e. more traumatic tissue destruction) than in NSI-566RSC-engrafted rats (i.e. mostly subacute tissue degeneration), suggesting a possible healing response via structural improvement in the cell injected rats (Wirth 2001). Traumatic tissue destruction, however, is somewhat similar to subacute degeneration only apart from a greater degree of actual volume loss, i.e. with a substantial narrowing of the circumference of the spinal cord at that site. The traumatic tissue destruction as observed mostly in vehicle-injected rats is therefore difficult to consider separately from subacute tissue degeneration after (voluminous) cell therapy has been applied. Another possible positive effect of NSI-566RSCs in this study seems to be the prevention of a glial scar and syringomyelia, as any increase in cell density near the injury site of vehicle control animals was mainly attributed to astroglial cells/scarring, while in NSI-566RSC-engrafted animals cell density increases were generally due to the presence of grafted cells filling up the cavity. This indicates a possible limiting effect on the glial scar and syrinx formation in NSI-566RSC-engrafted animals.

In addition, with respect to the results of NSI-566RSC on the neurological function assays, it should be noted that while positive treatment effects appear to be approaching significance in some cases, the effect sizes would be small. Gait and activity analyses provide many variables for analysis, but only those of relevance in spinal cord injury were analysed. CatWalk gait-analyses prove especially useful to test spinal cord function through objectively scoring of coordinated locomotion (i.e. smooth transitions between front and hind paws/limbs, with in-line placements of the front and

hindpaws) (Hamers 2006). These measures reflect the function of descending tracts, and hence, neuroprotection and or regeneration. This measure is best represented by the variables regularity index and hind paw base of support (Hamers 2001, Hamers 2006). Group comparisons for gait-analyses require similar speed and hindpaw work in animals between groups. These factors were controlled through the measurement of run duration, and the stride length, and stance and swing duration of the hindpaws (Hamers 2006). Activity analyses were used to assess differences in variables relevant to spinal function, i.e. movement leading to actual displacements, in terms of distance moved, frequency of such movement, and velocity/durations. None of the motor analyses showed significant differences between groups. Last, no effects on hindpaw sensory function (i.e. pain perception) were noted. Due to the lack of control animals/measurements, any trends in differences in sensory function could reflect either an effect in hyperalgesia, allodynia and/or hypesthesia.

The data from this study and the abovementioned efficacy and safety studies were used for IND application and successfully led to IND approval of NSI-566RSC. This study can function as a reference guide in the development of future safety studies of cell-based therapies. Nonetheless, it remains important to keep supervising drug authorities (e.g. Office of Gene and Cell Therapy/CBER/FDA) involved at an early phase of a product development program, to enable early identification of potential individual issues and solutions.

VIII. Immunosuppression for human cell-based therapies in immunocompetent rats

Effective long-term immune-suppression in rats by subcutaneously implanted sustained-release tacrolimus pellet: effect on spinally grafted human neural precursors survival.

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Abstract

Achievement of effective, safe and long-term immunosuppression represents one of the challenges in experimental allogeneic and xenogeneic cell and organ transplantation. The goal of the present study was to develop a reliable, long-term immunosuppression protocol in Sprague-Dawley (SD) rats by: A) comparing the pharmacokinetics of four different subcutaneously delivered/implanted tacrolimus (TAC) formulations, including: 1) castor oil/saline solution, 2) unilamellar or multilamellar liposomes, 3) biodegradable microspheres, and 4) biodegradable 3-month lasting pellets; and B) defining the survival and immune response in animals receiving spinal injections of human neural precursors at 6 weeks to 3 months after cell grafting. In animals implanted with TAC pellets (3.4 mg/kg/day), a stable 3-month lasting plasma concentration of TAC averaging 19.1 ± 4.9 ng/ml was measured. Analysis of grafted cell survival in SOD+ or spinal trauma-injured SD rats immunosuppressed with 3-month lasting TAC pellets (3.4-5.1 mg/kg/day) showed the consistent presence of implanted human neurons with minimal or no local T-cell infiltration. These data demonstrate that the use of TAC pellets can represent an effective, long-lasting immunosuppressive drug delivery system that is safe, simple to implement and is associated with a long-term human neural precursor survival after grafting into the spinal cord of SOD+ or spinal trauma-injured SD rats.

Introduction

One of the essential requirements for successful translation of experimentally-defined cell-based replacement therapies which utilize the allogeneic or xenogeneic cell grafts into clinical practice is the development of safe and effective immunosuppression protocols that will permit long-term survival and maturation of grafted cells. Current clinical and experimental immunosuppression protocols typically use single or combined immunosuppressive drug regimens with drugs delivered orally, intraperitoneally, intravenously or subcutaneously in a single daily dose or divided into multiple daily doses [see reviews (Barraclough 2011, Halloran 1996, MacGregor 1995, Wente 2006)]. While in human clinical settings a targeted plasma concentration of a variety of immunosuppressant drugs can effectively be achieved by a drug dose titration, to accomplish comparable consistency in targeted plasma levels in animal studies remains a major challenge.

Besides cyclosporines, mycophenolate mofetil (MMF), rapamycin or prednisolone, TAC (FK-506, Prograf) represents an immunosuppressant of choice and is frequently used as a solo therapy or in combination with other immunosuppressive drugs (i.e., MMF) (Hefferan 2011, Reis 1998, Xu 2010) [see reviews (Lama 2003, Su 2011)]. TAC couples with immunophilins, proteins termed FK-506 binding proteins (FKBPs) (Siekierka 1989, Thomson 1995). The formation of a pentameric complex comprised of TAC, FKBPs, calcineurins A and B and calmodulin results in the inhibition of the phosphatase activity of calcineurin (Halloran 1996, McKeon 1991). The action of transcription factors requiring dephosphorylation for transport to the cell nucleus is inhibited and leads to suppression of T-cell proliferation and function (Thomson 1995).

In human clinical allogeneic organ transplantation, the recommended concentration of TAC in blood is in the range of 10-20 ng/ml (Pirsch 1997, Przepiorka 1999, Staats 2004) and is effective in maintaining long-term survival of transplanted solid organs (such as kidney, bone marrow or liver) with tolerable side effects typically presented as nephrotoxicity, neurotoxicity, gastrointestinal toxicity or drug-induced diabetes (Vicari-Christensen 2009). In experimental allograft or xenograft animal studies that use rodents (mice, rats) or minipigs as recipients, TAC is typically administered using a chronically implanted intravenous catheter, intraperitoneally or subcutaneously, with doses ranging from 0.05-3 mg/kg/24 hrs (Gold 1995, Hefferan 2011, Saxena 2007, Tze 1992, Usvald 2010). However, despite the use of such aggressive immunosuppressive protocols, experimental xenograft studies are frequently hampered by inconsistent graft survival particularly seen in long-term survival studies. It is believed that the oscillation in plasma drug concentrations and/or insufficient target plasma levels may in part account for inconsistent graft survival. In addition, the requirements of BID (from latin "bis in die": two times a day) injections in order to achieve satisfactory TAC levels and to

minimize toxicity make this approach 1) labor intensive, 2) frequently associated with side effects resulting from repetitive animal injections (such as local inflammatory changes and infection), and 3) associated with systemic side effects such as nephrotoxicity and hepatotoxicity [see reviews (Finn 1999, Gijzen 2010, Teh 2011)].

To extend the half-life of administered drugs in general, several longer-releasing formulations were developed. First, the use of TAC-loaded liposomes has been shown to provide moderate prolongation of the TAC half-life in the whole blood of naïve rats in comparison with conventional i.v. injections of TAC diluted in saline (Ko 1994, McAlister 1998). Second, the use of biodegradable microspheres was shown to provide a relatively stable level of TAC in whole blood for up to 10-21 days after single s.c. administration (Miyamoto 2004, Wang 2004). Third, the use of implantable biodegradable pellets has been successfully used to deliver a variety of synthetic drugs or hormones in human patients and in animal experimental models and showed up to 3-6 months of stable drug release after a single pellet implantation (Jockenhovel 1996, Packard 1992, Srinivasan 2002, Studd 1987). To our knowledge, no immunosuppressive pellet formulation has been reported to be successfully used in rodent or other animal models of xenogeneic neural precursor transplantation.

Accordingly, the goal of the present study was two-fold. First we characterized the pharmacokinetics of four different subcutaneously delivered/implanted TAC formulations, including: 1) castor oil/saline solution, 2) unilamellar or multilamellar liposomes, 3) biodegradable microspheres, and 4) biodegradable 3-month lasting pellets. The optimal TAC formulation, as defined by simplicity of TAC delivery and stable/predictable blood TAC concentration was then selected and used in the second component of our study. The primary goal of the second part of the study was to validate the level of functionally effective immunosuppression in a separate group of SOD1^{G93A} transgenic or spinal trauma-injured SD rats implanted with 3-month lasting TAC pellets and grafted spinally with human fetal spinal stem cells (hSSC) or human ES-derived neural precursors (ES-NPC). The survival of grafted cells was determined at 1-3 months after grafting using human-specific antibodies and confocal microscopy.

In addition, the potency of TAC pellet-induced immunosuppression was validated by quantitative analysis of the circulating T-cell population (CD45, CD4, CD8) and by qualitative and quantitative analysis of the infiltrating T-lymphocytes (CD45, CD4, CD8) in cell-grafted spinal cord regions.

Our results indicate that s.c. implanted 3-month lasting biodegradable TAC pellets represent an effective, safe and simple method to achieve long lasting and effective immunosuppression as

evidenced by 1) consistent xenograft survival and cell maturation, 2) near complete suppression of grafted site T-cell infiltration, and 3) suppression of circulating blood T-cell concentration.

Material and Methods

All procedures were approved by the Institutional Animal Care and Use Committees by the University of California, San Diego and by the Czech Academy of Sciences. Adult Sprague-Dawley albino rats (Velaz Praha, Czech Republic and Harlan Industries, Indianapolis) and SOD1^{G93A} ALS rats (SOD+) (UCSD colony, Dr. D. W. Cleveland, San Diego, California; 49–57 days old) were used in experiments. Animals were housed in standard cages with free access to food and water.

Animal experimental groups were divided into 2 principal studies: 1) TAC pharmacokinetic study, and 2) spinal grafting of human neural precursors in TAC pellet-immunosuppressed animals.

TAC pharmacokinetic study

Four different TAC (Prograf®, Astellas Pharma, Deerfield, Illinois, USA) vehicle-delivery systems were used and delivered into the subcutaneous space (see Table VIII-1 for summary).

Group	Route	Vehicle	Dose	Frequency	n	Time points (detection of blood- levels)
No.1	s.c.	powder dissolved in castor	3	every 24h	4	2,9,24,72,120 hours
	injection	oil/saline solution	mg/kg			
No.2	s.c.	powder dissolved in castor	1.5	every 12h	4	2,12,14,24,120 hours
	injection	oil/saline solution	mg/kg			
No.3	s.c.	unilamellar liposomes	3	once, at beginning	4	2,12,24,48,72 hours
	injection		mg/kg			
No.4	s.c.	multilamellar liposomes	3	once, at beginning	4	2,12,24,48,72 hours
	injection		mg/kg			
No.5	s.c.	microspheres	10	once, at beginning	3	2,9,24 hours, 2,4,7,10,13,16,19,22 days
	injection		mg/kg			
No.6	s.c.	microspheres	20	once, at beginning	3	2,9,24 hours, 2,4,7,10,13,16,19,22 days
	injection		mg/kg			
No.7	s.c.	3 months lasting pellet	1.8±0.15	once, at beginning	4	5,13,20,29,50,56,71,90 days
	implant		mg/kg/day			
No.8	s.c.	3 months lasting pellet	3.43±0.21	once, at beginning	3	5,7,10,17,26,38,59,74, 90 105, 120, 140 days
	implant		mg/kg/day			
No.9	s.c.	3 months lasting pellet	5.1±1	once, at beginning	6	10,27,42,53 days
	implant		mg/kg/day			

Table VIII-1 Summary of experimental groups used in tacrolimus PK study.

1) TAC castor oil/saline solution (Groups No. 1 and No. 2):

Because the hydrophobic nature of TAC powder and its poor solubility in water solutions (e.g., saline) (Kino 1987), TAC powder was dissolved in a mixture of 100% ethanol (8% of total volume), castor oil (2% of total volume) and sterile saline for injections (90% of total volume; **Fig. VIII-1 G**). Two dosing designs were studied. In the first dosing design, animals (n=4; Grp. No. 1) received 3 mg/kg of TAC in 24-hr intervals for a total of 5 days. Blood samples for TAC measurement were collected at 2, 9, 24, 72 and 120 hrs. At 24 and 72 hrs the blood samples were collected just before subsequent TAC injection. In the second dosing design, animals (n=4; Grp. No. 2) received 1.5 mg/kg of TAC in 12-hr intervals for a total of 5 days. Blood samples for TAC measurements were collected at 2, 12, 14, 24 and 120 hrs. At 12 and 24 hrs the blood samples were collected just before subsequent TAC injections.

2) TAC liposomes (Groups No. 3 and No. 4):

Two structurally different liposome designs (unilamellar or multilamellar; **Fig. VIII-1 I, J**) were used (Encapsula NanoSciences LLC, TN). In the first group, TAC-loaded unilamellar liposomes (n=4; 3 mg/kg; Grp. No. 3) were used. In the second group, TAC-loaded multilamellar liposomes (n=4; 3 mg/kg; Grp. No. 4) were used. In both groups, TAC liposomes were injected as a single bolus. Blood samples for TAC measurements were collected at 2, 12, 24, 48 and 72 hrs.

3) TAC microspheres (Groups No. 5 and No. 6):

TAC-containing microspheres were prepared from tacrolimus powder and poly (D,L-lactide-coglycolide) copolymer (Resomer LG 503H, Aldrich) adopting the procedure previously described (Wang 2004). The tacrolimus content in the resulting dry TAC microspheres was 45 mg TAC/g of microspheres as determined by HPLC. Rats were injected with a single bolus of TAC-containing microspheres at a dose of 10 mg/kg (n=3; Grp. No. 5) or 20 mg/kg (n=3; Grp. No. 6). Blood samples for TAC measurements were collected at 2, 9, 24 hrs and at 2, 4, 7, 10, 13, 16, 19, and 22 days.

4) TAC releasable pellets (Groups No. 7, No. 8 and No. 9):

TAC-containing 3-month releasable pellets (45, 65 or 90 mg/pellet; Innovative Research of America, FL, USA or Tacropellet, MD, USA; **Fig. VIII-1H**) were implanted subcutaneously in the interscapular region of the neck in isoflurane (2%)-anesthetized rats. Animals with three different body weight ranges (120-350 g) were employed resulting into 3 different dosing groups releasing 1.8 mg/kg/24 hrs (n=4; Gr. No. 7), 3.4 mg/kg/24 hrs (n=3; Grp. No. 8) and 5.1 mg/kg/24 hrs (n=6; Grp. No. 9) of TAC. Blood samples for TAC measurements were collected periodically between 5-140 days after TAC pellet implant (see **Table VIII-1** for details).

Blood collection and TAC analysis:

In all experimental groups, blood samples were collected from the saphenous vein in fully awake restrained animals. The lateral aspect of the hind leg was shaved, cleaned with 70% EtOH and disinfected with Chlorhexidine. The leg was immobilized in the extended position by applying gentle downward pressure immediately above the knee joint. Petroleum jelly was applied on the site in order to visualize the saphenous vein and to optimize the blood flow. A 20 gauge needle was used to puncture the vein. We first punctured the vein proximal to the ankle and then moved up if additional punctures were necessary (no more than three needle punctures were attempted in any of blood collection sessions). A maximum of 200 µl of blood was collected per time point. Blood was collected into EDTA tubes and kept at -20°C until analysis using 2 equivalent validated assays: chemiluminescent microparticle immunoassay (CMIA) and liquid chromatography/mass spectrometry (LC/MS/MS).

Chemiluminescent microparticle immunoassay (CMIA). CMIA – ARCHITECT TAC (Abbot Architect, Chicago, Illinois, USA) assay was used according to the manufacturer's protocol. The detection limits of quantification for this method were 2 ng/ml (min) and 30 ng/ml (max), respectively. If the concentration of TAC in the sample exceeded the 30 ng/ml limit, blood was diluted with saline (in a 1:1 ratio) and the measurement repeated. The CMIA method was used for quantification in groups 1, 2, 3, 4 and 8.

High performance liquid chromatography – mass spectrometry (LC/MS/MS).

A guard column C18, 4 x 2.0 mm (Phenomenex, Torrance, California, USA) equipped with ABI 4000 QTrap linear ion trap mass spectrometer (AB Sciex, Concord, Canada) was used. Whole blood samples were prepared as follows: 40 µl of sample (EDTA whole blood) was mixed with 140 µl protein precipitation solution (conc. ascomycin and zinc sulfate dissolved in methanol). After centrifugation (17,000 x g for 5 min.), 40 µl of the supernatant was transferred to autosampler vials

for injection into the LC/MS/MS system. Guard column C18 was washed for 1 min. (isocratic flow rate, 600 µl/min.) with a mixture of methanol containing 2.5 mM/l ammonium acetate and distilled water containing 2.5 mM/l ammonium acetate (ratio 75:25). Thereafter, the mobile phase was changed to 2.5 mM ammonium acetate dissolved in methanol (100% of the volume, flow rate 600 µl/min., time 1.50 min) and TAC/ascomycin were eluted to the detector. The column was reconditioned with methanol containing 2.5 mM/l ammonium acetate (75% of the volume, flow rate 600 µl/min., time 1.50 min). MS/MS analysis was performed in multiple reactions monitoring mode using transitions m/z 821.6>768.5 for TAC and m/z 809.5>756.5 for ascomycin. System control and data acquisition were performed using Analyst 1.2 software (Applied Biosystems/MDS Sciex) for automated data processing. The detection limits of quantification for this method were 2 ng/ml (min) and 50 ng/ml (max), respectively. LC/MS/MS method was also used for determination of concentration of the TAC in whole blood in groups 5, 6, 7 and 9.

Assessment of Tacrolimus pellets – induced side effects:

Animals were evaluated daily for the presence of motor dysfunction (upper extremity motor function assessment was used in paraplegic spinal trauma animals), agitation, jumpiness, and tactile allodynia. In addition, animals were monitored for the presence of other potentially TAC-treatment-related signs of toxicity including: scruffy coat, porphyrin (an indicator of stress, known as “red tears”, often mistaken as blood), weight loss, hypothermia, pale eyes (a reliable indicator of anemia), soft stool, diarrhea, lethargy, vocalization, and swollenness of the pellet implanted subcutaneous site (potential indicator of a local cyst formation).

Spinal cord grafting of human fetal tissue-derived stem cells or embryonic cell line-derived neural precursors

To assess the effectiveness of immunosuppression in TAC pellet-immunosuppressed rats, human fetal spinal cord derived stem cells (hSSC) or human embryonic cell line (HUES-7)-derived neural precursors (HUES7-NPC) were employed for intraspinal grafting in transgenic SOD+, (n=10) rats or in SD rats with previous L3 compression injury (n= 4) (see **Table VIII-2** for experimental cell grafting groups).

Derivation of both cell lines was described in detail in our previous studies (Johe 1996, Kakinohana 2012, Usvald 2010, Yuan 2011). Briefly, human fetal spinal stem cells (Neuralstem, Inc., Rockville, Maryland, USA) were derived from the cervical-upper thoracic region of spinal cord tissue obtained from a single 8-week human fetus after an elective abortion. One day prior to each surgery day, one cryopreserved vial of the previously prepared neural precursors was washed, concentrated in hibernation buffer, and shipped from the cell preparation site (Neuralstem, Inc., Rockville, MD, USA)

to the surgery site (UCSD, San Diego, CA, USA) at 2-8°C by overnight delivery. Upon receipt the following day, the cells were used directly for implantation without further manipulation. Before and after implantation, the viability of cells was measured with trypan blue (0.4%; Sigma). On average, 88-93% viability was seen.

Rat strain	Group	Animal ID	Grafted cell line	Survival time after cell grafting (days)
SOD1 (ALS)	No.7	4746	HUES7	77
SOD1 (ALS)	No.7	4753	HUES7	33
SOD1 (ALS)	No.7	4750	NSI	77
SOD1 (ALS)	No.7	4752	NSI	77
SOD1 (ALS)	No.9	5038	HUES7	40
SOD1 (ALS)	No.9	5081	HUES7	32
SOD1 (ALS)	No.9	5041	NSI	38
SOD1 (ALS)	No.9	5076	HUES7	32
SOD1 (ALS)	No.9	5095	NSI	70
SOD1 (ALS)	No.9	5104	NSI	37
SD (SCCI)	-	149	HUES7	105
SD (SCCI)	-	153	HUES7	105
SD (SCCI)	-	155	HUES7	105
SD (SCCI)	-	160	HUES7	105

Table VIII-2: Experimental cell grafting groups. SCCI = spinal cord contusion injury

The human embryonic stem cells (hESCs; HUES-7 line; Melton Laboratory, Harvard University, Massachusetts, USA), were cultured on a mitomycin C-treated mouse embryonic fibroblast (MEF) feeder layer in HUES hESC medium. Columnar rosettes were manually isolated from induced embryoid bodies and passaged every 3 days to remove contaminating cells. In this stage, NPC were harvested and FAC-sorted (Fluorescence-activated cell sorting-FACS) and CD184+, CD44-, CD271-, CD24+ cell populations further expanded on PLO/L- coated plates using modified N2 media. For cell growth, 10 ng/ml bFGF as the sole mitogen was added. NPC were expanded for 10-20 passages and frozen aliquots prepared from passage 15-20. On surgery day, one cryopreserved vial of the previously prepared passage was thawed, washed and concentrated in hibernation buffer. Before and after implantation, the viability of cells was measured with trypan blue (0.4%; Sigma). On average, 85-95% viability was seen.

Spinal cord cell-grafting procedure

SOD+ rats: animals weighing 267 ± 20.9 g (Group No. 7, age 57 days; $n=4$; **Table VIII-1** and **Table VIII-2**) and 203.6 ± 44 g (Group No. 9, age 49-52 days; $n=6$; **Table VIII-2**) received spinal grafts of hSSC or HUES7- NPC at 13 days after TAC pellet implantation. To implant cells, the previously described technique was used (Kakinohana 2004). Rats were anesthetized with isoflurane (2% maintenance in room air), placed into a spinal unit apparatus (Stoelting, Wood Dale, Illinois, USA) and a partial Th12–L1 laminectomy was performed using a dental drill (exposing the dorsal surface of L2–L5 segments). Using a 33-gauge needle connected to a microinjector (Kopf Instruments, Tujunga, California, USA), the spinal cord was injected with $1 \mu\text{l}$ (approx. 15,000 cells per injection) of the hSSC or HUES7- NPC cells in hibernation buffer. The duration of each injection was 60 s followed by a 30 s pause before needle withdrawal. The center of the injection was targeted into the base of the ventral horn. 10 injections (approx. 800 μm rostrocaudally apart) were made on the left side of the lumbar spinal cord. After injections, the incision was cleaned with penicillin-streptomycin solution and sutured in two layers. Animals were injected with analgesics and antibiotics and were allowed to recover. For the first 14 days after transplantation, additional immunosuppression was performed as previously described (Hefferan 2011) with daily injection of mycophenolate mofetil (MMF, Cellcept, Roche Pharmaceutical, Nutley, New Jersey, USA, dose 30 mg/kg). Animals were allowed to survive until they reached the endstage of the disease (Group 7; 33-77 days after cell grafting, for details see **Table VIII-2**) or until they lost 10% of their bodyweight caused by the progression of the disease (Group 9; 32-70 days after cell grafting, for details see **Table VIII-2**).

Spinal cord (L3) contused rats: four female SD rats ($n=4$; Group No.10; BW: 205 ± 11.9 g) were anesthetized with isoflurane (2% maintenance in room air), placed into a spinal unit apparatus (Stoelting, Wood Dale, Illinois, USA) and a partial Th13 laminectomy was performed using a dental drill (exposing the dorsal surface of L3 spinal segment). Spinal cord compression injury was induced by placing a 3.2 mm acrylic rod (weight=32 g) for 15 min on the dorsal surface of the exposed L3 segment. After compression, the incision was cleaned with penicillin-streptomycin solution and sutured in two layers. Animals were injected with Depomedrol (methylprednisolone, 10 mg/kg) and antibiotics (Cefazoline, 10 mg/kg) and were allowed to recover. Three days after injury animals were re-anesthetized, previously injured L3 spinal segment exposed and received spinal grafts (total of 5 injections; $1 \mu\text{l}$ per injection, approx. 400 μm rostrocaudally apart) of HUES7-NPC targeted into the epicenter and just above and below the injury. The following immunosuppression protocol was used: for the initial 14 days after cell grafting, animals received combined immunosuppression composed of daily single s.c. injection of MMF (30 mg/kg) and BID injections of TAC (1.5 mg/kg/12 hrs). At 14 days, animals were implanted with TAC pellets delivering the TAC dose of 4.9 ± 0.27

mg/kg/day. Because our initial PK study showed that on average 10-15 days is required to achieve a targeted plasma TAC concentration (i.e., above 15 ng/ml), animals continued to receive BID injections of TAC (1.5 mg/kg/12 hrs) for an additional 12 days. MMF treatment (30 mg/kg/day; s.c.) was only used for the initial 14 days after cell grafting. In this experimental group, animals survived 105 days after cell grafting (for details see **Table VIII-2**).

Naïve non-immunosuppressed SD rats: four female SD rats (n=4; Group No.11; BW: 367 ±14 g) received lumbar spinal grafts of hSSC (total of 5 injections; 1 µl per injection, approx. 400 µm rostrocaudally apart; approx. 15,000 cells per injection) as described. No immunosuppression was performed and animals survived for 14 days.

Perfusion, fixation and immunofluorescence staining of spinal cord sections

Rats were deeply anesthetized with pentobarbital and phenytoin and transcardially perfused with 200 ml of heparinized saline followed by 250 ml of 4% paraformaldehyde in PBS. The spinal cords were dissected and post-fixed in 4% formaldehyde in PBS overnight at 4°C and then cryoprotected in 30% sucrose PBS until transverse or longitudinal sections (40-µm-thick) were cut on a cryostat and stored in PBS. Sections were immunostained overnight at 4°C with the following primary human-specific (h) or non-specific antibodies made in PBS with 0.2% Triton X-100: mouse anti-nuclear matrix protein/h-nuc (hNUMA; 1:100; Millipore, Temecula, California, USA), rabbit anti-human neuron-specific enolase (hNSE, 1:500, Chemicon), mouse anti-human synaptophysin (hSYN, Chemicon; 1:2000), rabbit anti-human glial fibrillary acidic protein (hGFAP, 1:500, Origene, Rockville, Maryland, USA), goat anti-doublecortin (DCX; 1:1000; Millipore), goat anti-choline acetyltransferase (CHAT, 1:50, Chemicon), mouse anti neuronal nuclei antigen (NeuN, 1:1000, Chemicon), mouse anti-CD4 and anti-CD8 antibodies (1:500; AbD Serotec, Raleigh, North Carolina, USA), mouse anti-CD3 and anti-CD45 antibodies (1:1000, e-Bioscience, San Diego, California, USA), rabbit anti-Iba1 (IB1) (1:1000, Wako, Richmond, Virginia, USA), mouse anti-RT1B (MHC class II) (1:500, BD Biosciences, San Diego, California, USA), mouse anti CD11b (1:200, BD Biosciences, San Diego, California, USA) and donkey anti-rat IgG (1:500, Invitrogen). After incubation with primary antibodies, sections were washed three times in PBS and incubated with fluorescent-conjugated secondary donkey anti-mouse, donkey anti-rabbit or donkey anti-goat antibodies (Alexa Fluor 488, 546 or 647, 1:250, Invitrogen) and DAPI for general nuclear staining. Sections were then mounted on slides, dried at room temperature and covered with a Prolong anti-fade kit (Invitrogen).

Fluorescent microscopy and leukocyte quantification

Fluorescence-stained sections were analyzed using a Leica DMLB Microscope with a Zeiss Axiocam MRm monochrome camera and Olympus FV1000 confocal deconvolution microscope. Images were captured and analysed using Stereo Investigator software (MBF Bioscience, Williston, Vermont, USA) and Olympus Fluoview FV10-ASW (Olympus Corporation, Tokyo, Japan). Z-stacks, three-dimensional, and orthogonal views were generated in Volocity High Performance 3D Imaging Software. All image manipulations were limited to brightness/contrast, and were performed in a standardized manner for all images. Images were assembled into figures using Adobe Illustrator (Adobe Systems, Inc., San Jose, CA).

Semi-quantitative evaluation of grafted spinal cord tissue infiltration with different types of leukocytes in animals immunosuppressed using TAC biodegradable 3-month lasting pellets was performed using 40- μ m-thick coronal sections. Quantification was performed in 5 sections per animal in TAC dose groups of 1.8 mg/kg/day and 5.1 mg/kg/day (see **Table VIII-1**, Groups No. 7 and No. 9; n=3 for each group).

The number of different classes of leukocytes positive for CD45, CD8/CD45, CD4/CD45, RT1B/CD45 and CD11B were counted and analyzed separately with respect to the location of positive cells in: 1) the core of the graft; 2) the pial surface of the graft, and 3) the spinal cord parenchyma outside of the graft. The degree of leukocyte infiltration was graded as follows: 0- absence of immune cells, *- 1-10 cells, ** - 11-50 cells, *** >51 cells per section.

Quantitative analysis of grafted cell survival

Quantitative analysis of grafted cell survival was performed in animals immunosuppressed with 1.8 mg/kg/day of TAC or 5.1 mg/kg/day of TAC (TAC pellet groups No. 7 and 9; see **Table VIII-1 & 2**) and grafted with HUES7-NSCs or hSCC (n=2 for each TAC dose and cell line).

For quantification, 5 transverse spinal cord sections were selected (minimum 500 μ m apart) from the cell-grafted segments from each rat. An image of virtual tissue of the whole hNUMA-stained section was captured at 10X using Olympus Imer M2 microscope equipped with MBF Stereo Investigator System. The total number of NUMA+ nuclei was then counted using ImageJ "Analyze Particles" plug-in function after setting an identical detection threshold for all analyzed sections. The number of counted cells was then averaged and expressed as number of cells counted per section for each TAC dose and grafted cell line analyzed.

All images used for quantification (i.e., leukocyte-stained and hNUMA+ stained sections) were examined by a blinded observer.

Flow cytometry

Peripheral blood from rats (saphenous vein) was collected into microtainer EDTA tubes (BD Biosciences, San Jose, CA, USA). Red bloods cells were lysed using RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). The lysed blood was stained with antibodies directed to rat CD4 , CD8, CD45, and CD3 (Biosciences, San Diego, CA, USA). A minimum of 10,000 live events (defined by FSC and 7-AAD) was acquired on a six-color BD FACSC. Analysis was done using FACSDiva software. CD4+ and CD8+ cell percentages was measured using a T cell lineage gate defined by 7AAD- CD45+ CD3+ small lymphocytic cells.

Results

Pharmacokinetic profile after subcutaneous TAC delivery (Fig. VIII-1)

Four different TAC vehicle-delivery systems were used for subcutaneous delivery (see **Table VIII-1** for details). 1) TAC-containing castor oil/saline solution: the whole blood TAC concentration in animals receiving a single bolus of TAC (3 mg/kg) was on average 39.5 ng/ml at 9 hrs after injection and decreased to 9 ng/ml at 24 hrs. With continuing daily injections at the same dose (i.e., 3 mg/kg), the plasma concentration of TAC was 12.2 and 7.2 ng/ml at 72 and 120 hrs, respectively (i.e., at 24 hrs after the previous injection) (**Fig. VIII-1A**); the whole blood TAC concentration in animals receiving a single bolus of TAC (1.5 mg/kg/BID) was 11.9 ng/ml at 12 hrs after injection. With continuing injections every 12 hrs at the same dose (i.e., 1.5 mg/kg), the blood concentration of TAC was on average 13.5 and 14.9 ng/ml at 24 and 120 hrs, respectively (i.e., at 12 hrs after the previous injection delivered at 12 hrs and 108 hrs, respectively) (**Fig. VIII-1B**). 2) TAC-containing unilamellar or multilamellar liposomes: the blood TAC concentration in animals receiving a single bolus of unilamellar or multilamellar liposomes-containing TAC (3 mg/kg) was on average 52.9 (unilamellar liposomes) and 52 ng/ml (multilamellar liposomes) at 12 hrs and decreased to 6.1 (unilamellar) and 3.6 ng/ml (multilamellar) at 72 hrs, respectively (**Fig. VIII-1C**). 3) TAC-containing microspheres: the whole blood TAC concentration in animals receiving a single bolus of TAC-containing microspheres (20 mg/kg or 10 mg/kg) showed a biphasic TAC release profile. The first peak was measured at 4 days after delivery and was 19.6 (20 mg/kg dose) and 6.4 ng/ml (10 mg/kg dose), respectively. The second peak was measured at 12 days after delivery and was 12.6 (20 mg/kg dose) and 8.3 ng/ml (10 mg/kg dose), respectively. The TAC concentration at 19 days after delivery was on average 1.5 and 1.7 ng/ml in 20 mg/kg and 10 mg/kg group, respectively (**Fig. VIII-1D**). 4) TAC-containing pellets: groups of animals were implanted with 3-month releasable TAC pellets releasing TAC at 1.9, 3.4 or 5.1 mg/kg/day. In the 1.9 mg/kg group (n= 4) the peak value of 11.1 ng/ml was measured 13 days after pellet implantation and then gradually decreased to 2.8 ng/ml at 90 days. In the 3.4 mg/kg

Figure VIII-1. Blood TAC pharmacokinetics after subcutaneous injections of four different TAC formulations. TAC was injected every 24 hrs for 5 days (3 mg/kg) (a) or every 12 hrs for 5 days (1.5 mg/kg/12 hrs) (b). Blood was collected prior to subsequent TAC injection (arrows). Second, TAC loaded unilamellar or multilamellar (c) liposomes were injected subcutaneously as a single bolus (arrow) (3 mg/kg) and TAC concentration measured periodically for 72 hrs. Third, another

two groups of animals were injected with single bolus of biodegradable TAC microspheres in the dose 10 mg/kg or 20 mg/kg (d). Note the lack of TAC plasma concentration “overshoot” during the first 9 hrs after delivery and a relatively stable plasma TAC concentration for up to 15 days after a single TAC microspheres bolus injection. Fourth, the biodegradable 3-month releasable TAC pellets delivering 1.88 mg/kg/day, 3.42 mg/kg/day or 5.1 mg/kg/day of TAC were implanted s.c. and the TAC plasma measured for up to 4.5 months (e, f). Note a progressive increase in TAC concentration during the initial 3-4 weeks after TAC pellet implantation and then followed by a relatively stable and dose-dependent long-lasting TAC release. Arrows indicate the TAC pellet implantation. Each time point is represented as the mean $\pm$ SD of TAC concentrations. Macroscopical and microscopical images of different TAC formulations including castor oil-saline (g), 90 mg pellet (h), unilamellar (i) or multilamellar (j) liposomes or microspheres (k).

group (n=3) the peak TAC concentration was measured at 38 days after pellet implantation and was on average 29.4 ng/ml. At 3 months the TAC levels were 19.1 ng/ml. In the 5.1 mg/kg group (n=6) the peak of TAC concentration was detected at 27 days and was 46 ng/ml. At 53 days the TAC concentration was 41.7 ng/ml (**Fig. VIII-1E, F**). Macroscopical and microscopical images of all TAC formulations used in this study including castor oil-saline, 90 mg pellet, unilamellar or multilamellar liposomes and microspheres is shown in **Fig. VIII-1G-K**.

Tolerability of SD rats to long-term TAC pellet-induced immunosuppression

Animals implanted with 3-month releasable pellets (dose 1.9 mg/kg/day) showed good tolerability for up to 3 months after pellet implantation and no detectable clinically-defined side effects were noted.

In the animal group implanted with 3.4 mg/kg/day TAC pellets, good tolerability was seen for up to 3 months without any prophylactic antibiotic treatment. Three animals were allowed to survive for an additional 2 months while a progressively decreasing TAC blood concentration was monitored. At the end of 5 months, 2 animals succumbed to infection, which was determined to be of gastrointestinal origin.

In the animal group receiving the highest TAC dose (5.1 mg/kg/day), good tolerability was seen for up to 6-7 weeks after pellet implantation. From the total number of 60 animals so far tested in our laboratory with this dose, 3 animals showed nephrotoxicity at 7-8 weeks. Clinically, these animals showed increased agitation and tactile allodynia. In all 3 animals plasma TAC levels were higher than 60 ng/ml. Once these symptoms were identified, the animals usually died within 2-3 days. Postmortem necropsy showed clear kidney atrophy and the presence of blood in the urine (i.e., hematuria). An additional 4 animals were found dead without any pre-clinical signs of toxicity

between 3-8 weeks after pellet implantation. In 6 of 44 implanted animals, a cyst partially filled with serous fluid and surrounding the pellet implanted-subcutaneous region was identified.

Figure VIII-2: Immunofluorescence examination of the presence of grafted human ES-derived neural precursors (HUES7-NPC) and immunological response in the spinal cord tissue of SOD+ rats implanted with 3-month lasting TAC pellet (1.9 mg/kg/day). TAC releasable pellets were implanted subcutaneously in SOD+ rats (n=4; 60-65 days of age) 13 days prior to grafting with HUES7-NPC. After cell grafting, animals were allowed to survive until they reached the endstage of the ALS disease (approx. 77 days after cell implantation). Immunohistochemical staining with human specific antibodies (hNUMA-human nuclear

protein, green; hGFAP-human astrocytes, cyan) and doublecortin (DCX, red) revealed consistent presence of well-engrafted cell populations in the spinal parenchyma (a-d). Colocalization of hNUMA (red) with NeuN (green) can be seen in a dense population of hNUMA+ grafted cells (e). Triple labeling with DCX, hSYN and NeuN antibody showed numerous DCX+ processes projecting towards large α -motoneurons and co-expressing hSYN punctate-like immunoreactivity (f, g). The immunosuppressive effect of a given TAC dose (1.9 mg/kg/day) was further tested by identifying the presence of infiltrating T-lymphocytes. A high density leukocyte population (CD45, red) in the vicinity of grafted human cells (hNUMA, green) was observed (h-j). Co-staining with markers of T-lymphocytes (CD4 and CD8, red) and microglia (IB1, green) revealed the presence of T-lymphocytes and activated microglia in the areas of grafted cells (k-m). The presence of cytotoxic subpopulation of T-lymphocytes in the regions containing hNUMA+ grafted cells (red) was confirmed by colocalization of marker CD8 (green) with general leukocyte marker CD45 (white), (n). VH – ventral horn, VF – ventral funiculus. Scale bar a-n: 30 μ m.

Survival and maturation of spinally grafted human fetal spinal cord stem cells (hSSC) or human ES-derived neural precursors (HUES7-NPC) in SOD+ rats and TAC-dose dependent suppression of T-cell infiltration (Figs. VIII-2 & 3).

To validate the effectiveness of TAC pellet-induced immunosuppression in an experimental xenograft design, transgenic SOD+ rats were implanted with TAC pellets and then received spinal grafts of hSSC or HUES7-NPC 13 days after TAC pellet implantation. Based on the initial pharmacokinetic study, 3 groups of transgenic SOD+ rats previously implanted with TAC pellets delivering either 1.9 mg/kg/day, 3.4 mg/kg/day or 5.1 mg/kg/day of TAC were used for cell grafting. In addition, a group of SD rats with previous L3-4 compression injury were used. Spinal trauma animals were grafted with HUES7-NPC. Animals in all experimental groups survived between 32-105 days after cell grafting (see **Table VIII-2** for experimental groups). In general, independent of the TAC dose group and grafted cell line, comparable long-term engraftment and maturation of grafted cells was seen in SOD+ animals.

In the 1.9 mg/kg/day TAC group (SOD+ rats) grafted with HUES7-NPC, triple-staining with human-specific nuclear marker (hNUMA), early postmitotic neuronal marker DCX and human-specific GFAP antibody (hGFAP) of transverse spinal cord sections taken from grafted spinal segments showed well engrafted hNUMA+ cell populations composed of DCX+ neurons and hGFAP astrocytes at 77 days after grafting (**Fig. VIII-2A-D**). Double-staining with NeuN (neuronal nuclei antigen) and hNUMA showed that the majority of hNUMA+ cells in the gray matter acquired NeuN immunoreactivity (**Fig. VIII-2E**; insert). Confocal analysis of DCX/hSYN (synaptophysin)/NeuN-stained sections showed a dense population of hSyn+ terminals residing in the vicinity of ventral α -motoneurons and were associated with DCX+ processes (**Fig. VIII-2F, G**).

Figure VIII-3: Immunohistochemical examination of the presence of grafted human fetal spinal stem cells (hSSC) and immunological response in the spinal cord tissue of SOD+ rats immunosuppressed with 3-month lasting TAC pellet (5.1 mg/kg/day). TAC releasable pellets were implanted subcutaneously in the SOD+ rats (n=6; 60-65 days of age) 13 days prior to grafting with hSSC. Animals were allowed to survive until 10% of bodyweight loss caused by the progression of the ALS disease (i.e., 32-70 days after cell

implantation). Immunohistochemical staining with human specific antibody hNUMA (human nuclear protein, green) and doublecortin (DCX, red) revealed consistent presence of high density grafts in the targeted spinal parenchyma in all animals (a, b). Double staining with human-specific synaptophysin (hSYN) and GFAP antibody (hGFAP) revealed a dense hSYN punctuate-like immunoreactivity and numerous hGFAP+ grafted astrocytes (c). In addition to a dense population of DCX+ grafted neurons the presence of human-specific enolase+ neurons (hNSE; late neuronal marker) can be seen (d). The immunosuppressive effect of the administered dose of TAC (5.1 mg/kg/day) was further validated by identifying the presence of T-lymphocytes in human cell-grafted spinal cord sections. Only occasional presence of CD45+ (red) leukocytes was seen; CD45+ elements can preferentially be seen at the periphery of hNUMA+ grafts (e-g). Co-staining with CD45 and CD8 antibody shows near complete lack of CD8+ immunoreactivity in CD45+ cells in cell-grafted spinal cord regions and only occasional double stained CD4/CD8/CD45+ cells can be identified (h-j), while the majority of CD45+ cells are CD8 negative (k; yellow arrows). VH – ventral horn, VF – ventral funiculus. Scale bar a,c: 100 μ m, b,d,e,k: 30 μ m, h:10 μ m.

The degree of T-cell infiltration was probed by staining with CD45, CD4 and CD8 antibodies (see **Table VIII-3** for quantitative analysis). High-density CD45+ cell populations were identified typically at the core or at the periphery of cell-grafted regions (**Fig. VIII-2H-J**). In the same areas CD4/8+ lymphocytes surrounded by activated IB1 immunoreactive microglial cells were seen (**Fig. VIII-2K-M**). Double-staining with CD45 and CD8 antibody showed a clear population of CD45/CD8+ cells in the vicinity of hNUMA+ grafted cells (**Fig. VIII-2N**; yellow arrows).

In the 5.1 mg/kg/day TAC group (SOD+ rats) grafted with hSSC, double-staining with hNUMA and DCX antibody showed a robust cell engraftment with high population of hNUMA/DCX+ grafted neurons throughout the grafted region and extending from the dorsal horn to ventral gray matter at 70 days after cell grafting (**Fig. VIII-3A**). Numerous solitary hNUMA/DCX+ neurons which migrated from the core of the graft were also identified (**Fig. VIII-3B**; inserts). Staining with human specific synaptophysin antibody showed a wide-spread hSYN punctate-like staining pattern throughout the grafted region (**Fig. VIII-3C**). In the same areas a high density of hGFAP+ astrocytes were also seen (**Fig. VIII-3C-insert**). Double staining with DCX and human-specific NSE antibody (hNSE) showed that in areas with high density of DCX+ processes solitary hNSE+ neurons were also present (**Fig. VIII-3D**).

Quantitative analysis of hNUMA+ cells in 1.9 mg/kg/day TAC group showed on average 2712 ± 320 cells in HUES7-NPC-grafted animals (n=2) and 1772 ± 629 cells in hSSC-grafted group (n=2). In the 5.1 mg/kg/day TAC group on average 2750 ± 527 cells were counted in HUES7-NPC-grafted animals (n=2) and 2164 ± 638 cells were counted in the hSSC-grafted group (n=2).

Figure VIII-4: Immunofluorescence examination of the presence of grafted human ES-derived neural precursors (HUES7-NPC) in TAC pellet (4.9 mg/kg/day)-immunosuppressed SD rats with previous L3 contusion injury. Animals (n=4) received spinal grafts of HUES7-NPC at 3 days after spinal injury and were allowed to survive 105 days after the cell grafting. Immunohistochemical staining with antibodies against human neuronal specific enolase (hNSE, red), human cell nuclei (hNUMA, blue) and doublecortin (DCX, red) on the longitudinally-cut spinal sections revealed continuous graft survival and extensive neuronal differentiation in cell-grafted peri-injury regions (a-d). Staining against the geneal leukocyte marker (CD45, green) and microglia (Iba1, red) revealed the absence of leukocytes and the presence of necrotic bodies (non-specific staining, green, yellow arrows) associated with the cytoplasm of microglial cells (e-g). cc = central canal, la = lateral, ro = rostral, ca = caudal. Scalebar a: 100 μm; b-d: 10 μm; e, f: 30 μm.

In both TAC dose groups (3.4 and 5.1 mg/kg/day), staining with CD45, CD4 and CD8 markers showed a similar pattern. The number of CD45+ stained cells was relatively low (if compared to 1.9 mg/kg/day TAC group; see **Table VIII-3** for quantitative analysis) with the majority of CD45+ cells typically found at the periphery of individual grafts (**Fig. VIII-3E-G**). Staining with CD4/8 antibody showed only sporadic presence of CD4/8+ cells (**Fig. VIII-3H-J**) and the majority of CD45+ cells found in grafted DCX+ regions were CD8 negative (**Fig. VIII-3K**; yellow arrows).

immunological profile	Dose of Tacrolimus		1.8mg/kg/day			5.1mg/kg/day (##)		
	cell type		G-core	G-pia	SC-par	G-core	G-pia	SC-par
CD45+	leukocytes		***	***	***	*	**	*
CD8+CD45+	cytotoxic T-cells		**	***	*	0	*	0
CD4+CD45+	helper T-cells		***	***	**	*	*	0
RT1B+CD45+	B-lymphocytes		**	***	**	*	*	0
CD11b+	granulocytes+ monocytes		**	***	*	*	*	0

Tab.3 Quantitative/qualitative analysis of leukocyte infiltration in transverse spinal cord sections with identified human cell grafts at 77-90 days after cell grafting; 0 = absence of immune cells, *-1-10 cells, **-11-50 cells, *** >51 cells per section; G = core- core of the graft, G = pial surface of the graft, SC-par = parenchyma of the spinal cord outside of the graft, ## = in animals injected with 3.4mg/kg/day Tac dose a comparable suppression of leukocyte infiltration as seen in 5.1mg/kg/day group was identified (not shown).

Survival and maturation of spinally grafted HUES7-NPC in adult SD rats with previous lumbar (L3) spinal contusion injury (Fig. VIII-4)

To characterize the survival of spinally grafted human ES-derived neural precursors, adult SD rats with previous L3 spinal contusion injury received spinal grafts of HUES7-NPC at 3 days after injury and survived for 3 months. The dose of TAC delivered by implanted TAC pellet was 4.9 mg/kg/day. Immunofluorescence analysis of hNSE/hNUMA/DCX triple-stained sections showed an advanced stage of neuronal maturation as evidenced by an intense hNSE immunoreactivity (**Fig. VIII-4A**; inserts). Numerous double stained hNSE/DCX solitary neurons which migrated outside of the grafts were also identified (**Fig. VIII-4B-D**). Similarly as in SOD+ rats immunosuppressed with a high TAC dose, staining with CD45 antibody revealed only occasional presence of CD45+ cells at the periphery of individual grafts (**Fig. VIII-4E-G**). Co-staining with CD45 and CD8 antibody showed only occasional presence of CD8+ cells (not shown).

Figure VIII-5: Effect of TAC pellet-induced immunosuppression of circulating T-cells and the spinal infiltration with IgG+ cells, B-cells and granulocyte/monocyte in cell-grafted animals. In TAC pellet-immunosuppressed animals (5.1 mg/kg/day), a significant suppression in the number of circulating blood CD4 and CD8 T-lymphocytes was measured using flow cytometry (a-c), (* $p < 0.05$; T-test). Staining with anti-rat IgG (fluorescence-tagged), anti MHC class II (RT1B) and with anti-CD45 antibody in the 1.9 mg/kg/day TAC group showed a high density of IgG+ cells at the periphery of grafted regions (d). In addition, an overall increase in the density of IgG staining in the same areas was seen (d; blue dashed area). Triple staining with CD11b, anti-rat IgG and CD45 antibody showed co-expression of CD11b in anti-rat IgG+ cells (f; yellow arrows) but no clear colocalization with CD45 marker was detected (f; lower panel). In 5.1 mg/kg/day TAC group a near complete lack of anti-rat IgG, RT1B and CD45+ cell was seen (g) and only sporadic occurrence of RT1B/CD45+ cells was seen at the periphery of grafted regions (h; yellow arrows).

Effect of TAC pellet-induced immunosuppression on circulating blood T-cell population and IgG immunoreactivity in spinal human cell-grafted regions (Fig. VIII-5)

To further probe the effect of TAC pellet-induced immunosuppression on T-cell activity, we quantified the density of circulating T-cell population in blood in naïve, SOD+ TAC non-treated and SOD+ TAC-treated (5.1 mg/kg/day) animals. In comparison to TAC non-treated animals, immunosuppressed rats had a significantly decreased number of circulating CD45, CD4 and CD8 cells (**Fig. VIII-5A-C**), ($p < 0.05$; t-test).

To identify the presence of IgG-secreting cells or soluble IgG in cell-grafted spinal cord regions, sections taken from all 3 TAC dosing groups were stained with anti-rat IgG (fluorescence-tagged) and then further stained to detect MHC class II (R1TB) and CD45 to identify B cells. In 1.9 mg/kg/day TAC group numerous IgG+ cells were identified in grafted regions. Similarly as for the CD8 population, the IgG+ cells were typically localized at the periphery of individual grafts. Double staining with anti-rat MHC class II antibody and anti-rat IgG showed the B-lymphocyte phenotype in small subpopulation of IgG+ cells (**Fig. VIII-5D**; yellow arrows). In addition to IgG presence on B-lymphocytes, an overall increase in the density of IgG staining in cell-grafted regions was seen (**Fig. VIII-5D**; blue dashed area). Since the area had minimal R1TB staining, it is possible that deposited IgG is detected in this area. Similarly, double staining with RT1B and CD45 antibody showed only sporadic presence of RT1B/CD45-double-stained cells in cell-grafted areas (**Fig. VIII-5E**).

Triple staining with macrophage and monocyte marker CD11b, anti-rat IgG and CD45 showed a clear colocalization of CD11b and anti-rat IgG immunoreactivity but with relatively weak CD45 staining pattern in the same CD11b/anti-rat IgG+ cells (**Fig. VIII-5F**; yellow arrows).

In 3.4 and 5.1 mg/kg/day TAC groups, only sporadic IgG, RT1B or CD45-stained cells were identified as well as the density of the IgG staining in cell-grafted regions was below detection threshold (**Fig. VIII-5G, H**) and was similar to that seen in control cell-non-grafted animals (not shown).

Rejection of spinally grafted human fetal spinal cord stem cells (hSSC) in the absence of immunosuppression (Fig. VIII-6)

To validate the importance of immunosuppression in providing grafted human cell survival, we next grafted hSSC into lumbar spinal cord in adult SD rats without immunosuppression. Analysis of spinal cord sections at 14 days after cell grafting showed complete grafted cell rejection and intense infiltration of CD4/8 cells at the injection site (Fig. VIII-6 A, B, C, D).

Figure VIII-6: Rejection of spinally grafted hSSC in SD rats in the absence of immunosuppression. Adult SD rats received 10 bilateral injections of hSSC into lumbar spinal cord and survived for 14 days. No immunosuppression treatment was used in any animal. Triple staining of transverse spinal cord sections with DAPI and hNUMA and CD4/8 antibody showed increased cellularity in cell grafted region (a; yellow dashed circle) but with no grafted cell survival as evidence by the lack of hNUMA+ cells (c). Instead an intense accumulation of CD4/8 cells in previously cell-grafted regions can be seen (b, d; yellow arrows). Scale bar a: 100 μ m.

Discussion

Experimental and clinical evidence for need of continuing immunosuppression in xenogeneic CNS grafting design

Extensive previous experimental animal but also clinical data show that xenogeneic neural grafts are rapidly rejected in non-immunosuppressed animals and/or human patients. For example, it was demonstrated that grafted porcine dopaminergic neuroblasts (derived from embryonic day 26-27) are rejected within days to weeks after intracerebral grafting in rats in the absence of immunosuppression (Brevig 2000). It was also demonstrated that the porcine xenografts undergoing rejections are infiltrated with CD8 lymphocytes and are stained positive for IgM and complement component (C3) (Barker 2000). Similarly, in clinical Parkinson's disease trial, rafting of porcine embryonic dopaminergic neurons showed very poor grafted cell survival at 7 months (one postmortem patient analyzed) after grafting despite continuing immunosuppression with cyclosporine (5 mg/kg) (Deacon 1997). In the same study, lymphocyte infiltration was also seen in the cell-grafted region. These data are consistent with our previous reports which demonstrate no or poor graft survival after spinal grafting of human spinal stem cells in SOD+ rats or SD rats with previous spinal ischemic injury if animals were immunosuppressed with TAC as a monotherapy (1.5 mg/kg, q12h or 1 mg/kg, qd, respectively) (Hefferan 2011, Kakinohana 2012). Similarly, grafted cell rejection was associated with an intense CD4/8 T-lymphocyte infiltration in previously cell-grafted regions. Jointly, these data show that in order to achieve satisfactory and long-term grafted cell survival using xenogeneic grafting design, continuing and aggressive immunosuppression which is effective in suppressing T-cell activity is needed.

Stable and controllable blood TAC concentration and tolerability of SD rats to long-term TAC pellet-induced immunosuppression

In our initial phase of the study, we have compared the plasma kinetics of four different TAC formulations after subcutaneous delivery. Three primary characteristics of the PK data and associated animal manipulations (such as repetitive animal injections) were considered in defining the optimal formulation to be used in the subsequent long-term spinal cell grafting studies and included: 1) the stability of plasma TAC concentration over a 24-hr period, 2) required frequency of injections to achieve a targeted plasma TAC concentration, and 3) potential side effects associated with a high plasma TAC concentration or repetitive animal injections.

The rationale for the selection of specific time points for TAC measurements (i.e., more frequent measurements in castor oil/saline, liposomes and microspheres formulations-injected animals and less frequent in TAC pellet-implanted animals; see **Table VIII-1** for summary) was based on expected

differences in TAC kinetics among all formulation and by the need to identify potential blood toxic TAC levels.

First, in castor oil-saline as a vehicle or liposome (unilamellar or multilamellar) TAC-injected animals, a comparable kinetics profile was seen during the initial 12 hrs after 3 mg/kg TAC injections with the peak concentrations (40-60 ng/ml) measured during 2-12 hrs after injections. In contrast to the castor-oil-saline TAC (3 mg/kg) group in which the TAC levels dropped to 10 ng/ml at 24 hrs after single injection, in liposome-TAC injected animals (3 mg/kg) on average 27 ng/ml TAC was measured at 24 hrs and was still around 11 ng/ml at 48 hrs after injection. In animals injected BID with the castor-oil-saline formulation (1.5 mg/kg/12 hrs) for 5 days, the measured peak TAC concentration was seen at 2 hrs (around 23 ng/ml) and stable levels around 13 ng/ml were then measured between 1-5 days if measured at the end of 12 hrs (i.e., at 12 hrs after previous TAC injection). These TAC pharmacokinetic data are similar as reported from other laboratories using mice or rats (Ko 1994, McAlister 1998, Yamauchi 2004).

From the perspective of conducting long-term immunosuppressive therapy, these data demonstrate that, should a castor-oil vehicle system be used for TAC delivery, the BID delivery regimen has the most favorable safety profile (as defined by the peak concentration) and is relatively stable through TAC levels. While more labor intensive, a stable targeted through TAC levels can be achieved and readily adjusted if needed. The use liposome-TAC formulation provides an extended half life, however, a relatively high peak concentration (i.e., above 50 ng/ml) and the cost (approx. \$1,600/6 animals) to prepare these formulations appears to limit its routine use at present.

Second, TAC microspheres were used and animals received a single subcutaneous injection of either 10 or 20 mg/kg TAC-containing microspheres. In both doses, a biphasic release profile was measured with the first peak seen at 4 days and the second peak at 12 days after injection. In the 20 mg/kg group, the peak measured at 4 days was around 20 ng/ml and the levels were still around 10 ng/ml at 15 days after a single injection. This TAC kinetics profile is similar if not identical as reported in other studies which employed the same TAC microsphere formulation. In contrast to the castor-oil and liposome formulation, no initial TAC plasma concentration "overshoot" was seen at 2 hrs after injection and levels were around 12 ng/ml at 9 hrs after injection (in the 20 mg/kg group). The use of TAC microspheres appears to provide a clear advantage over castor-oil and liposome formulation, permitting stable TAC plasma levels for up to 15 days after a single subcutaneous injection. Moreover, additional injections can readily be added should a rapid and continuing stable increase in TAC concentration be desired.

Third, TAC pellets containing different concentration of TAC and delivering 1.8 mg/kg, 3.4 mg/kg or 5.1 mg/kg of TAC/day for up to 3 months were used for subcutaneous implantation. In higher concentration groups, a progressive increase in plasma TAC concentration was seen during the initial 30 days after pellet implantation and then remained relatively stable for an additional 1-2 months. While the pellets are designed for 3-month continuing TAC release, we have observed continuing TAC release exceeding 4 months (n=3) with the TAC levels around 13 ng/ml measured at 5 months after pellet implantation.

With respect to toxicity, no detectable side effects were seen in animals receiving 1.9 and 3.4 mg/kg/day doses and surviving between 1-3 months. From a total of 60 animals receiving the 5.1 mg/kg/day TAC dose, 4 animals died from apparent kidney toxicity at 1.5-2 months. These animals showed signs of agitation, tactile hypersensitivity as well as scratching behavior 3-4 days before death. We speculate that these behavioral signs could be the result of progressive kidney failure and increased creatine levels. Increased creatine levels have been reported in patients receiving higher dose of TAC after solid organ transplants (Finn 1999, Teh 2011).

Defining the optimal TAC immunosuppressive regimen to permit long-term survival and maturation of spinally grafted human neural precursors in rats

As demonstrated in our current study independent of the daily TAC dose delivered (i.e., 1.9, 3.4 or 5.4 mg/kg/day) in the form of a TAC pellet, consistent survival of grafted ES-derived NPC or human fetal spinal cord stem cells was seen at intervals 30-90 days after grafting. This was expressed as the presence of high density DCX or NSE immunoreactive-grafted neurons in targeted spinal cord regions. In addition, high density neuronal processes derived from grafted human neurons expressing human-specific synaptophysin and projecting toward host interneurons and α -motoneurons were identified. Comparable grafted cell survival and maturation was seen in both SOD+ rats and in SD rats with previous L3 contusion injury. In our previous experiment, we demonstrated consistent xenograft survival three weeks after the transplantation of hSSC into the spinal cord in SOD+ rats treated with daily i.p. injection of TAC (3 mg/kg/day) combined with daily i.p. injection of Mycophenolate mofetil (30 mg/kg) (Hefferan 2011).

As shown in the present study, the SOD+ mutant rats show a significant increase in circulating CD4/8 cells if compared to naïve SD rats and this increase in CD4/8 T-lymphocytes was completely blocked by high dose (5.4 mg/kg/day) TAC treatment (**Fig. VIII-5; a-c**). In addition, several previous studies have demonstrated significant spinal inflammatory changes in symptomatic SOD+ rats characterized by intense activation of microglia and astrocytes in areas of previous α -motoneuron loss (Hall 1998, Hefferan 2012). The characteristics of this model and requirement of continuous high dose TAC

immunosuppression in order to provide long-term grafted cell survival further emphasize the importance of aggressive immunosuppressive therapy to achieve consistent cell survival in this neurodegenerative model.

Similarly, in our recent study, we demonstrated consistent cell survival for up to 2 months after spinal grafting of HUES7-NPC after using 1 mg TAC/day dose delivered s.c. as a bolus in castor-oil preparation in SD rats with previous spinal ischemic injury. However, no cell survival was seen if animals were allowed to survive for total of 4 months (Kakinohana 2012). In another study, on average 28-day survival of allogeneic islet grafts in mice after single injection of TAC loaded microspheres (dose 20 mg/kg) or continuing graft survival (>100 days) after repeated injections of TAC loaded microspheres (dose 10 mg/kg; 7-day intervals) was described (Wang 2004). Jointly, these data show that the TAC dose of around 3 mg/kg/day and divided into two 12-hr doses (if delivered as a bolus s.c injection) or in a form of continuously TAC releasing pellet is required for long-term effective immunosuppression to permit xenograft survival in rats.

Translated to plasma TAC concentration, TAC levels of ≤ 15 ng/ml were measured in 1.5 mg/kg/12 hr (castor-oil formulation) or in 3.42 or 5.1 mg/kg/day (pellet groups), i.e., the TAC dosing regimen which was associated with the most potent suppression of T-cell infiltration in the cell-grafted regions. Comparable TAC plasma levels were shown to be required to permit long-term survival of grafted pancreatic islet cells in mice (Wang 2004) or to lead to life-long survival of transplanted solid organs in human patients (Pirsch 1997, Przepiorka 1999, Staats 2004, Vicari-Christensen 2009).

TAC dose-dependent suppression of T- and B-lymphocyte activation and proliferation

Quantitative analysis of T-cells (CD8, CD4) in grafted spinal cord regions showed a near complete absence of this cell population in 3.4 and 5.1 mg/kg/day TAC group if analyzed at 77-90 days after cell grafting. Similarly, the analysis of CD8 and CD4 cells in circulating blood showed a significant decrease in the 5.1 mg/kg/day TAC group if compared to non-immunosuppressed SOD+ rats. In contrast to the 3.4 and 5.1 mg/kg/day TAC groups, a clear population of CD4/8 cells was seen in 1.9 mg/kg/day TAC-treated and cell grafted animals. Interestingly, a substantially higher density of CD8 population was seen on the dorsal surface of individual grafts facing the pial membrane. We speculate that the presence of grafted cells in heavily vascularized but BBB-lacking pial arterial system is more susceptible to extravasation of the circulating T-cell population and the resulting T-cell mediated response. We also hypothesize that even if a satisfactory cell survival was seen in the 1.9 mg/kg/day group at 77 days after cell grafting, a progressive grafted cell rejection will likely develop should the same level of low level immunosuppression continue over an extended period of time. We also saw small lymphocytic IgG+ MHC class II+ CD45+ B cells in the graft surface. We

speculate that these cells are releasing graft-specific antibodies that are bound the the graft or to CD11b+CD45- microglia cells.

Limitation of subcutaneous TAC pellet-induced immunosuppression

Several technical issues need to be considered once a pellet immunosuppression regimen is going to be implemented into any rat xenograft-immunosuppression study. 1) Based on our PK study, it takes on average 7-15 days after pellet implant before targeted (≤ 15 ng/ml TAC) plasma levels are achieved in 200-350 g rats. Thus, the TAC pellet needs to be implanted before cells are transplanted and the plasma level of TAC validated. However, because consistent and predictable plasma TAC concentrations were measured in our initial PK study in all 3 TAC pellet dosing groups, we have currently implemented less vigorous TAC plasma monitoring and the TAC concentration is only measured 1-2 times during the course of the 2-3 month post-pellet implantation period. Consistent with our initial PK TAC data, expected TAC concentrations were measured in more than 30 animals. 2) Should there be a desire to remove already implanted TAC pellets to achieve an abrupt termination of immunosuppression, the tissue surrounding the pellet implanted region needs to be excised in block. From our experience, the pellet identification is substantially obscured as soon as 7-14 days after implantation because of its structural disintegration. 3) Similarly, should there be a need to increase the dose of delivered TAC, an additional pellet(s) can be implanted. Should this be the case, TAC plasma monitoring is recommended to be performed in 5-7 day intervals for at least 2-3 weeks after additional pellet implant to identify potential unwanted toxic TAC concentrations. However, as shown in our current study, the plasma TAC concentrations up to 45-50 ng/ml were well tolerated for up to 3 months and only a fraction of animals displayed systemic side effects. We are currently testing the tolerability of repetitive TAC pellet implant (3.5 mg/kg/day dose group) and have not seen any detectable side effects at 4.5 months of continuous immunosuppression at this dose (unpublished observation).

In conclusion, we demonstrated that by using an implantable 3-month lasting TAC-releasing pellet it is possible to achieve functionally effective immunosuppression in SD rats as defined by long-term survival and maturation of spinally grafted human neural precursors derived from human fetal spinal cord or from embryonic stem cell. Plasma TAC concentration of ≤ 15 ng/ml was found to be required to lead to a near complete suppression of T-cell activity in the human cell-grafted spinal cord region and in circulation blood and was readily achieved by 7-10 days after pellet implantation. Jointly, these data demonstrate that the use of implantable TAC pellets can represent an effective long-lasting immunosuppressive drug delivery system which is technically simple to implement, is safe for

an extended period of time (4.5 months) and is associated with consistent and long-term human neural precursor survival after grafting into the spinal cord of SOD+ or spinal trauma-injured SD rats.

IX. Dissertation discussion

Dissertation discussion

In this chapter the results of our studies related to the research questions, as addressed in Chapter 1, will be discussed. First, the reasons for the wide variance of pain prevalence in spinal cord injury (SCI) patient populations are explored (see below). Since most problems related to SCI still both have a chronic and therapy-resistant character, effective treatments are urgently needed (see below). Development of treatment strategies requires further fine-tuning of animal research for application in the clinical reality. Therefore the differentiation between spinal-reflex-mediated functionality and supra-spinally mediated functionality in rodent SCI is of great importance and needs first to be addressed (see below).

Spinal Cord injury and its varying pain prevalence

The first research question of this thesis was related to the large variance in reported SCI pain prevalence figures and therefore formulated as follows: “Is it possible to identify SCI population characteristics in published studies that can explain the differences in reported pain prevalence rates in SCI patients?”. The answer to this research question can be subdivided into: **A)** there is a negative, and independent, relationship between reported pain prevalence rates and the study characteristics: 1) *‘Strictness of the pain definition (mild, moderate or strict)’*; more strict relates to lower pain prevalences’, and 2) *‘Whether or not a study was primarily a SCI pain study (yes vs. no)’*; primary pain studies reported higher pain prevalences figures was noted, and **B)** a positive relationship for the population characteristics *‘depression prevalence’* and *‘time interval of pain measurement after the initial SCI’*; SCI populations with high depression rates and longer time intervals showed higher pain prevalences, but (in)dependence of the latter two variables could not be tested and is, indeed, possible (**Chapter 2**). Our meta-analysis was based on 82 studies (see **Chapter 2**), which reported on pain prevalence in SCI patient populations. To correct for the unwanted relationship between reported SCI pain prevalences and the study characteristics *‘retrospective data’*, *‘year of publication’*, and *‘response rate’*, we excluded all retrospective studies, the studies published before 1960, and three studies which had reported both an extreme prevalence rate and a doubtful response rate (69 studies remained of the initial 82).

It should be noted that meta-analyses (of pooled data) do not prove any causal relationship, but only provide supporting evidence for such a correlation. A major complication in our, and most other, meta-analysis is the fact that correlations are based on regression analyses which might be sub-optimal due to the use of aggregate/pooled patient data and not individual patient data.(Stroup 2000) It is easily understood that in case a study reports high pain prevalence and, at the same time,

a high depression rate within its studied pooled patient population, it still is not possible to determine whether the individuals with pain and those with a depression are the same. An increase in the confidence on such correlations can be obtained through the inclusion of both a larger number of studies (with varying reported prevalence rates) and study samples in which all or most patients have a particular characteristic (e.g. a depression).

Hence, studies reporting on the pain prevalence of categorical subpopulations in their analysis, e.g. in patients all having a complete lesion, have also been included as such into our meta-analysis (for example: Anke et al. 1995 (Anke 1995)). Furthermore, our meta-analysis allows the study of categorical subpopulations reporting on pain prevalences of SCI patient which were either 1) having complete or incomplete injuries, 2) working or not working, 3) having traumatic or non-traumatic injuries, or 4) tetraplegic or paraplegic. However, these clinical patient characteristics did not result in any gain or loss of the already identified correlations with pain prevalence rates, making it unlikely that a marked relation between SCI pain and such clinical patient characteristics exists. However, with regard to the determinant '*depression*' which did show a relation with pain prevalences; no studies were noted that report a subpopulation in which all individuals either report or do not suffer from a depression. Nonetheless, wide varieties in depression rates have been reported by the different studies, ranging from 2.2% up to 65.4% (**Chapter 2**).

Using categorical subpopulations one could also calculate relative risks for reporting pain for patients presenting a specific characteristic (compared to the ones not presenting that characteristic). Indeed, studies investigating pain predictors typically only report relative risks, not pain prevalences. These relative risks can also be used for a meta-analysis on pain determinants. Since our research question basically entails the identification of pain determinants, as any relationship between pain prevalence and a population characteristic is indicative for a pain determinant, one could argue that a meta-analysis of risk factors for SCI pain should have been more appropriate instead of a meta-analysis for SCI pain prevalence rates. We decided not to perform a meta-analysis of risk factors for SCI pain because the studies reporting relative risks for pain after SCI normally only do so for a limited amount of risk factors. Also, not many studies report risk factors, and the ones reported practically only involve patient-related determinants (and no methodological factors). Moreover, using risk factors no pain prevalence determinants have yet consistently been identified (Margot-Duclot 2009). Importantly, when using relative risks to determine factors influencing pain prevalence in a meta-analysis, one limits the possibility to analyze other pain determinants than the ones for which the relative risks are given (typically only a limited amount of patient-related determinants). Moreover, it is not unlikely that such other, underlying, determinants

for pain prevalence might include methodological factors. The latter is particularly evident as our meta-analysis showed strong relationships between reported pain prevalences and methodological factors (e.g. use of retrospective data, difference in pain definition, and whether or not a study was a pain study).

By any means, from our meta-analysis on SCI pain prevalence rates we may conclude that pain is a major problem in the SCI population, as projected averages indicate that *more* than half of SCI patients has pain complaints.

Spinal reflex mediated functionality and supra-spinally mediated functionality in rodent SCI models

In order to explore the effectiveness of treatment approaches focused at the detrimental effects of SCI, animal models are of great scientific value. However, investigating SCI treatments in animal models is controversial and has been the subject of an intense scientific debate since many years. For instance the subjectivity of pain in humans, let alone animals is something that is difficult, if not impossible, to catch in hard scientific data. Also (loco-) motor function differs significantly between humans and animals. An important underlying cause might be the difference in supraspinal ('conscious') CNS involvement for (sensi-)motor reflex function (Baastrup 2010b). This aspect is addressed in **chapter 3** and **4** in order to answer our second research question: "Can we differentiate between spinal reflex mediated functionality and supra-spinally mediated functionality in rodent SCI models?".

With respect to sensory function we were able to demonstrate that while stimulus-evoked hindpaw withdrawal reflexes remained intact or even augmented after SCI (as we noticed *decreased* thresholds for paw withdrawal/jumping/kicking behavior), the evoked escape response paradigm revealed *increased* thresholds for below-level stimuli (**chapter 3**). Below-level paw withdrawal responses are thus poor outcome measures for pain/hypersensitivity tests in rodent SCI models, as they are mediated by spinal functional autonomy. Also, increased below-level as well as spinally mediated withdrawal reflexes did not coincide with muscle stretch hyperreflexia (as measured in the calf/gastrocnemius muscle; (Baastrup 2010a, Baastrup 2010b, Marsala 2005) and see **chapter 3**). This suggests that withdrawal reflexes do not correlate with spasticity, which then makes it more likely that paw withdrawal reflexes (after SCI) relate best to clinical syndromes like spasms and/or clonus, and thus reflect functionality based on intact autonomic spinal circuitry (i.e. spinal reflexes).

Regarding the motor function we were able to demonstrate that spinal autonomic functionality is also likely to mediate a large amount of the basic forward locomotion function in rats (see **chapter**

4). Motor tests which require more supraspinal control, i.e. ladder climbing, motor evoked potentials, and backwards locomotion (on a rotating rod) showed relatively more impairment after moderate or severe SCI than the basic locomotion tests like open-field, gait-analyses, and the rotating rod. However, these tests were still not able to significantly detect chronic deficits after mild thoracic spinal cord injury. Only one locomotion test, the backwards locomotion test, was able to show significant impairments, and thus sensitivity for loss of (even some) supraspinal control, in mild SCI at six weeks post injury (see **chapter 4**).

These findings are important as it is likely that rodent SCI models can be made more sensitive to treatment effects on restoration of across-lesion connectivity/functionality when outcome measures which are more supra-spinally mediated (such as escape responses and backwards locomotion ability), are used instead of the conventional sensory and motor outcome tests. For patients restoration of across-lesion connectivity and functionality is likely much more relevant than exploiting spinal autonomic function. We therefore conclude that the use of supra-spinally mediated outcome measures, such as escape responses and backward locomotion ability, can render rodent SCI models more sensitive to detect repair of across-lesion connectivity/functionality.

As the absence of chronic severe locomotor/sensorimotor deficits in rats can be largely ascribed to recovery mediated by the spinal autonomic circuitry, it is argued that this network should ‘simply’ be trained to regain adequate functionality in human SCI patients. In this perspective, wheelchair immobilization could have an inhibiting effect on the retraining of spinal autonomic circuitry. This was recently found to be the case in SCI rats, in which lower body wheelchair immobilization (in rats) led to a vast reduction in recovery of hindpaw function. This indicates that SCI-rats normally highly train their spinal circuitry by moving around in their cages (Caudle 2011). Indeed, it is thought that sensory information associated with phasic limb movements, loading, and paw or foot contact retrains spinal circuitry below the injury (de Leon 2001). Without doubt, some of such spinally mediated sensorimotor functionality is also present in humans (Dietz 2000). However, improvements in walking ability for SCI patients through step training are variable at best and far less than expected based on animal studies (Dobkin 2007). This might partly be due to the fact that the extensive self-training, as is observed in SCI rats, is difficult to translate to humans. Nonetheless, the biomechanical properties of bipedalism in humans as compared to quadrupedalism in rats is also much more likely to require a far more complex control, necessitating supraspinal involvement for the multisensory integration and planning to execute motor commands and maintain posture and balance for walking, standing, and sitting (Dobkin 2007). Therefore, the lack of sensitivity for supra-spinally mediated functionality of most rodent SCI outcome tests makes them not ideal to test

therapeutic efficacy for human SCI treatments. In view of the fact that the backwards locomotion test did show significant locomotion deficits even in mild SCI animals (see **chapter 4**) and up to six weeks, we like to state that backwards locomotion is a promising behavioral test for across lesion connectivity and re-connectivity. Which specific spinal motor and/or sensory tracts are most responsible for rat backwards locomotion, remains to be investigated. Interestingly, the corticospinal tract is typically regarded of little importance to basic hindlimb locomotion in rats and other quadrupeds (Drew 2002, Muir 1999), while it has a more predominant role in human voluntary motor functions (including gait) (Barthelemy 2011). This raises the question what the role of the corticospinal tract is for backwards locomotion. If the corticospinal tract is significantly involved in backwards locomotion, then this may result in testing backwards locomotion on a treadmill as a better outcome measure than forward locomotion, in terms of clinical relevance. In view of this, the persistent general belief that treadmill tests per se (like rotating rod tests) less optimally represent voluntary locomotion, as compared to open-field or runway tests, must be questioned. Hence, it is recommended that treadmill testing allows more stringent control and acquisition of testing parameters, for example: speed, partial external weight support, and as we show, direction of locomotion. The use of an automated treadmill with video capturing and some limited fixed restraining of the animal could significantly improve this method.

Another aspect important to consider in view of assessing supraspinal response thresholds in SCI rats and treatment effects for below-level sensation, is the fact that both increased thresholds (hyposensitivity) as well as decreased thresholds (hypersensitivity) are both undesirable outcomes and that baseline thresholds (i.e. normosensitivity) is the aim. In other words: the closer to the original sensory baseline thresholds of an individual animal, the better the treatment. This seems obvious, but widely used analyses to compare treatment groups (such as repeated measures ANOVAs) tend to ignore this aspect. As group means are used for the analyses, any therapeutic difference between groups in, for example, a better recovery of hyposensitivity in one group might be due to a few animals which developed thresholds below their baseline values (and thus became hypersensitive). The latter might suggest that the recovery is worse, which consequently leads to misinterpretations. We here want to state that an index for the outcome of sensory function should include the following two aspects and as a consequence becomes more appropriate. First, baseline sensory thresholds of individual animals must be used to define hypo- and hyper-sensitivity. Secondly, changes of sensory thresholds are relative to baseline values in a logarithmic relation between stimulus intensity and perceptive thresholds, as generally used is a concept generally in

most psychometric tests (Chaplan 1994, Mills 2012). Logarithmic transformation of thresholds makes that a relative increase, for example, a twofold increase in perceptive thresholds would always equal a similar (i.e. a twofold) decrease, in absolute terms.

Here we suggest implementing the abovementioned concepts in future studies. The outcome then is based on a single, easy to use, outcome variable, the ‘Improvement Index’, which we will further explain below:

$$Improvement\ Index_i = abs\left(\log\left(\frac{x_{i,pretreatment}}{x_{i,baseline}}\right)\right) - abs\left(\log\left(\frac{x_{i,result}}{x_{i,baseline}}\right)\right)$$

Here, $x_{i,t}$ is the average AL-ER threshold of animal i at time point t . To improve between-study comparability (regardless of the chosen base of the logarithm) and to help interpret the values of the Improvement Indices, we suggest to also report the reversed log transformations of the groups mean Improvement indices, which, with a log base of 10, is simply calculated by: $10^{Mean\ Improvement\ index}$. Reversed log transformed Improvement Indices can also be plotted on a secondary y-axis (for example, see **Fig.IX-2B**), to give an idea of the effect size or the net improvement ratio).

Figure IX-2: Example graphs, showing fictive data, to illustrate differences between classical repeated measures analyses (A) and Improvement Indices analyses (B) to explore differences in the treatment effect on sensory thresholds.

In order to further illustrate the added value of the Improvement Index an example will be given: starting from three experimental SCI treatment groups, each containing four th.SCCI rats (Experimental groups A, B, and C in **Fig.IX-2A**). These experimental groups all have shown post-injury hyposensitivity, when compared to the non-treated control group based on standard statistical analyses (Bonferroni post-hoc: $P < 0.001$, for all). Moreover treatment groups A, B, and C do not statistically differ (Two Way Repeated Measures ANOVA: $P = 1.00$). However, one can easily deduce from the graph (**Fig IX-2A**) that the outcome of the treatment in group A is better than in groups B and C, as only group A has no remaining individuals with hyper- and hypoalgesia. If all individual measurements would merely be logarithmically transformed (which becomes obligatory definitely for data on von Frey mechanical hypersensitivity (Chaplan 1994, Mills 2012)), the differences become more apparent, although still no significance is reached (Two Way Repeated Measures ANOVA: $P = 0.56$). Hence, based on this type of analyses the conclusion is still justified that treatment in group A, B and C are equally effective, thereby completely disregarding the fact that 50% of the animals in group B, and especially of group C, become hypersensitive while the other 50% only partly recovered from their hyposensitivity. With the use of the individually based Improvement Indices and statistical analysis of between-group differences (One-way ANOVA & Bonferroni post-hoc) mean differences between treatment groups is statistically significant ($P < 0.05$, 95% confidence intervals: group A vs. B: 0.01-0.46, group B vs. C: 0.001-0.40). Hence, Improvement Indices form a better basis for correct evaluation of treatment results in tests based on sensory thresholds.

The analysis and outcome based on Improvement Indices might have substantial positive impact on the future success of studies into this topic, but it requires quite an elaborate explanation before it can be used and allows appropriate interpretation by the reader. Instead, we have used the percentage of the maximal possible effect (%MPE), with logarithmically transformed sensory thresholds. Although this method of %MPE does not use individual baseline and pretreatment values, it does use the group's average baseline and pretreatment value to express the treatment efficacy. Furthermore, %MPE does not discriminate between hyper-, hypo-, and normo-sensitivity. In the specific case of **chapter 6**, the use of %MPE did not result in different conclusions as compared to those after the use of Improvement Indices. Nevertheless, Improvement Indices can yield very different, and more realistic, results when compared to %MPE and classic comparisons of the mean, especially in case overall treatment effects result in normo-sensitivity, as in such situations the differences between all conditions (hypo-, hyper-, and normo-sensitivity) become relatively small.

Spinal Cord Injury research and effective therapies

The ultimate goal of animal SCI research is to find effective therapies to test possible effective therapies to improve behavioral outcome related to pain and functional disabilities. It is therefore that we addressed the third research question: “does transplantation of OECs or HSSCs into the lesion site provide functional improvement in an experimental SCI model”.

In this thesis we focused our efforts on the use of cell (transplantation) therapies. Two different cell types were used for transplantation; rat olfactory ensheathing cells (OEC) (**Chapter 5**) and human spinal stem cells (HSSC) (**Chapter 6**), because both hold great promise in restoration of neurotrauma such as SCI (as detailed in Introduction (**Chapter 1**)).

Olfactory Ensheathing Cells (OEC)

With respect to the implantation of 3D OEC collagen scaffold complexes into a hemisected spinal cord no functional improvement was noted during a follow-up period of 10 weeks (**chapter 5**). The acute grafting of a structured 3D collagen scaffold seeded with OEC into a 2mm-long thoracic spinal cord hemisection rat model, did not result in beneficial nor detrimental effects in terms of gait and withdrawal reflexes. On the other hand, intraparenchymal injection of HSSC into and around a lumbar (L3) spinal cord compression injury 3 days post lesion resulted in some functional improvements in terms of gait, sensory function, and spasticity (stretch hyperreflexia), as measured up to 8 weeks post injury/grafting. Hence, small but significant functional improvements in terms of sensory function, spasticity and gait were observed after the implantation of intraparenchymal injected HSSC cells into the compressed spinal cord.

The absence of a functional therapeutic effect of our 3D OEC scaffold in an SCI model seems to be related to the inability of OEC to occupy and populate the entire injury site, even in the presence of a state-of-the-art 3D collagen scaffold which provides a bridging opportunity for the OEC. Other studies showing efficacy of OEC therapy in rodent models typically use relative small/gap-less injuries (Li 1997, Li 1998), of which could thus explain why these models did show a functional improvement after OEC therapy. Nevertheless, even our SCI model used is a far less challenging representation of the one found in the human condition (which include random cavitations and uneven lacerations). At the end of our 10 week 3D-OEC scaffold post-grafting experiment, the OEC's could be detected (as they had been obtained from GFP-transgenic rats) and we found the majority of these grafted cells at the border of the graft. Here, the OEC may have exerted a facilitating effect on axonal growth, as neurofilament positive staining was noted within the graft (**Fig. IX-3**). However it needs to be stressed that neurofilament-immunoreactivity was also observed after implantation of in non-OEC-seeded scaffolds. Clearly, as stated before, the increased neurofilament ingrowth into

the graft is not accompanied by any behavioral improvement (at 10 weeks post injury). These rather disappointing results may relate to an insufficient *in vivo* survival or migration capability of OEC upon transplantation. *In vitro*, we observed OEC throughout the (pre-implanted) scaffolds, including the presence of OEC within the scaffold center (**chapter 5**). Hence, possible difficulties in optimal nutrient supply to the scaffold-center, necessary for OEC survival *in vivo*, might underlie the absence of these cells at this location. Unexpectedly, the center of the graft was occupied by neurofilament positive fibers, irrespective of the use of OEC-seeded or non-seeded scaffolds. Therefore, the presence of axonal profiles, throughout and including the center of the graft is not likely related to interactions induced by transplanted OEC.

Figure IX-3: Artists drawing of a section of an injured spinal cord, after formation of an injury cavity and glial scar.** Left (A): schematic representation of the clinical problem after SCI (see thesis introduction; chapter I). Shown are severed axons which (endogenously) attempt sprouting/regrowth(+) after injury. However,

this sprouting is inhibited(-), mainly due to the formation of an astroglial scar^{**}. Sprouting of primary afferents~ causes part of the spinal (spastic) hyperreflexia[#] and possibly also hypersensitivity (/hyperalgesia). Right (B): acute grafting of 3D OEC collagen scaffolds. A few neurofilament-immunoreactive nerve fibers were found into the graft, but this was not significantly altered by the presence of OEC in the scaffold, at best indicating a minor prevention of the inhibitory effects of the glial scar formation by the scaffold (and OEC?). Also, the center of the scaffold provided little trophic support, as the center of the scaffolds were found to be mostly unpopulated by OEC and, although to a lesser extent, by neurofilaments.

Furthermore, acute post-injury grafting fully exposes the OEC to the secondary injury cascade which follows directly after the primary injury (see **chapter 1**), which then further increases the nutritional and metabolic stress exerted upon them. An early timing of engraftment and nutrition-providing/facilitating capabilities (e.g. collagen density) of the graft are thus issues which could remain to be important factors responsible for our negative results. Timing of the grafting might thus be a critical factor in survival of the transplanted cells and thus in the effect and induction of axonal regrowth.

In contrast, any cell-based therapy used in preliminary studies in human SCI patients typically report improved therapeutic outcome when grafting is performed early after injury (Sykova 2006, Yoon 2007). The time-frames referred to in these clinical studies are very difficult to relate to the experimental findings. In general engraftment into the lesions of human SCI patients rarely occurs within the first weeks after injury, and is more often performed many months, up to years, after injury. Moreover, currently published clinical studies on cell-based therapies in SCI typically lack a sufficient number of study subjects and an (appropriate) control group. Interestingly, a first randomized double-blinded controlled (good quality) study of OEC grafted in non-modeled canine SCI (34 pet dogs) showed some locomotor improvement in OEC treated animals, which was not observed in control (vehicle-injected) animals, even though the treatment was initiated at least more than a year after SCI (Granger 2012, Ito 2006, Krudewig 2006). The OECs used in these canine experiments were harvested from the olfactory mucosa. This is comparable to the OECs used in a fairly successful non-randomized clinical study in SCI patients where gain with only minor pain problems (without any novel persistent pain problems) was shown (Lima 2006). In our experiments we used OEC collected from the olfactory bulb, closely mimicking the OECs used in a clinical study which showed mostly negative results (Huang 2012). Although the present available clinical OEC studies are of low quality in terms of methodological design (e.g. blinding, size, and selection of control groups), the results are suggestive for a possible important difference in clinical impact if the

OECs implanted are either harvested from the nasal mucosa or the olfactory bulb itself. Another advantage of OEC collected from the olfactory mucosa is that they are much more easily obtained for autologous transplantation, which aids greatly in circumventing host vs. graft immune response problems (as discussed in **chapter 8**).

From this we tentatively conclude that there might be a subgroup of OECs which is able to facilitate functional locomotor recovery. These transplanted OECs might induce neuronal plasticity including axonal regrowth as has been shown in well controlled experimental studies (Li 1997, Novikova 2011). Although (OEC-filled) scaffolds might eventually facilitate across lesion functional (re-) connectivity in a perfectly specific fashion, it does not necessarily provide an answer to problems secondary to the spinal hyperexcitability found after SCI. These problems are believed to be the basis of many clinical problems, such as pain, spasticity, spasms, and (autonomic) hyperreflexia. In fact, an increased neuronal plasticity might induce worsening of secondary problems after SCI (see Introduction **Chapter 1**). Even if perfect long tract bridging and reconnection can one day be facilitated, it remains an open, and yet mostly theoretical question, whether the problem of hyperexcitable propriospinal circuitry would then also be treated or that it allowed easy facilitation of increased pain and hyper-reflex states. In this context it is important to realize that the onset of problems related to spinal hyperexcitability are unlikely to be solely a direct consequence of a loss of spinal-supraspinal connections. Most secondary problems such as spasticity and pain are likely to be attributed to spinal plasticity, as they develop over time, up to months or years after the primary insult (Hiersemenzel 2000, Siddall 2003). The largest window of opportunity for OEC-therapy thus lies in the early phase after SCI, so it might prevent the onset of secondary complications.

Human Spinal Stem Cells (HSSC)

Intraparenchymal injection of HSSC into and around a lumbar (L3) spinal cord compression injury 3 days post lesion resulted in significant improvements both on reconnection and regeneration of injured fibers as well as on hyperexcitability of segmental networks and development of spasticity and central pain. This is in contrast to the possible effects of OEC, which seem more limited to the improvement of reconnection (see above). It has been shown that HSSC engraftment into an animal model of spasticity ameliorates the spinal hyperexcitability and (due to that) locomotion recovery (Cizkova 2007). Furthermore, HSSC have also shown to be able to regenerate/relay long tracts across spinal lesions (Bonner 2011).

Our HSSC transplantation into a traumatic SCI rodent model also resulted in amelioration of spasticity (i.e. stretch evoked muscle hyperreflexia) in SCI animals, which was possibly due to an increased inhibitory input on the hyperexcitable spinal (reflex) circuitry (see **Fig. IX-4B**). The

observed coinciding improvement in locomotion, which consisted of a slight -but significant- improvement in hindlimb paw positioning, might either be due to a secondary effect of the improvement of the spastic-syndrome or to an improvement in supraspinally mediated locomotor control. The latter would suggest some across lesion reconnectivity; a feature which is also supported by our observations as reported in **Chapter 6**: 1) the observation of an improvement in the SCI-induced below-level hyposensitivity and 2) the wide-spread neuronal differentiation, cavity-filling, and host-graft synaptogenesis of transplanted HSSC (see **Fig. IX-4**). These observations which are indicative for improved across lesion connectivity, however, remain merely circumstantial evidence, as we have found a) only slight but non-significant improvements in cortical evoked hindlimb MMEPs, b) no difference in (myelinated) axon survival in the spinal white matter. Furthermore it should be noted that the sensory improvements could also indicate the onset of a state of hypersensitivity (hyperalgesia?) rather than an improvement of hyposensitivity. Nevertheless, we observed an amelioration of the spinal hyperexcitability, without onset of below-level hypersensitivity in terms of absolute thresholds. Although we did not find evidence of therapy-induced increases in axonal density at the lesion site, it needs mentioning that improved rerouting through existing axons could also still be a mechanism for the recovery of behavior. It has been shown that spontaneous plasticity can occur in the injured rat spinal cord which can facilitate functional recovery across the lesion site through mechanisms of rerouting via still existing axonal pathways (Ballermann 2006, Courtine 2008).

In view of future clinical application of our HSSCs the observations on reconnectivity and regeneration of injured fibers as well as on hyperexcitability of segmental networks and development of spasticity and central pain are important but at least equally important, no tumor formation (see **chapters 7 and 8**) was observed, with at the same time a pronounced neuronal differentiation of the transplanted HSSCs (neuronal differentiation is regarded a rare quality among different neural stem cells) as well as astroglial and oligodendroglial differentiation with cavity-filling (and syrinx-preventing) potential.

In conclusion: spasticity, hyposensitivity, and paw placement are targeted by our HSSC treatment, without worsening of other outcome in the L3 SCI model. Specifically, HSSC-therapy did not show any signs or symptoms indicative for problems of hyperalgesia. We were not able to demonstrate any direct histological evidence of improved survival of axons or the formations of new neuronal relays across the injury site (although others did show that neuronal precursors do have this capacity (Bonner 2011, Lu 2012)). Nonetheless, similar to the evidenced amelioration in the L3-SCI-induced plastic changes by HSSC engraftment, HSSC could also have facilitated rerouting of lost across-lesion

connectivity through surviving axons by improving spontaneous plastic changes at both infra- and supra-lesional spinal segments.

Figure IX-4: Abstract drawing of a section of an injured spinal cord, after formation of an injury cavity and glial scar**. Left (A): schematic of the clinical problem after SCI (see thesis introduction; chapter I). Shown are severed axons, causing motor deficits and hyposensitivity, which are (endogenously) attempting sprouting/regrowth(+) after injury. However, the latter is inhibited(-), mainly due to the glial scar**. Also shown is the sprouting of primary afferents~, which causes part of the spinal (spastic) hyperreflexia# and possibly also hypersensitivity (/hyperalgesia). Right (B): the treatment effect of our HSSC treatment, initiated at 3 days post-injury. HSSC 1) filled up most of the cavity, 2) decreased hypoesthesia, and aberrant primary afferent sprouting, 3) led to some improvement stretch hyperreflexia and gait, and 4) were shown to differentiate in I) neuron with synaptic (incl. GABAergic) connectivity on host neurons, II) astrocytes, and III) oligodendrocytes, at 2 months post grafting/injury.

Possible future research goals

Although our OEC transplantation approach in a rat SCI model did not show any added benefit, the scaffold itself did seem to facilitate some neurofilament outgrowth. It is therefore imaginable that bare scaffolds (or perhaps even seeded with OEC from the nasal mucosa; see IX.3.1) eventually prove useful. Furthermore, adapting the shape (and method of delivery) of scaffolds for use in human injuries, which consist of cavities and non-smooth lacerations, should be prioritized.

The opportunities of effective therapies and SCI research seems to lie mainly in improving/preventing depression (**chapter 2**) and in the amelioration of local plasticity, both by improving aberrant sprouting or activity and (possibly) facilitating signal relay or rerouting (see above). Regarding the latter, future pharmacological studies can provide better evidence as for which pathways are most involved in which therapeutic effect, and help selecting and optimizing cell-based therapies. For instance, such a study can answer the question whether it is indeed the GABA-ergic neuronal activity which improves the spasticity, and to what extent. It is also in this perspective of local effectiveness that it was less surprising that we noted benefits in the L3 SCI model (**chapter 6**), but not in the thoracic SCI model (as in **chapter 7**), for the HSSC treatment, as the thoracic SCI model requires the therapeutic effects to be carried out much further from the site of injury, site of cell-grafting, and site of outcome measurement (i.e. the hindpaws). Obviously, when ameliorating local plasticity is the main treatment effect, gaining effects on larger distances is more difficult, especially when the outcome measures (such as locomotor and sensory measures) are merely gross measures and are lacking the required sensitivity to measure small effects. We thus believe and emphasize that with the use of appropriate models cell-based therapies can -and should- be further optimized in such a way that they will prove to be both a real and a predictable benefit for specific problems in human SCI patients. Notwithstanding, appropriate outcome measures should also be used, for which spasticity measurements, supraspinally mediated sensory and motor tests, and ideally even measures of spontaneous pain, are desirable, but still heavily underused.

X. Published articles

List of published articles (December 2013)

1. [Chapter 6] van Gorp S, Leerink M, Kakinohana O, Platoshyn O, Santucci C, Galik J, Joosten EA, Hruska-Plochan M, Goldberg D, Marsala S, et al: **Amelioration of motor/sensory dysfunction and spasticity in a rat model of acute lumbar spinal cord injury by human neural stem cell transplantation.** *Stem cell research & therapy* 2013, **4**:57.
2. [Chapter 8] Sevc J, Goldberg D, van Gorp S, Leerink M, Juhas S, Juhasova J, Marsala S, Hruska-Plochan M, Hefferan MP, Motlik J, et al: **Effective long-term immunosuppression in rats by subcutaneously implanted sustained-release tacrolimus pellet: Effect on spinally grafted human neural precursor survival.** *Exp Neurol* 2013, **248C**:85-99.
3. [Chapter 5] Deumens R, van Gorp S, Bozkurt A, Beckmann C, Fuhrmann T, Montzka K, Tolba R, Kobayashi E, Heschel I, Weis J, Brook GA: **Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord.** *Behav Brain Res* 2013, **237**:185-189.
4. Kakinohana O, Juhasova J, Juhas S, Motlik J, Platoshyn O, Galik J, Hefferan M, Yuan SH, Vidal JG, Carson CT, Galik J, van Gorp S, et al: **Survival and differentiation of human embryonic stem cell-derived neural precursors grafted spinally in spinal ischemia-injured rats or in naive immunosuppressed minipigs: a qualitative and quantitative study.** *Cell transplantation* 2012, **21**:2603-2619.
5. Israel MA, Yuan SH, Bardy C, Reyna SM, Mu Y, Herrera C, Hefferan MP, van Gorp S, Nazor KL, Boscolo FS, et al: **Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells.** *Nature* 2012, **482**:216-220.
6. Jaken RJ, van Gorp S, Joosten EA, Losen M, Martinez-Martinez P, De Baets M, Marcus MA, Deumens R: **Neuropathy-induced spinal GAP-43 expression is not a main player in the onset of mechanical pain hypersensitivity.** *J Neurotrauma* 2011, **28**:2463-2473.

Submitted:

[Chapter 2] van Gorp S, Kessels AG, Joosten EA, van Kleef M, Patijn J: **Pain prevalences and their large heterogeneity after spinal cord injury: a systematic review.** *European J Pain*

[Chapter 3] van Gorp S, Deumens R, Leerink M, Nguyen S, Joosten EA, Marsala M: **Translation of the rat thoracic contusion model; part 1 – supraspinally versus spinally-mediated pain-like responses and spasticity.** *Spinal Cord*

[Chapter 4] van Gorp S, Leerink M, Nguyen S, Platoshyn O, Marsala M, Joosten EA: **Translation of the rat thoracic contusion model; part 2 – forward versus backwards locomotion testing.** *Spinal Cord*

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XII. Curriculum Vitae

Sebastiaan Franciscus Johannes “Bart” van Gorp was born in Breda, the Netherlands, in 1984 on Friday the 30th of March. From 1996 to 2002 he attended the Sint-Oelbertgymnasium in Oosterhout, Noord-Brabant. In 2002 he started university. First, Biomedical engineering at the Eindhoven University of Technology and, one year later, he transferred to the Maastricht University to study Medicine. During his six years of Medical training, Sebastiaan followed the International Track in Medicine and volunteered in research projects of the Department of Anesthesiology at the Maastricht University Medical Center. He also attended both a research internship at the Montpellier Neuroscience Institute in France and a course on Health Care Systems at Birmingham University in England. From 2009 to 2013 he was a PhD-student at the Department of Anesthesiology at the Maastricht University Medical Center, during which he worked for about two years at the University of California, San-Diego (United States) in the laboratory for neuroregeneration led by Prof. Martin Marsala. Since February 2013 he is working as an Anesthesiology and Reanimation resident at the Université Catholique de Louvain in Brussels, Belgium.

XIII. Summary

Current SCI treatment options are limited. Promising treatments to promote regrowth and regeneration in the injured spinal cord carry the risk of worsening outcomes, such as pain, which is likely due to further stimulating aberrant neural plasticity. However, little is known on the mechanisms of SCI pain or aberrant neural plasticity. Clues to its etiology might be found by analyzing differences between studies on different SCI populations, as widely varying pain prevalences have been reported. On the other hand, one can also accept the limited understanding of aberrant neural plasticity etiology and take a perhaps more pragmatic path by screening the currently most promising regenerative treatments, such as cell-based-therapies, in an adequate preclinical *in vivo* model. However, translation of positive preclinical results into the clinical setting has thus far been disappointing, which calls for a reevaluation of our preclinical tools.

Therefore, this dissertation 1) explores causes for the heterogeneity in SCI pain prevalences and searches for pain determinants, 2) explores tools (sensory and motor assays, and immunosuppression protocols) for the preclinical and translational evaluation of SCI treatments, and 3) investigates the relevance and feasibility of promising candidate cell-based therapies (olfactory ensheathing cells, OEC, and human fetal spinal cord-derived neural stem cells, HSSC) for SCI treatment in preclinical models. The research questions asked are:

1. Is it possible to identify which study and/or population characteristics show a relationship with the differences in reported pain prevalence rates in SCI patient populations? (chapter 2)
2. Can we differentiate between spinal reflex mediated functionality and supraspinally mediated functionality in rodent SCI models? (chapter 3 and 4)
3. Does transplantation of OEC or HSSC into the lesion site provide functional improvement in an experimental SCI model? (chapter 5 and 6)
4. Is long-term HSSC engraftment safe for clinical use and achievable with the use of slow-release immunosuppressive drug formulas? (chapter 7 and 8)

Ad 1) The answer is yes. Eighty-two studies reporting on SCI pain prevalence were examined. Study design related determinants of SCI pain prevalence reports were: pain definition strictness (mild, moderate, or high), primary study goal (pain study or not), data source (retrospective or not), and, in

a limited number of cases, response/attrition rates. While correcting for latter items, populated characteristics found to determine pain prevalence rates were both the proportion of patients with a depression and the average time after injury (positive correlations). Between-study heterogeneity may remain even after the identification/correction of abovementioned causes of heterogeneity. Hence, pain after SCI seems to relate to the duration of the injury and depression, yet, major causes of bias in reported pain prevalence are found to be related to the primary study goal (pain study or not), choice of pain definition, and the use of retrospective data.

Ad 2) The answer is yes. Reduced hindpaw withdrawal thresholds (after rat thoracic SCI), which are often believed to model below-level pain states in human Spinal cord Injury patients do not reflect any decrease in thresholds for pain perception (i.e. hyperalgesia), but instead, coincide with a decrease in below-level perception (i.e. hypoesthesia; which, indeed, is also more the rule than the exception in human SCI patients, and a limiting factor in recovery of locomotion). Yet, increased hindpaw withdrawal responses might still relate to another clinical problem in SCI patients; the “spastic”- or “upper motor neuron”-syndrome (which is thus far largely ignored in translational research). Hence, it was tested whether spasticity/muscle stretch hyperreflexia co-occurred with the increased hindpaw responses, but no such spasticity was observed. Therefore we suggest/discuss that hindpaw withdrawal responses are best interpreted as either 1) a different subset of clinical problems related to the spastic syndrome (i.e. spasms and/or clonus), or 2) as mostly a species-specific phenomenon. Also, widely-used existing locomotion tests can be questioned for their translational value, as, unlike human SCI patients, rats 1) easily recovers hindpaw walking ability after severe SCI, 2) can even do this without sensory perception (see above), 3) have a vastly easier (more stable) bodily position during walking (quadrupedalism vs erect bipedalism), 4) require merely simple ‘stepping patterns’ for normal/daily-life function, and 5) learn to walk much faster in the postnatal period. This indicates that rat locomotion has a low dependence on spinal integrity or supra-spinal input (i.e. depend on their “Central Pattern Generator” or “Spinal” walking), while humans are much more dependent on supra-spinal control. During preclinical therapy evaluations this difference should be kept in mind and an adequate/translatable testing paradigm which is sensitive for supra-spinal mediated motor function must be used. A novel locomotion test is proposed in this dissertation, i.e. backward walking on a rotating rod, and is shown to be more sensitive to SCI than existing tests, even in the chronic phase post SCI, and is likely to be more dependent on supra-spinal input.

Ad 3) The answer is ‘possibly not’ and ‘yes’, respectively. OEC are a promising graftable cell-based therapy, but only few studies have focused on experimental models with large cavitations (as occurs

in humans), which will require bridging substrates to transfer and maintain OEC within the lesion site. A state-of-the-art collagen-based multi-channelled three dimensional scaffold is used to deliver olfactory ensheathing cells to 2 mm long unilateral low-thoracic hemisection cavities in rats. Hyperreflexia of the hindpaws was monitored using the Von Frey hair filament test, while an extensive analysis of motor ability was performed. No substantial improvement or deterioration of motor functions was induced and there was no effect on lesion-induced hyperreflexia. On the basis of these data, it can be concluded that relatively large spinal cord lesions with cavitation may present additional hurdles to the therapeutic effect of OEC. Also, intraspinal grafting of clinical grade HSSC in a rat model of acute lumbar (L3) SCI was tested. Spasticity, lesion volume, various motor and sensory dysfunctions, and neural integration was assessed. Treatment led to a progressive improvement in lower extremity paw placement, amelioration of spasticity and thermal and tactile pain/escape thresholds. Near complete injury-cavity-filling by grafted cells and development of putative GABA-ergic synapses between grafted and host neurons was observed.

Ad 4) The answer is very likely 'yes'. Achievement of effective, safe and long-term immunosuppression represents one of the challenges in experimental allogeneic and xenogeneic cell and organ transplantation. A reliable, long-term immunosuppression protocol in rats was developed by: 1) comparing the pharmacokinetics of four different subcutaneously delivered tacrolimus formulations (tacrolimus is a widely clinically used immunosuppressive agent which inhibits T-lymphocyte signal transduction and IL-2 transcription), and 2) defining the survival and immune response in animals receiving spinal injections of human neural precursors. It is demonstrated that use of TAC pellets can represent an effective, long-lasting immunosuppressive drug delivery system that is safe, simple to implement and is associated with a long-term spinal graft survival in SCI or SOD+ rats. Furthermore, especially the potential tumorigenic property of many contemporary experimental stem cell therapies requires special preclinical safety assessments, but no standard guidelines or any stem cell safety studies have yet been published. A preclinical assessment of the safety of HSSC therapy for a phase 1 clinical trial application is described. The design of the reported safety study is the result of a dialogue between researchers in this field and the national drug authority (the Food and Drug Administration). The negative results found for tumorigenicity, toxicity, and worsening outcomes, in conjunction with data from HSSC efficacy/therapeutic studies have resulted in the approval of the currently ongoing clinical trial of spinally applied HSSC in human patients.

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