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ASSESSMENT OF RAT SPINAL CORD INJURY MODELS

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Assessment of rat spinal cord injury models

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Life is like a box of chocolates. You never know what you’re gonna get.

To my parents with love

致敬爱的父母

ABSTRACT

Traumatic spinal cord injury (SCI) is a complicated and devastating condition, causing different extents of motor, sensory and autonomic dysfunctions. In addition, there is a risk for secondary complications after SCI including posttraumatic syringomyelia (PTS) that can cause further functional loss. Since there is no available effective treatment, tremendous efforts have been made to develop new therapeutic strategies to promote functional recovery after SCI. In experimental research, it is important to utilize model systems, including in vivo models that mimic the clinical situation to develop new treatments. Furthermore, multiple tests should be applied to comprehensively evaluate the models and novel treatments.

We developed two assessment tools for swimming and wading in rats with SCI, with the sensitivity to injury severities from the most severe to the very mildest. Both the Karolinska Institutet Swim Assessment Tool (KSAT) and the wading scale consist of six parameters, reflecting different functional aspects. KSAT and Wading scores for four experimental groups of different injury severity consistently displayed the functional improvement after injury. The internal consistency, inter-rater and test-retest reliability were all very high. We also found a high correlation between KSAT/Wading score and spared white matter at injury epicenter, and between the KSAT, wading and BBB scores.

In addition, we studied kinematic analysis of swimming in two SCI models (mild contusion and compression SCIs) and a control group by a simple two-dimensional system representing the hindlimbs with 3 segments and 2 angles, analyzing six parameters. The results showed that three parameters Swim Speed, Stroke Time and Extension time/Flexion time changed significantly between week 2 and 8. The results of Swim Speed, Angular Velocity and Stroke Time at week 8 were highly correlated with spared tissue at injury epicenter, particularly in contusion SCI. However, for these parameters there was overlap between very mildly injured rats and controls, not achieving the same sensitivity as the KSAT score.

To study neural cell therapy of PTS, we developed a novel rat model mimicking the clinical situation. We used a combination of mild low thoracic contusion trauma and subarachnoid injection of autologous venous blood. The injured rats developed cysts that were extracanalicular, mainly rostral to the injury and lined with astrocytes. T2-weighted magnetic resonance imaging (MRI) scanned 20 weeks after injury showed hyperintense fluid-filled cysts and hypointense areas of tissue degeneration with iron-laden macrophages/microglia. However, the functional analysis did not reveal deterioration coinciding with cyst expansion. Under the guidance of MRI, human neural precursor cells (hNPCs) were transplanted into the cysts. The hNPCs survived, covered the surface of the cyst walls and migrated into surrounding tissue. Moreover, the cells partially obliterated the cysts and in some areas merged the walls of the cysts.

In conclusion, KSAT and wading scale were found to be reliable tools to assess motor activity in swimming and wading, while kinematic analysis did not prove to be very useful for functional testing. The new rat PTS model closely mimics the pathophysiological and anatomical features of the clinical situation. Using this model, transplantation of hNPCs was shown to be a potential treatment to obliterate cysts in PTS.

LIST OF SCIENTIFIC PAPERS

I. **Ning Xu**, Elisabet Åkesson, Lena Holmberg, Erik Sundström. A sensitive and reliable test instrument to assess swimming in rats with spinal cord injury. *Behav Brain Res.,* 2015, 15(291): 172-83.

II. **Ning Xu**, Sreenivasa Sankavaram, Lou Brundin, Elisabet Åkesson, Lena Holmberg, Erik Sundström. Detailed analysis of motor performance during swimming and wading in rats with spinal cord injury. *Manuscript*

III. **Ning Xu**, Raymond Mirasol, Lena Holmberg, Per Henrik Vincent, Eva-Britt Samuelsson, Erikur Benedikz, Bartosz Bezubik, Emilia Rothstein, Lars-Olof Wahlund, Åke Seiger, Elisabet Åkesson, Scott Falci, Erik Sundström. Transplantation of human neural precursor cells to a rat model of post-traumatic syringomyelia. *Submitted Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
bFGF	Basic fibroblast growth factor
BBB	Basso, Beattie, and Bresnahan
CPG	Central pattern generator
CSF	Cerebrospinal fluid
CNTF	Ciliary neurotrophic factor
EMG	Electromyograhpy
EGF	Epidermal growth factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HE	Hematoxylin and eosin
hNPCs	human neural precursor cells
IH	Infinite Horizon
KSAT	Karolinska Institutet Swim Assessment Tool
LSS	Louisville Swimming Scale
MRI	Magnetic resonance imaging
NF	Neurofilament
PTS	Post-traumatic syringomyelia
SCI	Spinal cord injury
SE	Standard error of the mean
SD	Standard deviation

1 INTRODUCTION

1.1 OVERVIEW OF TRAUMATIC SCI AND PTS

Traumatic SCI refers to trauma to the spinal cord, resulting in physical, psychological and social well-being problems. Regarding global epidemiology of SCI, it is usually not accurate due to pre-hospital mortality and incomplete data from many countries. The prevalence and incidence differ between countries and regions, with the highest reported rate in United States [1]. The most common causes are traffic accident, followed by falls, violence and sports. The mean age at SCI is between 15 and 35 years old, but there is a trend towards older age over the last decade [2]. Males are more commonly affected with varying male-female ratio in different countries [3].

Immediately after SCI patients are subject to intensive care and surgery to secure the vital status, stabilize the vertebral column and remove bone fragments of foreign bodies impacting on the spinal cord to minimize secondary damage to the spinal cord and associated medical comorbidity that can worsen the medical condition [4]. The American Spinal Injury Association Impairment Scale or International Standards for Neurological Classification of Spinal Cord Injury is applied to assess the injury severity as well as the motor and sensory deficits from the acute stage and onward. The neurological status is defined by the upper level of functional loss, and if the loss is functionally incomplete or complete, which determinate the long-term prognosis. Importantly, functionally complete injuries are not necessarily anatomically complete. In the acute stage, most or all functions below the level of injury are often lost due to spinal shock, but some functions usually recover as the spinal shock disappear, and more slowly as plasticity, reorganization and compensatory mechanisms set in. Few patients experience complete neurologic recovery by hospital discharge. The annual mortality rate of SCI populations was reported to be 3.6 times higher than general population [5], the most frequent causes of death being cardiovascular disease, pulmonary disease and neoplasm [6]. The importance of evaluating and monitoring remaining autonomic functions are highlighted and use of the International Autonomic Standards is recommended [7].

Post-traumatic syringomyelia is a late complication of SCI, characterized by fluid filled cysts which expand rostrally or caudally, and lead to worsening of the neurological symptoms. The clinical incidence of PTS was earlier claimed to be 0.3-3.2% [8, 9], but as the clinical use of MRI increases, rates of 12-22% or higher have been reported [10-15]. Several predisposing factors such as higher age, higher spinal level (cervical and thoracic), displaced fractures, spinal instrumentation and complete injuries have been proposed to associate with earlier onset of syringomyelia [10], but a definitely causative relationship has not yet been confirmed.

The clinical symptoms of PTS are not specific for diagnosis and include pain, abnormal sensations, spasticity, motor and sensory loss. In some cases, symptoms may be missing completely with no detectable functional deterioration in spite of significant loss of neural

tissue [16]. MRI is the most effective way to diagnose this condition, but artifacts from spinal instrumentation may interfere, and sensitivity may not be sufficient to detect small changes over time.

1.2 PATHOPHYSIOLOGY OF TRAUMATIC SCI AND PTS

In SCI, two distinctive pathophysiological processes can be identified: the primary and secondary phase of injury. The primary injury refers to the mechanical damage to the spinal cord, leading to numerous loss of tissue components, massive hemorrhage and ischemia [17]. It involves a number of mechanisms, the most common of which are contusion, laceration of the spinal cord by foreign objects or bone fragments, and prolonged compression induced by dislocated vertebrae and edema. This damage takes place immediately after SCI and per definition not treatable. However, while the fundamental damage to the spinal cord may be deleterious per se, the following secondary injury determines the neurological deficits and the final outcome.

The secondary phase is characterized by a continuation of events during the primary phase-edema, hemorrhage, ischemia and cell death, leading to a variety of interconnected biochemical processes. Several cellular responses such as increase of intracellular calcium levels, mitochondrial dysfunction, arachidonic acid metabolism and activation of inducible nitric oxide synthase [18] are associated with the formation of reactive oxygen and reactive nitrogen species. Free radicals invoked widespread lipid peroxidation as well as oxidative and nitrative damage to proteins and nucleic acids, leading to cell membrane lysis, collapse of ion gradients and cell death. The increase of extracellular glutamate levels may also induce excessive activation of glutamate receptors, which can be detrimental to vulnerable neurons and oligodendrocytes through flux of sodium and calcium [19]. Activated peripheral and resident inflammatory cells [20] including microglial cells, astrocytes, monocytes and neutrophils, with increased production of chemokines and cytokines may exaggerate the damage to the spinal cord tissue. Neurons and oligodendrocytes undergo necrosis and apoptosis, and many spared axons demyelinate and either degenerate or remain as malfunctional axons. In this process, some microcysts rose in the cord and gradually coalesced, which was considered a pre-factor of PTS [21]. After the degenerative phase, the lesion is completed by the formation of scar tissue at the injury site, composed of abundant glial cells [22], pericyte-derived stromal cells [23], invading fibroblasts and Schwann cells, and a modified set up of extracellular matrix proteins [24]. An important feature of the glial scar is the inhibition of axonal regrowth by secretion of chondroitin sulphate proteoglycans [25] and other neurite inhibitory molecules [26-29]. There is limited regeneration and remyelination occurring after SCI, plasticity in the spinal cord and brain is probably the main factor responsible for functional recovery.

Concerning PTS, post-mortem analysis has shown that the typical features are that the syrinx is extra-canalicular, the syrinx is formed in the neural parenchyma and not through widening

of the central canal. The margins of the cysts are composed of densely packed astrocytes [30], and ependymal cells in some cases [31]. The fluid in the cysts is believed to be similar to the cerebrospinal fluid (CSF) and extracellular fluid, but the composition of the fluid has not been studied.

Regarding the pathophysiological mechanism of PTS, pathological changes both inside and outside the spinal cord after trauma probably contribute to the development of the cysts. Among them, tethering of the cord by strands of connective tissue formed through inflammation in the arachnoid membrane is generally considered as the main cause of the PTS. Consequently, the perturbation CSF in the subarachnoid space led to a dynamic change of CSF flow and pulse pressure. The intramedullary pulse pressure theories [32, 33] were come up to explain the accumulation of extracellular water and distension of the cysts. Alterations in the parenchyma by myelomalacia, including compromised blood-spinal cord barrier [34] leading to fluid leakage, disruption of ependymal cells [35] affecting the balance between the spinal cord and central canal, disruption of the perivascular space and up-regulation of Aquaporin-4 [36] influencing the water transport, also can potentially play roles in the development of the cysts.

1.3 TREATMENT FOR TRAUMATIC SCI AND PTS

Depending on the stage of SCI, different interventions have been suggested to reduce the damage to the spinal cord and promote functional recovery, but none of them could induce robust functional recovery.

Before reaching hospital, regardless of the clinical symptoms of the patient, spinal immobilization is usually maintained to prevent secondary injury. This involves application of a cervical collar, and the patient being placed on a backboard stabilizing the head and body. The efficacy of this treatment was still not clear [37-39]. In addition, the potential adverse effect, such as discomfort, increased respiratory effort and skin ischemia [40] should always be kept in mind.

After being transferred to the emergency unit, surgery with the aim to stabilize the spine and decompress the cord takes place. Increasing evidence [41-43] suggests the safety and feasibility of early surgical intervention. The best timing and indication for surgery still need to be explored. Regarding acute pharmacological treatments, numerous drugs have been studied [41]. Methylprednisolone was introduced as acute treatment in many clinics as a result of the two National Acute Spinal Cord Injury Studies [44, 45]. The treatment was later abandoned in most clinics as a result of critical evaluation of the effectiveness and the risk of side effects [46-48]. Hypothermia represents another approach to limit the secondary injury of SCI, potentially having several neuroprotective mechanisms, including slowing down the rate of metabolism and decreasing the generation of free radicals [49, 50], inhibiting excitotoxicity and neural cell apoptosis [51-53], ameliorating inflammation [54, 55], preserving the blood spinal cord barrier and preventing edema [56-58], inhibiting astrogliosis

and increasing angiogenesis [59, 60]. However, there are no definitive recommendations in terms of therapeutic window, temperature and duration. A clinical trial on hypothermia is about to start at the Miami Project to Cure Paralysis/Miami University Medical School.

Rehabilitation remains the most important and effective treatment for SCI. Once the patients′ neurological condition is stabilized, extensive passive exercises are applied to prevent complications may otherwise happen long term, including decubitus ulcer, muscle atrophy, pain, contractures and stiffness [61-64]. In the chronic stage, usually occupational therapists plan personal physical training program, depending on the patients′ interests, attitude, education level and social and cultural background. The primary goal is realization of independent mobilization and restoration of normal psychological and emotional state. This strategy is mainly based on the plasticity of the spared neural networks and the central pattern generator (CPG) theory. There is strong evidence proving the existence of CPG in animals [65, 66] and also indirect proof demonstrating its existence in humans [67, 68]. There is a large number of training methods and different training paradigm. Depending on the ability of body weight support, training may be done over ground, on a treadmill, or in water. Although functional improvement of different nature such as motor strength, gait parameters and spasticity has been reported in clinical studies [69], there is still not conclusive evidence for the optimal training. Recently epidural lumbar stimulation, developed and optimized to enhance the effect of locomotor training, has shown promising results [70], but clinical trial is still needed before it can become clinical practice.

Stem cell therapy has received a lot of attention in the last two decades. Stem cells derived from different sources have been shown to survive and differentiate into different neural cells after transplantation, and more importantly promote functional recovery after SCI in experimental studies [71-73]. Several mechanisms, including neuroprotection, immunomodulation, remyelination, promotion of regeneration and plasticity have been proposed, and gained support from numerous studies. Several clinical trials have indicated that various types of cell transplantation therapies do not have obvious safety issue, but we still lack evidence for its efficacy of promoting functional recovery [74]. Successful stem cell-based therapy for SCI may require better understanding of the detailed biological properties of those stem cells, and improvement in cell survival, differentiation and functional integration.

For patients with PTS, published results indicate that surgical removal of the tethering between the spinal cord and the vertebral column is the most effective, sometimes combined with a shunting procedure to drain the syrinx fluid. Success rates from PTS surgery vary between hospitals [75, 76] but they remain too low in many sites [77]. Regarding alternative treatments, a clinical pilot study with only two cases [78] demonstrated that human fetal spinal cord tissue transplanted to the syrinx survived for at least 18 months, and obliterated the syrinx at the site of the graft, indicating that transplantation of cells or tissue may represent a feasible and safe treatment for PTS.

1.4 ANIMAL MODELS FOR TRAUMATIC SCI AND PTS

Not only due to the availability and easy management, but also to the similarity in pathophysiology and functional changes after SCI [79], rats are still the most favored animals in SCI research. Depending on the functions studied, lesions can be made at different levels of the spinal cord. Cervical injury models may be considered the most clinically relevant model of tetraplegia, and translation of recovery of forelimb function would have a tremendous impact on the quality of life for these patients. However, for obvious reasons experimental research is usually limited to unilateral injuries [80]. Low thoracic injury models are most commonly used in SCI research, representing a highly relevant tool to investigate white matter function, injury effects on limb coordination, consequences of SCI on bladder and bowel functions etc. Analysis of hindlimb functions acutely and after recovery benefit from the close correlation between spared white matter at the injury epicenter (i.e. the number of spared axons) and the functional performance. In addition to location, different types of lesions such as contusion, compression, partial or complete transection and chemical lesions have been developed to model different aspects of SCI.

1.4.1 Contusion SCI models

Allen [81] first used weight-drop technique to produce contusion injury in dogs, later adapted to rats. A defined weight was dropped from a certain height inside a guiding tube perpendicular to the exposed spinal cord. The weight strikes the dorsal surface of the cord directly or through an impounder plate, and the energy is transmitted to the spinal cord tissue, causing a contusion injury. By application of different weight or height, different injury severity can be achieved. However, the technique resulted in inconsistent cord injures in rats [82] since the impounder contact area was an important variable for the injury outcome [83].

A more sophisticated device, named the New York University – Multicenter Animal Spinal Cord Injury Study device was developed by Gruner [84]. It consists of dropping the fixed weight (10 g) from four different heights (6.25, 12.5, 25, 50 mm) that standardized grades of contusive SCI. Two important improvements were achieved with this device, reduction of the risk of multiple injuries caused by drop-weight bounce, and monitoring of the physical parameters such as velocity and tissue placement. In the past decade, the capability of this device to consistently produce different degrees of injury severity and functional outcomes has been validated. However, the duration of impact is still not under control.

With the aim to obtain better reproducibility, Noyes and collaborators designed an electromechanical impactor, known as Ohio State Univerisity device [85], later developed into the Infinite Horizon (IH) impactor. The key improvements involve immediate retraction of the impacting rod immediately after the strike, stabilization of the spine by clamping, and using a computer feedback-controlled electromagnetic impactor to control all physical parameters of the contusion, as well as the displacement of the cord. The limitations are the difficulty of fixing the spinal column, and the precise identification of the zero distance

between the impactor and the dorsal spinal cord surface, as the spinal cord is moving during breathing. Nevertheless, these devices enable monitoring of biomechanical properties that should improve reproducibility, and allow exclusion of suboptimal impacts.

1.4.2 Compression SCI models

To more closely mimic the ventral compression commonly observed in clinic, the use of modified aneurysm clip was first described in 1978 [85]. After exposure of the spinal cord, the clip is positioned with one blade below the ventral cord and the other one above the dorsal cord. Then it is followed by closure of the clip inducing pre-set forces and continuation of the compress for certain period (e.g., 60 second). The alternative is use of calibrated forceps [86] to induce defined displacement. In addition, a small inflatable balloon affixed to the end of a catheter could be inserted into the epidural or subdural space to compress the cord in rat [87], and this procedure had been refined in other species [88-90]. In this model, both the contusion and compression mechanisms are involved, distinctively the significant ischemia caused by persisting compression of the cord.

1.4.3 Laceration SCI models

Complete and partial transection models are mainly used to investigate neuronal regeneration after SCI, or to analyze the role of specific spinal tracts in certain functions. They are not suitable for studying the complex pathophysiological processes in SCI since they are too different from the clinical situation. They are also utilized to study bridge interface approaches by different types of hydrogel scaffold or peripheral nerve transplants, usually in combination with neural trophic factors or stem cells [91-93]. Complete transection model has the advantage of easy operation and strictly consistent functional outcome, but high mortality is a critical issue. The alternative to study axonal growth after incomplete injuries is to use different tracers like fluorogold [94], Mini-ruby BDA [95] and cholera toxin B, or different types of viruses [96, 97] to label the regenerating axons. The users have to be aware of that artifacts might be caused by tracer leakage into the surroundings, and unexpected uptake of tracers after lesions.

Since functional recovery in incomplete transection models is robust, it has been extensively used to investigate the plastic changes in the injured spinal cord. In a corticospinal tract transection model, Bareyre FM and colleagues found that the injured spinal cord spontaneously formed a new intraspinal circuit [98], which contributed to the functional recovery. Rossignol and colleagues [99] further proved that plastic changes in CPG below the lesion are also involved in functional recovery after lateral hemisection lesions. It can also be used to study the role of different spinal tracts. However, sometimes it is difficult to confirm that the targeted tract is completely severed.

1.4.4 Chemically induced SCI models and others

These models serve our understanding of complicated biochemical cascades in secondary injury phase. They focus on specific aspects of secondary injury with the attempt to explore

the cellular or molecular mechanisms involved in SCI and further develop therapeutic drugs. Clinical translations of the findings in these models may be more difficult and should be taken in account.

Microinjection of phospholispase A_2, enzymes that release precursors of inflammatory mediators-fatty acid, cause a dose dependent inflammation, followed by other pathological events such as demyelination, degeneration and immune cell activation. Administration of oxidants, like the herbicide paraquat [100], hydrogen peroxide and $FeCl_2$ [101] or peroxynitrite donors [102] to the spinal gray matter could mimic oxidative damage. Excitotoxicity could also be created by several excitatory amino acid, including glutamate, glutamate and aspartate, N-mehtyl-D-aspartate and Kainate [103] which cause direct damage to neuron and oligodendrocyte. Intra-parenchymal application of lysolecithin or ethidium bromide [104] in combination with X-ray irradiation to prevent remyelination is method to study degeneration and demyelination.

Ischemia in the rat spinal cord can be induced by intravenous injection of non-toxic photosensitive dyes, like Rose Bengal [105] or Erythrosine B [105], with irradiation of the target area using regular light or laser light for selective time period [106].

Current syringomyelia animal models are based on the injection of kaolin into subranchnoid space to induce aranchonoiditis [107], usually combined with injection of the glutamate agonist quisqualic acid to cause excitotoxic injury in the parenchyma or with traumatic injury. A major concern is that the permanent presence of kaolin represents a situation very different from the clinical situation. A consequence is a chronic inflammatory state that probably has effects on the host tissue and transplanted cells used to study potential treatments.

1.5 FUNCTIONAL ASSESSMENT OF TRAUMATIC SCI RAT MODELS

Ideally, assessment of SCI models should include motor, sensory and autonomic functions. At present, experimental studies often focus on assessment of motor function. Because of the high popularity of low thoracic models, most of the behavioral tests are designed to evaluate hindlimb functions. Regarding the sensory tests, there are a few testing approaches available for rats and the evaluation is dependent on their behavior, not subjective feelings. Another complicating factor is sensory responses are potentially affected by two processes that may be simultaneously present, acting in opposite directions. The loss of sensory functions will result in decreased responses to sensory stimuli, while development of allodynia and similar pathological responses to injury results in increased responses. Assessment of autonomic functions are not common in rats, but with small scale sensors monitoring blood pressure and heart rate in awake animals, these clinically very relevant studies are becoming more common. Some of those commonly used functional testing methods were introduced below.

1.5.1 BBB and BBB sub-scoring scales

The Basso, Beattie, and Bresnahan (BBB) scale, which was developed from Tarlov′s open field test, is one of a few thoroughly validated tests for assessing hindlimb function in rats. This rating system covers the entire range of hindlimb function from complete paralysis to normal locomotion. In the lower range of scores (0-7 and 8-10) it concerns the extent of joint movements and body weight support. In the middle range (11-14), it assesses the frequency of the forelimb-hindlimb coordination, which was considered the most meaningful and important modification of Tarlov′s open field test. In the higher range, there are several discrete parameters representing different functional aspects. It is originally designed for contusive injury, and successfully applied in compression and hemi-section injuries later on [108]. It is a hierarchical scale and each point represents a specific stage of recovery of walking ability. However, the relationship between injury severity and functional score is not linear and animals tend to cluster at ratings of 8 and 14 [109]. Nevertheless, the BBB scale has become the most widely used and standardized locomotion test in SCI research.

Due to the design of BBB scale, some functions are not scored until the animal recovered to a certain level. For instance, before the rat recovers consistent forelimb-hindlimb coordination, different functional attributes in the higher range of the BBB scale could not be scored. However, interventions might improve one of the functional aspects without affecting others, which may not be detected by the BBB scale. In response to this drawback, a BBB sub-scoring scale was established to increase the sensitivity of the BBB scale [110]. The four behavioral parameters in the higher range, including paw position, toe clearance, trunk instability and tail position, are scored separately and added to an aggregate score. Obviously this is not a fundamental resolution for this problem.

1.5.2 Swim scales

Swimming as a different movement pattern from walking has several advantages in assessment of locomotion functions. First, weight bearing is avoided in swimming that might make the severely injured animals more easily reveal some aspects of motor functions affected by weight bearing. Consequently, less sensory input is involved in swimming, probably reducing the impact of sensory loss on the functional score. Second, the unique bipedal movement in swimming, characterized by forelimbs tucked under the chin and strict left-right alternating hindlimb movement at high frequency, serves a suitable model to investigate right-left hindlimb coordination. Third, the training effect achieved by the spontaneous walking in the home cage, and the lack of control over individual differences in this training may obscure treatment effects. For obvious reasons this effect is not an issue in swim testing. Rats are natural swimmers and readily to swim after a few days adaptation.

The Louisville Swimming Scale (LSS) is the only validated measurement for assessment of swimming ability in SCI rats [111]. The parameters in LSS, Forelimb dependency, Hindlimb Movement, Hindlimb Alternation, Trunk Instability and Body Angle, represent important aspects of swimming behavior. However, this scale has important drawbacks. The inability to

separate the functional status after mild injures from normal rats, and the sensitivity to assess functional recovery in severely injured rats with BBB scores below 8 were not investigated. Liebscher et al [112] proposed four parameters, including Forelimb usage, Hindpaw distance, Hindlimb stroke and Tail movement, but the definitions are ambiguous and the basic property of the test, such as reliability, validity and sensitivity were not analyzed. Zörner and colleagues investigated a wide variety of parameters, but they did not use the data to suggest a method to reliably assess rats with different injury severities [113].

1.5.3 Beam walk, ladder walking and grid walk

There are also several commonly used tests based on different types of walking and climbing. These tests were designed to evaluate the fine movement in which ascending pathways are involved, so they could be considered as sensory-motor tests. In contrast to the BBB scale, they are effective in detecting both forelimb and hindlmb deficits after SCI, and also applied in assessment of brain injury [114]. Since animals have to be able to do some stepping to perform these tests, they are useful only for moderate to mild injuries. Pre-training should be carried out until the animals readily finish the task with no mistakes, and sometimes reward is utilized to increase the motivation.

Regarding beam walk test, animals are allowed to spontaneously traverse elevated wooden, plastic or metal beams of different width, and the whole process can be video recorded for detailed analysis. One or multiple beams with variable difficulties were usually adopted. The performance of the rat is evaluated by different scoring systems, including simply qualitative measures (yes or no), semi-quantitative measures defined with several rankings and kinematic measurements including the traverse time, foot stepping angle, rump height index and foot fall number [115, 116]. This test is very sensitive to detect minor deficits of paw placement and body balance. Successful accomplishment of the task rely not only on the spinal neural circuitry, but also the input from supra-spinal pathways, including cortico-, rubro- and especially vestibulospinal tracts [117].

Ladder walking, originally designed by Metz and colleagues to assess forelimb and hindlimb placing, stepping and inter-limb coordination, is considered to be more sensitive than beam walk for mild injuries [118]. This task requires the animal to walk a horizontal ladder with variable spacing between each rung. Similar to beam walk, both qualitative and quantitative data can be collected by foot placement scale, forepaw digit scale and the number of errors and the average time crossing the ladder. A modification of the test was done by adding upward and downward ladders to discern other aspects of locomotion disabilities, to establish a comprehensive assessment system for skilled walking based on the foot fault scoring system and the analysis of complete stepping sequence. The challenging nature of this test was indicated by a few mistakes made also by normal rats, which might reduced its reproducibility and practical use.

In grid walk test, different sizes of grid with regular or irregular holes are elevated above the ground, and the number of missteps is counted when the animals go across the grid. Several

researchers proposed that grid walk with irregular holes might improve the testing effectiveness by avoiding habituation to the fixed spacing. In order to obtain reliable results, it is necessary to run at least three trials. The missteps are counted when the entire paw with the heel appears below the grid. Thus, functional deficits are shown as the total number of missteps, percentage of missteps or representative scores. It is usually applied to assess the deficits of forelimb-hindlimb coordination, which are mediated by propriospinal pathways and long ascending and descending pathways in the ventrolateral funiculi. It is worth noticing that walking speed and stress can influence the outcome of this task.

1.5.4 Gait analysis

Gait disorders have been reported in many neurodegenerative diseases. In SCI research, footprint analysis was originally developed to precisely evaluate inter-limb coordination and walking pattern. It provides more gait attributes than BBB scale, such as stride length, print area, contact area, swing duration and base of support. Thus, this method generates a wide variety of quantitative data, which may be useful to discriminate minor functional changes after mild injuries, to detect functional changes after interventions. Its application is limited to animals with weight bearing and some levels of coordination.

Originally, rats were placed on a runway ending in a dark box, and footprints were collected using ink or developer to dip the paws before walking on a white paper or X-ray film [119]. This type of method had several disadvantages: time consuming, high variability, acquisition of unclear footprint sometimes, examiner bias, and bias caused by negative factors, like foot contractures, autotomy and excrements on the track. With the purpose of automated gait analysis, Hamers developed the computer assisted CatWalk system [120]. The animal walked in a corridor on a glass walkway with a high-speed color camera placed below. The footprints were obtained by diffraction of light at the site of contact between the paw and the glass surface, called Illuminated Footprints Technology^{TM}. This method not only enabled fast and accurate gait analysis, but also captured more functional aspects of locomotion. Later on, it was realized that data varied depending on the walking speed, and it was recognized that animals had different spontaneous walking speed. This was addressed by the development of the DigiGait^{TM} and Treadscan systems using transparent treadmills to keep the animal walking at a fixed speed. Krizsan-Agbas D and collaborators [121] highlighted the importance of using multiple speeds for gait analysis to accurately reveal functional deficits. Animals may not reveal their real functional outcome when forced to walk at their uncomfortable speed.

1.5.5 Ground Reaction Force

This is a kinetic measurement to calculate the forces exerted through the limbs and paws on the ground during locomotion. It is usually used in unilateral SCI lesions [122-124] to investigate the role of specific limbs in postural control and locomotion. Interestingly, one study showed that the rats had more weight on the affected hindlimb than on other limbs [125]. This method can also be used to study spinal reflex and sensory discrimination during

skilled reaching [126]. A force transducer with sufficient sensitivity and suitable size for rodents is now commercially available. The advantage of this method is generation of unique data regarding behavioral compensation, which could not be obtained by other methods. However, it can only be applied in rats with weight support and the ability to perform stepping.

1.5.6 Von Frey test

This is a test of the mechanical nociceptive thresholds. It evaluates the reaction to mechanical stimuli caused by the application of the von Frey filaments - nylon threads of varying diameters that produce pre-specified forces when applied as hard as is necessary to bend them slightly. Commonly in this test the animal stands on elevated mesh platform, and the examiner poke the plantar surface of the paws with the filaments. Retraction or licking of the limb and vocalization are the signs of discomfort used to define the threshold. To make the right judgment, a minimum of five tests for each filament is required with a short 3-5 min rest period after each test. It can also be applied to other parts of the body such as the trunk. It is widely used to study skin areas with normal responsiveness, as well as hyper- or hyposensitive areas. A problem is that a SCI can cause both loss of sense of pressure and allodynia, i.e. pathological pain responses to otherwise non-painful stimuli.

1.5.7 Hotplate test or Hargreave's test

These tests evaluate the thermal sensitivity to pain resulting from exposure to heat. There are different variations of hot plate equipment available [127]. The animal is placed in a cylinder, with a heating plate as the bottom. The temperature of the heating plate is maintained at a fixed temperature, usually around 50 degree. The latency to nociceptive response is recorded, defined as the time elapsed until the animal licks or flicks a paw. If there is no response within 30 seconds, the test is terminated to avoid injury. Each animal is tested at least three times. Thus animals may display a behavioral tolerance phenomenon, characterized by decreased latencies and reduced sensitivities to heat stimulation [128]. A development of the method more often used today is the Hargreave's test or the plantar test. Instead of a hot plate, a light beam is focused on the sole of a paw to heat it. When the paw is lifted, the light is turned off, and the delay automatically recorded. Also in this setup the heat source is automatically turned off after a defined time to avoid tissue injury.

1.5.8 Electrophysiology

Electrophysiological recording of muscle activity by electromyography (EMG) is an invasive method requiring implantation of electrodes into muscles. EMG provides direct and precise information about patterns and magnitudes of muscle activation during locomotion and any type of movement. This type of information is very important for the understanding of the mechanism of functional outcome, especially combined with corresponding kinematic and kinetic data. However, the fact that a particular movement is caused by activation of several muscles in concert with a complex interaction between various muscles complicate the understanding of data, and make it difficult to use EMG as outcome measures after SCI. For

example, it is difficult to assessment of single muscle contribution to a particular movement, although recordings of multiple muscle activity are possible.

A more useful related method in SCI research and also clinical practice is the recording of motor evoked potentials. They are elicited invasively by transcranial electrical stimulation of the motor cortex and recorded from muscles by EMG. In combination with immunohistochemical analysis, Cao and colleagues showed that the ventral and lateral tracts are necessary for transcranial magnetic motor evoked potentials responses [129]. Conversely, brain activities can be obtained in response to sensory stimulation, eliciting somato-sensory evoked potentials. But the power in discriminating injury severity and reflecting course of recovery is very limited. In addition, blood oxygen level dependent functional MRI has been applied in rats to investigate the supraspinal neural plasticity, which plays a very important role in functional recovery in humans. Several investigators found the expansion of forelimb representations after thoracic SCI [112, 130]. Both tests have to be done under anesthesia and require expensive equipment.

2 AIMS

The main aims of my studies were to develop better assessment methods for rats with SCI than those available and to develop a clinically relevant rat model for PTS.

The specific objectives were as follows:

Paper I: To develop a validated instrument to assess swim performance in spinal cord injured rats.

Paper II: To evaluate kinematic analysis of swimming and an assessment tool for wading in rats with SCI.

Paper III: To establish a new rat model of PTS that can be applied to study neural cell therapy.

3 MATERIALS AND METHODS

3.1 HUMAN NEUROSPHERE CULTURE (PAPER III)

hNPCs were derived from human embryonic spinal cord (5.5-7 weeks of gestation). All the procedures were previously standardized in our laboratory and approved by the Regional Ethical Committee, Stockholm. The cells were cultured in DMEM/F12 medium (Life Technologies) supplemented with 0.6 glucose (sigma), 5nM hepes (Life Technologies), 2 μg/ml heparin (Sigma), 1%N2 supplement (Life Technologies), 20 ng/ml bFGF (R&D Systems), 20 ng/ml EGF (R&D Systems) and 10 ng/ml CNTF (R&D Systems) at a density of 40,000-50,000 cells/cm^2. The hNPCs were in vitro expanded as neurospheres at 37°C in 5% CO_2. The medium was changed twice a week. After 7 to 10 days of culture, neurospheres were passaged by enzymatic dissociation using TrypLE (Life Technologies) for 4 minutes at 37°C with gentle mechanical shaking, subsequently re-cultured in fresh medium.

To track the cells in vivo after xeno-transplantation, hNPCs at passage 5-10 were transduced to express green fluorescent protein (GFP). Briefly, hNPCs were seeded on a petri dish coated with poly-ornithin/laminin in hNPCs culture medium (as described above) with the addition of 2 μg/ml Polybrene and a replication incompetent lentivirus (Allele Biotechnology) which carries the GFP gene driven by the consitiutively active EF1α promoter. After 3-5 days, >98% infection rate was confirmed by flow cytometry (FACSCalibur, Becton-Dickinson). Then GFP labeled hNPCs, passage 1-2 were utilized for transplantation. The cell number of the neuroshperes to be transplanted was calculated as descried before[131]. Neurospheres with defined number of cells were prepared in the culture medium without the mitogens, bFGF, EGF and CNTF prior to transplantation.

3.2 EXPERIMENTAL ANIMALS (PAPER I-III)

In study (**I, II and III**), three-months old female Sprague-Dawley rats were used. They were housed in a standardized environment with water and food ad libitum, at a 12/12 light-dark cycle with lights on at 07:00, room temperature maintained at 22 degree and humidity at 45-55%. Each cage housed two or three rats. All the experimental procedures followed the guidelines of the Swedish animal protection legislation and were approved by Regional Ethics Committee on Animal Research, Stockholm, Sweden.

3.3 ANIMAL MODELS AND NEURAL CELL TRANSPLANTATION PROCEDURES (PAPER I-III)

Two types of SCI models (**Paper I-II**) and a PTS (**Paper III**) model were applied here.

Firstly, atropin (0.05 mg/kg, NM Pharma AB) was given 30 min before surgery, and followed by injection of a mixture of Hypnorm (fentanyl citrate, 0.22 mg/kg, and fluanisome, 6.8 mg/kg, Janssen Pharmaceuticals) and Dormicum (midazolam, 3.4 mg/kg, Hoffman-La Roche). The body temperature was maintained at 37 °C by a heating pad during the whole operation. 6 ml Ringer/2.5% glucose (Sigma) were applied regularly to all the rats before and after surgery to reduce the risk of dehydration.

Thereafter the back skin was incised and the muscles were separated to expose the lower thoracic vertebral column. The dorsal lamina of vertebra Th 9 was removed. After placing a few drops of Xylocain (lidocain hydrochloride 20 mg/ml, AstraZeneca), the exposed spinal cord was subjected to clip compression during 30 s by a clip with four different forces (20 g, 45 g, 75 g, 140 g) or contusion by an IH spinal cord impactor (Precision Systems and Instrumentation, LLC, 100 kdyn impact, dwell time = 0).

A new PTS model was developed. 10-30 µl of autologous blood was aspirated from the tongue vein by a heparinized 50 µl Hamilton syringe (Sigma-Aldrich) and slowly injected into the subarachnoid space under vertebra Th 8 after a contusion injury described above. A piece of Lyoplant (B/Brain Aesculap AG) was placed on the spinal cord to close the dura, and the wound was sutured in layers.

Postoperatively, voiding of the urinary bladders was performed twice a day until spontaneous micturition occurred. Temgesic (buphrenorphin, i.m. 7 µg/kg, Reckitt & Colman) was administered twice a day for four postoperative days and Borgal (trimetoprim sulfa, s.c. 15 mg/kg, Intervet International B.V.) was given if symptoms of urinary infection would appear. The rats used in the experiments in **paper I and II** were sacrificed 8 weeks after injury. In **paper III**, the rats were sacrificed at 2, 8 and 20 weeks after lesion, except the rats selected for hNPCs transplantation at 20 weeks that were sacrificed 5 weeks later (27 weeks after injury).

At the time of cell transplantation, the wound was reopened, the injured spinal cord was again exposed at vertebral T9. With the guidance of MRI images, the location of the cyst was identified. If necessary, a part of the vertebrae adjacent (rostral or caudal) to the injury site was removed to get access to the cyst. A glass capillary (0.3 mm end inner diameter) connected with Teflon tubing to a 10 µl Hamilton syringe using Teflon-tubing, was loaded with a suspension of 25-30 neurospheres, approximately 300,000 cells. When the cyst was punctured, the neurospheres were slowly injected into the cyst under visual monitoring. To avoid leakage of transplanted cells, the glass capillary was carefully removed, followed by positioning of a piece of Lyoplant over the injection site. The wound was closed carefully layer by layer. For immunosuppression, the rats were given ciclosporin (10 mg/kg s.c., Sandimmun, Novartis) 24 h before transplantation, and once a day until the termination of the experiment 5 weeks after transplantation.

3.4 FUNCTIONAL ASSESSMENT

Motor function was evaluated in five different movement patterns, including open field walking, swimming, wading, beam and grid walking. The mechanical and thermal pain thresholds were determined by von Frey filaments (Stoelting) and Plantar test (Hargreaves') analgesia meter (Ugo Basile) respectively.

3.4.1 BBB

The Basso, Beattie, Bresnahan (BBB) locomotor rating scale was used to evaluate open field walking during 4 minute periods on a 65 × 150 cm table. The experimental animals **(Paper I-III)** were tested and video recorded before SCI and regularly after the lesion. A blinded examiner scored all the subjects.

3.4.2 Swimming and Wading

To study swimming and wading function in SCI models we applied a 15 cm wide, 30 cm deep and 150 cm long Plexiglas swim tank. A partially submerged black platform with rough surface was positioned at the end of the tank, allowing the experimental rats to easily move out of the water after each run. The tank was filled to a depth of 20cm with warm tap water (28-32°C). For observation of function from below, a high quality mirror was placed below the tank at an angle of 45 degree. A High Definition video camera positioned in front of the tank recorded the events in the middle 60 cm of the tank at a frame rate of 120 s^{-1}. Motion sequences and individual frames were thoroughly analyzed by utilizing QuikTime player.

For assessment of wading, the depth of water was instead set at 6 cm to submerge half of the rat body when standing. A platform with the rat' home cage on top was placed at the end of the runway to attract the rat.

Evaluation and scoring of the parameters were based on observation of recorded videos. In **Paper I**, we studied various parameters and six of them were chosen to form a multiple item scale, the Karolinska Institutet Swim Assessment Tool (KSAT). In **Paper II and III**, KSAT was applied directly to score the animals.

3.4.3 Beam walk and Grid walk

For beam and grid walk testing, we applied six 1 cm long beams with varying width from 0.7 to 6 cm [132], a 150 × 20 cm grid with 3 × 3 cm holes, and an appropriate holder with enclosed wall which facilitate the subject to concentrate on the task.

All the experimental animals were pre-trained (before lesion) in beam and grid walk (**Paper III**) until they made no mistakes in either task. After lesions, the rats were regularly assessed as shown in **Paper III** with three trials each time. Scoring of beam walk was based on the following criteria: 0 = unable to transverse the beam, 1 = traversed the beam with > 5 misplaced steps, 2 = traversed with 1-5 misplaced steps and 3 = no misplaced steps. The average score for each beam was added to give a total score ranging from 0 to 18. For the

assessment of grid walk, the maximum number of mistakes, 47, was defined as a paw slipping through every hole of the grid.

3.4.4 Pain Assessment

To study two types of pain thresholds, the rats were first habituated to the test situation, kept in 10 x 20 cm transparent plastic cages on an elevated platform. For von Frey test, the platform was a steel wire mesh with 6 x 6 mm square openings through which the application of the von Frey filaments to the sole of the paw could be done. Starting with the thinnest filament, rats were tested by applying increasing pressure on the sole of the hind paws until an avoidance response occurred for a certain filament, and the next thicker filament. This was repeated on each hind paw until a response was achieved three times for the same filament, which was then defined as the response threshold. The pressure was applied for 10 seconds, or until the avoidance response occurred. In Hargreaves' test, the delay to lifting the paw in response to heat was recorded. The mean delay from 5 trials was calculated. All the rats were tested before surgery, and three times between 10 and 20 weeks after lesions.

3.5 MAGNETIC RESONANCE IMAGING

A set of equipment including a horizontal 9.4 T magnet (Varian, Yarnton, UK) with a 31 cm bore, a 72 mm volume coil and a four-channel phased array surface receive coil (RapidBiomed, Würtsburg, Germany) were used for MRI scanning. After anesthesia, the animals were placed in supine position with the lesion site centered on the surface receive coil. During the whole process, the core body temperature was maintained at 37°C and respiration rate was monitored by a warm air system (both equipment from SA-instruments, Stony Brook, NY, USA). The injured spinal cord were scanned through a gradient echo sequence, with sequence parameters effective repetition time =1.3 s, echo time = 4.35 ms, flip angle = 35°, nex = 4, axial slices of 0.5 mm thickness, field of view 40 mm × 30 mm^2, matrix 256 × 256, to produce axial T2 weighted images. Motion artifacts caused by pulsatile flow and respiration were minimized by employment of respiratory gating and spatial saturation band covering the aorta. Sagittal reconstructions and calculation of different measurements of the cysts were done by Image J software (NIH).

3.6 IMMUNOHISTOCHEMISTRY

The use of this method had several purposes: quantitative analysis of the lesion volume and spared white matter at injury epicenter (**Paper I-II**); characteristic analysis of the cysts formation (**Paper III**); investigation of the survival and differentiation of hNPCs (**Paper III**).

For general morphological analysis, tissue sections were stained with hematoxylin and eosin (HE). A modified Prussian blue staining was used to detect Fe^{3+}, indicating the presence of old hemorrhage. The course of inflammation after trauma and subarachnoid injection of blood was reflected by ED-1 immuno-staining. Glial fibrillary acidic protein (GFAP) was used to visualize astrocytes representing living spinal cord tissue, astrogliosis on the cyst margins and differentian of hNPCs. The tissue sections with the smallest cross-senctional area of spared tissue were stained with neurofilament (NF) to calculate the spared white matter at injury epicenter. The survival of hNPCs was identified by the human specific marker-HSP-27 and neural stem cell marker Nestin.

3.7 STATISTICAL ANALYSIS

Data were presented as scatter plots, mean values with standard errors of the mean (SEM) or standard deviation (SD). Statistical analysis was performed by PASW 18 Statistics software (SPSS) and Graphpad Prism software. The effects of injury severity, time and their interaction on the outcome of KSAT score, BBB score, Wading score, Beam walk score, mistakes in grid walk and kinematic parameters for swimming (**Paper I-III**) were evaluated by two-way repeated measures analysis of variance (ANOVA), accompanied by post-hoc Bonferroni test. The internal consistency, inter-rater and test-retest reliability of the KSAT scale and wading scale (**Paper I and II**) were analyzed by Cronbach's alpha reliability coefficient, intra-class correlation coefficient and the Pearson correlation coefficient. The relationships between the KSAT and BBB scores, compression force, spared white matter and lesion volume (**Paper I and II**), the relationships between wading scores and spared white matter at injury epicenter, BBB and KSAT scores and the relationship between kinematic parameters and spared tissue at injury epicenter (**Paper II**) were analyzed by linear and non-linear correlations.

4 RESULTS AND DISCUSSIONS

4.1 APPLICATION OF TRAUMATIC SCI MODELS AND DEVELOPMENT OF A NEW PTS MODEL

With regard to clinical relevance, traumatic SCI models are often better than models for other neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, the etiology of which are still not clear. Different types of trauma causing human SCI can be closely mimicked in experimental research, producing highly clinically relevant situations, and serving a comprehensive understanding of human SCI. The high similarity in pathophysiology and functional changes after SCI in rats and humans make the rat models suitable for investigation of functional recovery with or without interventions.

In **Paper I and II**, we utilized rat compression SCI model applying clips of four different closing pressures, 20 g, 45 g, 75 g and 140 g for 30 s. This resulted in rats with four different degrees of SCI severity, used to develop methods to assess swimming and wading. The results showed a diverse functional outcome in each group probably caused by unpredictable neural plasticity [133]. Despite the fact that the current clinical trials focus on severely injured patients, long-term investigations of severely injured animals are rarely performed. In the original 'BBB scale' paper [134], only one animal with a BBB score below 9 was studied, and the same problem also exists in the 'LSS scale' paper [111]. Thus, the validity of these assessment scales for severely injured rats is unclear. In our study, the majority of rats in the most severely injured group (140 g) had BBB scores below 8, eight weeks after SCI. The entire cohort of injured rats ranged from almost complete paralysis to near normal walking, and provide the necessary material to comprehensively study the functional change in swimming and wading behavior. Because severely injured rats had very limited hindlimb movements and middle-range injured rats usually suffered severe to moderate trunk rotation which led to the inaccurate calculation of kinematic parameters, our detailed analysis of swimming (**Paper II**) focused on two groups of mild compressive and contusive injury.

Compared with SCI models, there were much less options concerning available PTS models. As mentioned above, previously published PTS models were induced by injection of kaolin into subarachnoid space, combined with multiple intra-spinal injections of quisqualic acid [135] or trauma [136]. The injections of the excitotoxic injections of quisqualic acid and trauma were used to induce myelomalacia in the cord, supposed to create a site of cyst induction. Kaolin was introduced to maintain an inflammation of the arachnoid membrane, believed to be critical for the development of PTS in human beings [137]. Kaolin remains in the spinal cord for several months (unpublished observations). These models were mainly applied to understand the physical mechanisms of PTS, and the role of liquid flux in establishing and expanding the cysts. When it came to development of cell therapy for PTS, continued presence of kaolin or other foreign material is not appropriate since it probably

leads to a chronic inflammation in the tissue. The purpose of **Paper III** was to develop a PTS model without using foreign substances for cell therapy.

By a combination of contusion injury and subarachnoid injection of autologous blood, cysts formation was successfully induced in this new model. To determine the effective blood volume and time, we first applied 10 µl of blood and followed the rat for 12 weeks in the pilot study. The results from H-E staining showed that obvious cysts formation occurred in half of the rats, but with varying cyst lengths (1.5 to 6.7 mm) and width, ranging from involvement of small part of the central gray matter to the involvement of almost all gray matter over several spinal segments. We then increased the blood volume to 30 µl to more reliably achieve large cysts and followed the injured rats during 20 weeks. All rats had large cysts, which were identified by MRI at week 20 (Fig 1) and the following histological analysis later. More importantly, the pathological features of those cysts were very consistent with that observed in human PTS [30, 31], including large, irregular shaped cysts, single or multiple numbers, development in both rostral and caudal directions, located in dorsal part of the gray matter and thick cyst margins composed of astrocytes.

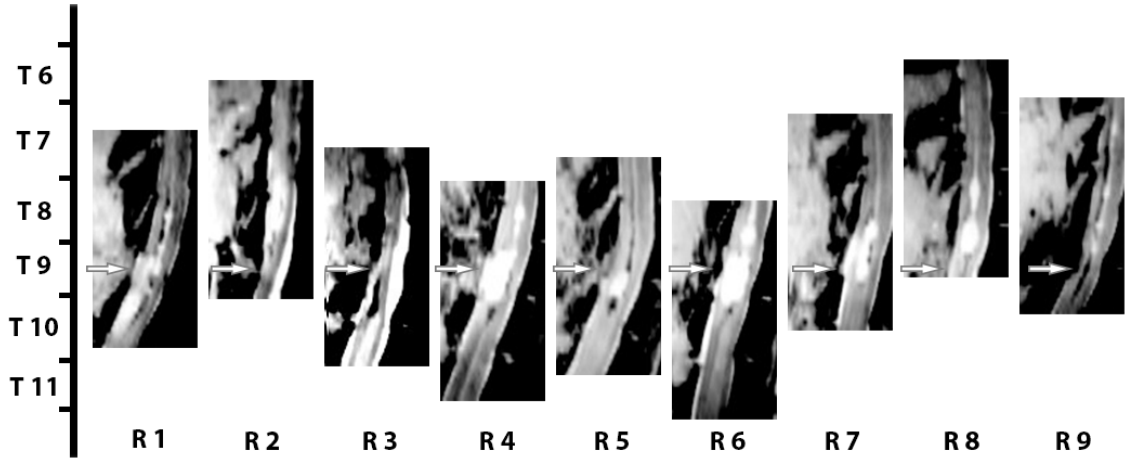

Figure. 1. Sagittal projections of T2-weighted MRI images of the thoracic spinal cord of 9 rats, 20 weeks after inducing post-traumatic cysts using mild contusive trauma and subarachnoid injection of 30 µl of blood rostral to the contusion. The arrows indicate the location of the contusion at vertebral level T9 (spinal segment T11). Light, hyperintense regions represent fluid-filled cysts.

By comparison between T2-weighted MRI images and the post-mortem tissues sections, we confirmed that hyper-intense regions corresponded to cysts, presumably filled with liquid before fixation. In the areas adjacent to the cysts, we also found T2-hypointense signals on MRI representing parts of cavities filled with ED1-positive macrophages/microglia and tissue debris. The hypointense MRI signal could be explained by the presence of hemosiderin deposits in corresponding cross sections, indicated by Fe-staining. Moreover, it is interesting to notice that in the central gray matter we found long and narrow T2-hypointense areas rostral and caudal to the cysts that was labeled by the Fe-staining, indicating previous hemorrhage in spinal parenchyma surrounding the central canal. Although the occurrence of hemorrhage around the central canal for several millimeters in a transection model was reported previously in a transection model, we have not observed any degenerative changes

in this regions in compressive or contusive SCI. Intra-parenchymal hemorrhage has been associated with PTS in several clinical studies [138, 139], probably by promoting myelomalacia [10], which as mentioned above is considered as a pre-requisite for PTS. Nevertheless, our results suggested that sub-ependymal hemorrhage is at least partially involved in the expansion of the cysts in the chronic stage.

Although clinical PTS could cause marked motor and sensory loss and neurogenic pain/allodynia in patients, none of these symptoms were apparent during evaluation of the progressive expansion of the cysts. We tried to detect the effect of cysts progression on motor and sensory function. Four different types of motor tests were used: BBB scale, which is most commonly used to assess low thoracic injury; KSAT scale with the advantage in assessment of the trunk muscle functions; Grid and Beam walk for evaluation of fine forelimb-hindlimb coordination. However, we found no worsening in motor functions during the cyst expansion (between 8 and 20 week) by any of the four tests. This is not surprising since clinically there is often no detectable functional change, even after extensive loss of neurons [16]. Overt motor symptoms are probably not seen until the expanding cysts reach the cervical segments. For technical reasons, sensory testing was limited to lumbar dermatomes of the hind-paws. Our results did not demonstrated any change in mechanical or thermal pain thresholds, while a transient lower pain threshold was reported by Seki and Fehlings [137] six weeks after the induction of cysts. However, they did not described which dermatomes were analyzed in their study.

We then investigated the possibility of experimental hNPCs therapy in this model. MRI scanning was used to determine the size and location of the cysts and further select cysts we considered large enough for transplantation. Using guidance by the T2-weighted MRI images, we transplanted hNPCs into the hyper-intense regions representing the fluid-filled cysts. The hypo-intense regions were avoided since they either contained tissue debris and phagocytic cells inside or did not even represent cysts. Five weeks after transplantation, we found large numbers of human derived donor cells in half of the rats. There are several explanations for donor cells missing in some rats. First, transplanted neurospheres may have been flushed out of the cyst through the slit immediately after injection, before they adhered to the cyst walls. Our in vitro data (unpublished) indicated it takes 2-3 hours to reach maximum adherence of hNPCs to human astrocytes, and there is a steady flow of liquid out of the cysts after the incision is made. However we do not know the actual adherence time in vivo. Second, the fluid in the cysts might contain some substances that are detrimental to hNPC. Third, as reported recently, immunosuppressive treatment may in some rats not be sufficient to suppress the immune response triggered by xeno-transplanted human cells [140]

In tissue sections, we further analyzed how the donor cells affected the cysts. The typical feature was that the grafted cells covered the cyst walls, while a large number of cells had penetrated the cyst wall and migrated into the parenchyma. In some rats the big cysts partially collapsed, probably already during surgery and then remained so. In these cases, we observed merged cyst walls with a thin sheet of human donor cells in between. Occasionally

neurospheres obliterated cysts that had not collapsed (Fig. 2). In some experimental rats, hNPCs completely obliterated a main branch of a large cyst over 0.6 mm, and partially obliterated an additional 0.7 mm length of the cyst. This shows that hNPCs have the capability to adhere to the cyst walls and obliterate cysts, and also migrate through the astrocyte layer of the cyst wall, into the surrounding gray matter.

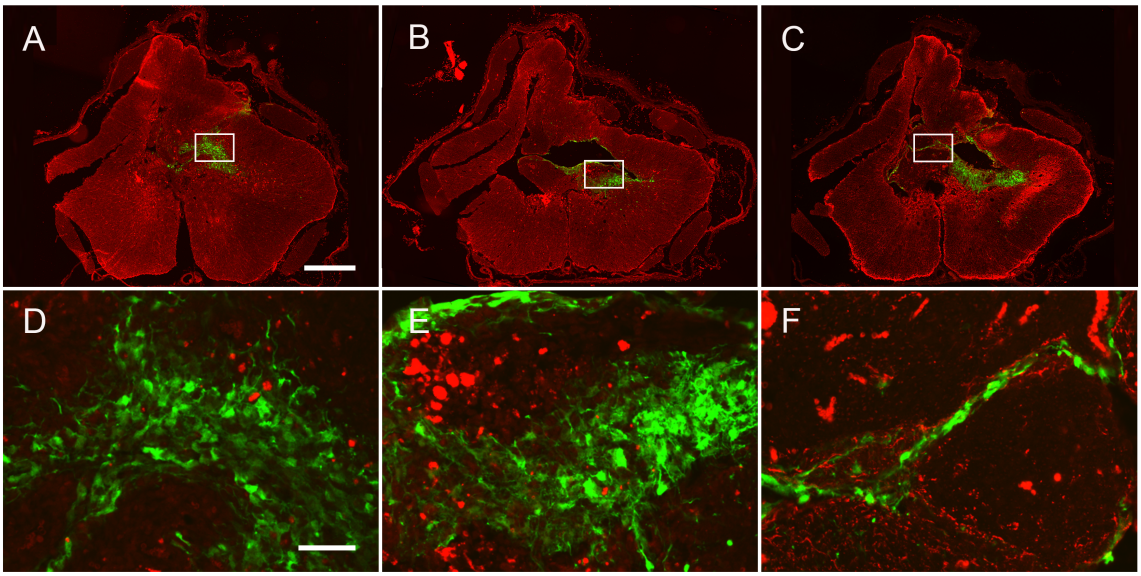

Figure. 2. Sections of a spinal cord showing, a large partly collapsed central cyst to which GFP-expressing hNPCs (green) were transplanted, immunostained for GFAP (red) to identify astrocytes in the host tissue and the graft. The boxed areas in the low magnification images (A–C) are shown at higher magnification (D–E). Grafted hNPCs were found in large numbers at the rostral end of the cyst (A, D), they covered most of the cyst walls, and had also migrated into the surrounding host parenchyma, forming large clusters of grafted hNPCs (B, E). Parts of the cyst had disappeared, and a thin membrane of grafted cells seems to mark the position of the cyst, probably representing the merging of opposing cyst walls lined with hNPCs. Scale bars represent 200 μm (A) and 25 μm (D).

4.2 DEVELOPMENT OF SWIMMING AND WADING FOR ASSESSMENT OF SCI RATS

As described above, current methodological studies of swimming in SCI rats are either incomplete or lack validity analysis. Furthermore, the importance of using wading for functional testing had been highlighted by Zörner and colleagues, in which not only the assistive weight support is provided but also certain levels of limb loading is preserved [113]. They suggested several parameters that may be useful for several SCI models but specific tests were not established. The aim of our study was to comprehensively analyze the swimming and wading performances after SCI of different severities, and evaluate possible parameters to develop reliable and validated tools for assessment of motor activities in SCI rats.

For swimming, we identified eight useful parameters after comparing normal and injured rats. Some parameters were used in previous studies but were modified by us to better reflect

functional recovery after SCI. After analyzing the contribution of each parameter to the total score, we selected six parameters to be scored and combined to generate a valid and reliable multiple item scale, the Karolinska Institutet Swim Assessment Tool (KSAT) (Fig 3). All eight parameters showed different improvement over time, reflecting functional recovery in different aspects. In most of the animal improvements stabilized 6 weeks after SCI. Although the Phase Lag parameter was sensitive to mild injury, evaluation of this parameter requires a laborious frame-by-frame analysis of high-speed videos and the variation was larger than that of hindpaw position, which is also sensitive to mild injury. For another mid-range injury sensitive parameter, Body Angle, we found that the improvement reflected by this parameter was mainly due to the recovery of hindlimb function. So we decided to exclude these two parameters from the KSAT scale.

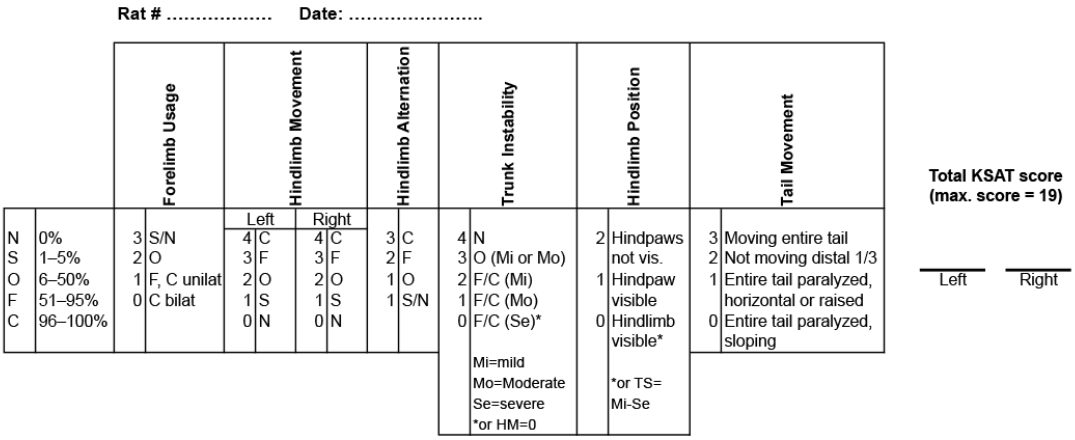

Fig. 3. The Karolinska Institutet Swim Assessment Tool (KSAT) scoring sheet for the six parameters Forelimb Usage (FU), Hindlimb Movement (HM), Hindlimb Alternation (HA), Trunk Instability (TI), Hindlimb Position (HP) Tail Movement (TM).

To facilitate wading analysis, first we divided one cycle of hindlimb movement into several sub-phases, and focused on the hindpaw movement. By applying a similar method as described above for the swim score, we established a wading scale composed of six parameters (Fig 4). Some of the parameters reflected similar pathological features as described in BBB scale, but with different definitions. Importantly, in contrast to the BBB scale, every parameter is scored for each rat.

Rat # **Date:**

Hindpaw Placement	Hindpaw Rotation	Hindpaw Toe Clearance
2 Plantar 1 Tiptoe or dorsal 0 No movement	3 No external or internal rotation 2 Rotation only at initial contact 1 None rotation both at initial contact and lift off 0 Rotation during the entire stance phase	3 None or seldom (0% or 1-5%) 2 Occasional (6-50%) 1 Frequent (51%-95%) 0 Consistent (96%-100%)
Hindpaw Lift	**Relative Stride Length**	**Paired Limb Movement**
2 > 3 cm 1 0.5 - 3 cm 0 < 0.5 cm	3 > 3/4 Hindpaw-Forepaw distance 2 1/2 - 3/4 Hindpaw-Forepaw distance 1 1/4 - 1/2 Hindpaw-Forepaw distance 0 < 1/4 Hindpaw-Forepaw distance	3 ≥ 10 2 6 - 9 1 2 - 5 0 0 - 1

Total wading score (max. score=16) Left: _____ Right:_____

Fig. 4. The wading scale scoring sheet for the six parameters Hindpaw Placement (HP), Hindpaw Rotation (HR), Hindpaw Toe Clearance (HTC), Hindpaw Lift (HL), Relative Stride Length (RSL), Paired Limb Movement (RLM).

Several behavioral tests lack reliability analysis, and some of these have nevertheless gradually been accepted in the field. An important issue concerning this kind of subjective instrument is the risk of high variation caused by variations in the definitions as applied by different examiners, the so called inter-rater reliability. The BBB showed high reliability when first established, and has since been widely used in SCI research. The definitions used in the BBB scale seem to be robust, but the published reliability data was based on staff trained using video recordings of different rats, videos that were also distributed to other researchers interested in using the BBB scale. It is not clear what the reliability is if staff with less training is included. For the KSAT and wading scales, the reliability was evaluated in three aspects: internal consistency, inter-rater reliability and test-retest reliability. We analyzed the inter-rater reliability by after giving the participants only written instructions. We believe the results were therefore more robust. The inter-item correlation analysis demonstrated a varying correlation relationship among each other (KSAT: 0.367-0.937 and Wading: 0.333-0.906). In the type of assessment scale we intended to create, this actually reflects that each parameter is sensitive to different injury severity, which is a necessary feature. Both the intra-class correlation from three blinded examiners evaluating certain number of rats (10 for swimming and 12 for wading) from different groups and test-retest reliability coefficient over one week (swimming: from week 7 to 8 and wading from day 56 to 57) was very high (KSAT: 0.995 and 1.000; Wading: 0.996 and 0.967). Thus, subjective assessment by KSAT and wading scale can produce reliable and consistent results.

The validity of the tests for low thoracic SCI is often evaluated by its correlation with spared white matter, since numerous studies have proved that the extent of spared white matter is strongly correlated with hindlimb motor functions evaluated by different methods [141-144]. Injured rats with only 10 percent of spared white matter remaining could perform consistent plantar stepping [145]. Although ventrolateral funiculi have long been considered to be essential for voluntary movement, their roles can be greatly compensated by other pathways after ventrolateral spinal injury [146]. Zorner and colleagues [113] observed massive functional deficits in swimming after ventral SCI at Th 8, while only minor functional loss was seen after dorsal SCI at Th 8. In our study, we also investigated the relationship between KSAT score/Wading score and spared white matter at injury epicenter. The KSAT score and Wading score was highly correlated to the spared white matter at injury epicenter by a one-phase association function (KSAT: $r^2 = 0.97$, $p < 0.001$ and Wading: $r^2 = 0.97$, $p < 0.001$). In addition to the loss of descending and ascending axons in the white matter, numerous motor neurons innervating the trunk muscle were also lost which may specifically affect the swimming performance in which the balance in water highly depends on trunk muscle function. However, we only found a low correlation between lesion volume and KSAT score by a curvilinear function ($r^2 = 0.56$, $p < 0.001$).

We next analyzed the relationship between each scales. Both the KSAT and wading scales showed strong linear correlation with the BBB scale (r^2=0.92, p<0.001; r^2=0.934, p<0.001) and an even higher correlation between the KSAT and wading scales (r^2=0.975, p<0.001). However, there are some differences. In comparison with the BBB scale (0-21), KSAT and wading scale have a slightly shorter range (0-19 and 0-16, respectively). The functional differences are small between the different BBB scores above 17, and similarly between those below 7. Our impression is that the interval difference between points in KSAT and wading scale were more similar. However, a Rasch analysis of the assessment scales is necessary to determine if they meet the requirements of invariance over the entire range of scores [147].

With regard to the sensitivity of the tests, we next evaluated the ability of KSAT, wading and BBB scales to detect improvement during the spontaneous recovery after SCI. The functional recovery process in each group was reflected by the increases in KSAT, wading or BBB scores during 8 weeks in the four groups of injured rats. We found steady increases in KSAT scores in the four injured groups, and similar patterns by BBB and wading scores in most of injured groups. The curves suggest more variability for the BBB score after mild injuries (**Paper I**) and for the wading score in several injury groups (**Paper II**). Furthermore, the results also indicated robust and progressive functional improvement after mid-range injuries (45 and 75 g) by KSAT, wading and BBB scale, minor functional improvements in the most severely injured group by KSAT and wading score while the increases in BBB scores were larger, limited functional improvement of BBB and wading score in the group with the mildest injuries, but a steady and progressive recovery by KSAT scale. Two-way repeated measures analysis of variance demonstrated significant main effects of time and injury on KSAT, wading and BBB score. There were also significant interactions between time and injury severity. The following post-hoc Bonferroni test showed that the four injured groups were significantly different when analyzed using the KSAT, wading or BBB scores. This suggested that the three scales could consistently detect functional improvements after SCI.

The clustering of rats at BBB score of 8-9 and 14-15 has been discussed earlier. The consequence is reduced sensitivity to improvements at certain levels of injury. In our study we also found that rats had a tendency to cluster at BBB scores between 14 and 15. To solve some problems with the BBB scale, the BBB sub-score was introduced, but it is only valid for rats with mild injury, and is actually a separate scale for mild injuries using the same parameters. Thus it is therefore not a complete solution to the drawback of the hierarchical design of the BBB score. We were trying to fundamentally resolve this problem by establishing a compound scale. Each compound scale consisted of a number of parameters each parameter was scored, and the scores added to give a total score. We found rats were evenly distributed along the range of injuries, there were no indications of threshold effects in the KSAT and wading scores, no accumulation of recovering animals at certain scores, and the clusters that appeared in the BBB scores were separated in the KSAT and wading scores. Larger groups of tested rats will of course be necessary to definitively exclude the risk of

clusters at certain KSAT and wading scores. Another advantage of the novel compound scale was that the recovery of the various functions was reflected by changes in several parameters. The potential issue of the compound scale is the risk of making more errors when each parameter was scored to generate a sum score. The extensive reliability analysis proved that this is not a problem for KSAT and wading scale.

The BBB, KSAT and wading scales all have the disadvantage of being ordinal rating scales, which produce semi-quantitative data. The scores are derived from observations of the spontaneous functional recovery process after SCI and introduction of a devised scoring system. However, it is still difficult to interpret the data regarding the functional difference between each point. While continuous kinematic measures generated quantitative data and more detailed analysis of behavior. Thus it is more sensitive to detect true functional recovery from substitution of function for a particular behavior. Therefore, we investigated several kinematic parameters for swimming. However, they turned out to be useless for functional testing. This is might not be surprising that several kinematic methods for walking did not present any advantages compared to the BBB scale in functional evaluation.

We found that exploration, which often was evident during walking, rarely occurred during swimming. Rats are natural swimmers but after the first days of adaptation to the test situation, there is no learning, in contrast to walking, which is spontaneously "trained" in the home cages. This suggests that swimming is a motor activity suitable for kinematic analysis. However, since moderate to severe trunk rotation appeared after moderate to severe injuries, which greatly affected the accurate calculation of kinematic measurements in the two-dimensional projection of the video footage, kinematic analysis of swimming was limited to rats with mild injury. As described in **paper I**, normal rats and those with mild SCI do not use forelimbs during swimming to compensate for insufficient hindlimb movements seen after more severe injuries. Thus assessment of hindlimb function in these rats could possibly be a reliable measure of recovery after SCI. Six kinematic parameters were analyzed at week 2 and week 8 in normal rats and in two groups of SCI rats (mild compression and contusion). The functional data was correlated with spared tissue at the injury epicenter after post-mortem examination. Two-way repeated measures ANOVA showed a significant main effect of time on KSAT scores and three kinematic parameters: Swim Speed, Stroke Time and the Extension time/Flexion time ratio, indicating that these parameters similarly to the KSAT score reflect functional recovery after SCI. Although we also found high correlations between spared white matter at the injury epicenter and Swim Speed, Angular Velocity and Stroke Time, there were some overlap between the injured rats and controls. Taken together, the kinematic parameters of swimming that we analyzed do not provide data that is superior to the other functional tests. There is an inherent advantage of using ratio scales rather than non-linear ordinal scales, speaking in favor of the type of measurements provided by kinematic analysis. However, our data show that the parameters studied are not good enough to replace the assessment scales used.

4.3 CHOICE OF ASSESSMENT METHODS

Currently, functional testing of rat SCI models often focus on motor functions, although assessment of sensory and autonomic functions is equally important. There is a wide spectrum of methodologies, including: i) the microscopic and histological examinations to study the anatomical and molecular basis, ii) the electrophysiological methods to investigate conductivity, processing and the final output of the neural signals, and iii) a plethora of behavioral tests to reveal the functional changes, the most relevant outcome of any treatments. A few issues need to be considered when selecting of functional-behavioral tests to be applied.

First, different types of motor activities with distinctive neural control should be included in the motor assessment of rat SCI models, since it helps to better understand the underlying anatomical and electrophysiological changes after SCI. A variety of behavioral tests based on different types of walking could give deep insight into this complex function. However, these tests all evaluate the same function – walking. Although there are obviously major similarities between rats and human beings, we can not be ascertain that an improvement in walking caused by a treatment in rats will also be translated into the situation in the spinal cord injured person. Vice versa, a treatment improving human walking may not necessarily show the same effect in rats with SCI. The predictive power of functional studies in rats with SCI should be stronger if several tests assessing different functions are used to evaluate a potential treatment. We therefore developed two reliable assessment tools based on swimming and wading, both motor activities with features that are different from walking. Using either of these methods together with the assessments of walking in an experimental treatment study would provide a much better basis for a decision if the treatment should be translated to clinical SCI.

Second, the sensitivity of tests to different injury severity is very important. Since different extent of functional change will occur in normal recovery after SCI, which may be significantly affected by various interventions, and it is necessary to be able to monitor motor functions during the whole recovery process. Both of the tests developed by us covered the entire range from complete paralysis to normal walking, and had the capability to reflect functional recovery in different lesion groups. But we also found that the wading scale was not accurate for functional testing at the early stage after SCI. Importantly, KSAT represents a better method to assess the functional recovery in mild injury, in comparison with other assessment methods.

Although numerous locomotor tests have been developed, few of them are useful for severely injured rats. The lack of weight support effectively excludes a number of tests based on walking and climbing. Two parameters, Hindlimb Movement and Tail Movement in the KSAT score, and several parameters in the wading scale were designed and shown to be sensitive to functional changes after severe injury. Our data did not show that they are superior to the lower range of the BBB scale. However, the functional differences between individual scores are also critical. For example, the change from 3 to 4 in BBB score is

defined as a change from extensive movement of two joints to slight movement of all three joints, a change that is not easily determined. Considering that the BBB scale is maybe the only validated assessment of ambulation that can be used in severely injured rats (although not really validated for this end of the injury spectrum), adding tests of swimming and/or wading is particularly important. It should also be recognized that clinical trials will with few exceptions involve persons with severe SCI.

At the other end of the spectrum many methods have been used to reveal the small deficits after mild SCI injury. Kinematic gait analysis is one of the methods that can provide quantitative measurements. However, all of these methods are limited to animals with weight-bearing capacity, and the ability to walk. Our hypothesis was that kinematic analysis of swimming would be a solution to this problem, and provide a quantitative assessment of animals from the most severe to the mildest injuries. The results showed that due to trunk rotation this method suffered from the same limitation, that accurate evaluation could only be operated in mild injured rats. Considering the conditions for clinical trials mentioned above, it might not be very relevant to study rats with mild SCI if a clinical trial is the goal. However, a clinical trial for PTS could possibly present such a situation if patients with severe non-motor symptoms and relatively mild paresis were included.

Finally, successful clinical translation will benefit from an understanding of the mechanism of treatment, and how it can affect functional recovery. However, it is not possible to predict which neural circuitry is repaired by various experimental treatments, how the treatment affect the complicated neural plasticity processes which is considered the main factor in spontaneous recovery, or what function that may be improved. The strategy to minimize the problem, and maximize the possibility to detect functional treatment effect is as mentioned to use multiple tests in experimental studies. It has become more common that the BBB scale is combined with other types of behavioral tests. It is also important to use uniform tests that allows for comparison between different treatments. We propose that the validated and highly reliable KSAT scale we developed is used as an additional assessment when the BBB scale or other test of ambulation is used for functional testing of rats with SCI.

5 CONLUSIONS AND FUTURE PERPECTIVES

In this thesis, I studied two motor activities different from open field ambulation, and evaluated the possibility to use them for functional testing of spinal cord injured rats. This resulted in two assessment tools, the KSAT scale and the wading scale. Furthermore, a new rat model of PTS that can be used to evaluate cell transplantation therapy was developed, and used in the first study ever on transplantation of neural stem/progenitor cells to PTS.

The KSAT scale turned out to be a sensitive and reliable scale to assess swim performance in spinal cord injured rats. Internal consistency, inter-rater and test-retest reliability were very high. KSAT scores accurately reflected the functional recovery after mild, moderate and severe SCI, and was highly correlated to injury severity and spared white matter at the injury epicenter. This method was developed based on data from rats with spinal compression injuries, its application in other types of SCI have to be explored in future studies. Kinematic analysis of swimming could only be used for mild SCI, and was still not found to be a sensitive and reliable method for functional evaluation. More accurate three-dimensional systems with better methods to track the movement of joints, particularly the knee joint, may result in a better assessment of swim performance in rat SCI models, and also improve the understanding of the effects of spinal lesions on hindlimb functions.

The wading scale was also found to be a sensitive tool for assessment of rats with SCI. High reliability was also shown for wading scale. Functional recovery in different severities was reflected in the wading scores. Wading is however not suitable for functional testing in the acute phase. We also found a high correlation between the wading score and spared white matter at the injury epicenter. Since scoring of both sides is done separately, the wading could also be used for unilateral or asymmetrical lesions, for which there are few optional tests.

Combining mild contusive SCI with subarachnoid injection of autologous blood consistently induced intra-spinal extracanalicular cysts, the characteristics of which were very close to clinical picture in PTS. By comparison between MRI and histology, we confirmed that the hyper-intense areas on MRI represents fluid-filled cysts, while the hypo-intense areas represent tissue containing iron-laden macrophages/microglia, either in tissue debris or in tissue. hNPCs transplanted to the cysts survived and had the capability to obliterate the cysts, showing a potential as a clinical treatment to treat human PTS.

I believe that cell transplantation for PTS has the capacity to obliterate the cysts, but in addition, the grafted cells can possibly also replace the lost interneurons to promote functional recovery. However, there are several issues that have to be clarified before a successful clinical translation, including identification of the number of cells suitable for certain sizes of cysts, promotion of the differentiation of hNPCs in vivo, specification of the effect and mechanism of hNPCs on the cysts. In addition, we should also explore sensitive motor tests for limb coordination to study the functional consequences of gray matter loss in rats PTS, and the potential of hNPCs to reverse the functional deterioration.

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