Reconnecting the CNS and PNS with Stem Cell Transplantation

NICLAS KÖNIG
Severe injury may result in disconnection between the peripheral and central nervous system. Regeneration of the central portion of sensory neurons into the spinal cord is notoriously poor in adult mammals, with low regenerative drive and an unpermissive central environment, most likely resulting in persistent loss of sensory function. A variety of strategies have been addressed to augment regeneration, including application of growth promoting factors, counteraction of inhibitory molecules, and provision of growth permissive substrates. Stem cells have been investigated in these contexts, as well as for the possibility of providing new neurons to act as a relay between the periphery and spinal cord. Here we have investigated different sources of neural stem cells for their ability to form neurons and glia after transplantation to the periphery; to project axons into the spinal cord; and to assist regeneration of surviving sensory neurons. These have been performed at two locations: the "dorsal root ganglion cavity", and the transitional zone following dorsal root avulsion. Neurons and glia were generated from mouse boundary cap neural crest stem cells and embryonic stem cell derived ventral spinal cord progenitors, and in addition to this, regeneration of sensory fibers was observed after transplantation of human fetal spinal cord derived progenitors and human embryonic stem cell derived ventral spinal cord progenitors. Further, delivery of neurotrophic factor mimetics via mesoporous silica nanoparticles proved a valuable tool for stem cell survival and differentiation. While technological advances make in vivo differentiation a realistic goal, our findings indicate that so far assisting regeneration of host sensory fibers to reconnect with the spinal cord by transplantation of stem cells is a more reliable strategy.

Keywords: stem cell transplantation, regenerative neurobiology, nerve injury repair

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To my family
List of papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

Forced Runx1 expression in human neural stem/progenitor cells transplanted to the rat dorsal root ganglion cavity results in extensive axonal growth specifically from spinal cord-derived neurospheres. *Stem Cells and Development* 2011, 20(11):1847-57


IV Trolle C, König N, Abrahamsson N, Vasylovska S, Kozlova EN.
Boundary cap neural crest stem cells homotopically implanted to the injured dorsal root transitional zone give rise to different types of neurons and glia in adult rodents. *BMC Neuroscience* 2014, 5;15:60

Human embryonic stem cell-derived progenitors assist functional sensory axon regeneration after dorsal root avulsion injury. *Accepted for publication in Scientific Reports* 2015

VI König N, Hoeber J, Trolle C, Garcia-Bennett A, Berezin V, Åkesson E, Kozlova EN.
Human spinal cord neural progenitors alone but not in combination with growth factor mimetic loaded mesoporous silica assist regeneration of sensory fibers into the spinal cord after dorsal root avulsion. *Manuscript* 2015

Neural crest stem cells from hair follicles and boundary cap have different effects on pancreatic islets in vitro. *International Journal of Neuroscience* 2014 [Epub ahead of print]

IX Schizas N, König N, Andersson B, Vasylovska S, Hoeber J, Kozlova EN, Hailer NP.
Neural crest stem cells protect spinal cord slice cultures from excitotoxic neuronal damage and inhibit glial activation. *Submitted to Neurotherapeutics* 2015

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### Abbreviations

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<td>Boundary cap</td>
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<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
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<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>bNCSCs</td>
<td>Boundary cap neural crest stem cells</td>
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<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
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<td>ChAT</td>
<td>Choline acetyl transferase</td>
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<td>CNS</td>
<td>Central nervous system</td>
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<td>CNTF</td>
<td>Ciliary neurotrophic factor</td>
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<td>CTB</td>
<td>Cholera toxin B subunit</td>
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<td>DCX</td>
<td>Doublecortin</td>
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<td>DRA</td>
<td>Dorsal root avulsion</td>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
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<td>DRTZ</td>
<td>Dorsal root transitional zone</td>
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<td>EB</td>
<td>Embryoid body</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ESC</td>
<td>Embryonic stem cell</td>
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<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
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<tr>
<td>NF200</td>
<td>Neurofilament 200kD</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
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<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>hfNSPC</td>
<td>Human forebrain-derived neural stem/progenitor cells</td>
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<td>hscNSPC</td>
<td>Human spinal cord-derived neural stem/progenitor cells</td>
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<td>Isolectin B4</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>RA</td>
<td>Retinoic acid</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>VGluT</td>
<td>Vesicular glutamate transporter</td>
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<td>VIAAT</td>
<td>Vesicular inhibitory amino acid transporter</td>
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<tr>
<td>VSFP</td>
<td>Voltage-sensitive fluorescent protein</td>
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Introduction

Comprising our brain and spinal cord, and linking these to the rest of the body, the nervous system is what allows us to think and act - essentially to be human. Injury to any part of this system may leave intractable deficits in both functionality and life quality. The study of nerve regeneration is both frustrating and enthralling. The study of stem cells is both controversial and revolutionary. Together, these form a landscape of immense potential and endless questions.

The debilitating consequences of nerve injury still pose great challenges for patients, clinicians, and researchers, and vast progress has been made in the field. Recovery of motor and sensory functions have distinct aims and outcomes, and very different prospects. Central sensory regeneration is thus not only less probable, but less addressed, and therein lies the importance of and inspiration for this work.
Background

The sensory system

The collection of ganglia and nerves that is the peripheral nervous system (PNS), functions as a relay between the central nervous system (CNS) – the brain and spinal cord – and the body (Fig 1A).

The PNS, with somatic and autonomic components, is thus responsible for functions such as voluntary control of muscles, gastrointestinal processes, hormone secretion, and much of the body’s sensory perception.

Highly specialized receptors of sensory nerve cells translate external inputs such as touch, sound, smell, as well as internal signals such as blood pressure, into electrical signals that can be transmitted via the spinal cord or cranial nerves to the brain.

Sensory neurons – or afferents – are activated by a single or multiple (polymodal) mechanisms, and these include the categories touch (mechanoreception), pain (nociception), stretch (proprioception), and temperature (thermoreception). Further, these may require a certain level of stimulation to be activated, so called threshold. Thus there are, for instance, low and high threshold mechanoreceptors (LTM and HTM), which will only fire within a range of pressures.

Axon function and survival are dependent on Schwann cells, the major glia of the PNS, which produce trophic factors and extracellular matrix components, creating a permissive growth environment. Schwann cells also myelinate certain sets of neurons with large or medium diameters, regulating the formation of Ranvier nodes, greatly increasing their speed of conduction. Taken together, the properties mentioned thus far distinguish sensory neurons as belonging to the class Aα (proprioception, mechanoreception), Aβ (proprioception, mechanoreception), Aδ (nociception, mechanoreception, thermoreception) – all of which are myelinated – or C (nociception, mechanoreception, thermoreception), which are unmyelinated. While most sensory neurons release the neurotransmitter glutamate – identified by the presence of vesicular glutamate transporter 1-3 (VGluT1-3; reviewed in [11]) – subsets corelease neuropeptides including calcitonin gene-related peptide (CGRP) and substance P, which are mostly used by nociceptors. These peptidergic neurons are thus distinguished from non-peptidergic neurons, which can be labeled with isolectin B4 (IB4). The cell bodies of afferents are collectively found in dorsal root ganglia (DRG), where they are surrounded by satellite cells, a specific type of Schwann cell. The impulse initiated at a sensory receptor is
carried along the peripheral branch of the axon and passes by the cell body in the DRG, continuing along the central branch in the dorsal root toward the spinal cord, giving the neuron a pseudounipolar form (Figure 1C). Afferents are organized in dermatomes, innervating adjacent segments of the body, and terminating at designated levels in the spinal cord. Whereas there are in the human cord 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal segment, as defined by the region of origin of spinal nerves, the numbers for mice and rats are 8 cervical, 13 thoracic, 6 lumbar, 4 sacral, and 3 coccygeal. Interspecies differences in anatomy include contributions to the sciatic nerve, which in mice are L3-L6, compared to L4-L6 in rats [90].

In consistently delineated patterns, cells of the spinal cord line up to receive and transmit information of particular modalities to and from select targets. The neuronal cell bodies are organized in such a way that they may be categorized into bands referred to as Rexed’s laminae (Figure 1C).

From the dorsal surface and inward, laminae are numbered I to VI in the dorsal horn, and VIII to IX in the ventral horn. Although vastly interconnected by interneurons and collaterals, the cells populating these bands grossly correspond to particular functions. Lamina I is also known as the marginal zone, receives input of pain and temperature sensation via Lissauer’s tract. Lamina II, or substantia gelatinosa, receives input from C- and Aδ fibers, continuing along the spinothalamic tract. Lamina VII and X make up the intermediate zone, accommodating the intermediolateral nucleus and Clarke’s nucleus at thoracolumbar levels, as well as the gray matter surrounding the central canal, respectively. Motor interneurons and the Commissural nucleus are located in lamina VIII of the ventral horn, whereas lamina IX contains lateral and medial motor neurons, Onuf’s nucleus at sacral level, and at cervical levels the nuclei of the phrenic and spinal accessory nerves.

The terminals at a given point in the spinal cord are of a wide origin: from sensory afferents entering via the dorsal roots, descending inputs from the brain, interneurons from the same and other side of the spinal cord, and collaterals from sensory fibers of adjacent segments.

The dorsal root transitional zone

Entry of sensory axons into the CNS is via the dorsal roots, crossing the sharply delimited CNS/PNS boundary within the dorsal root transitional zone (DRTZ) of each smaller rootlet. Central tissue protrudes into theses dorsal rootlets, and so this area is a composite of a thick layer of astrocytes (glia limitans) and a continuous basal lamina which also extends into the periphery.
Figure 1. An overview of the nervous system. (A) Division between central (blue) and peripheral (red) nervous system. (B) Cross section of spinal cord with surrounding vertebra. (C) Detailed view of spinal cord cross section. Roman numerals denote Rexed’s laminae. CNS = central nervous system, PNS = peripheral nervous system, DRTZ = dorsal root transitional zone, DH = dorsal horn, VH = ventral horn.
Sensory axon injury and regeneration

Following trauma to the nervous system, nerves can become crushed, stretched, or even transected. The cellular events and morphological changes that follow, as documented by Ramon y Cajal in "Cajal’s Degeneration and Regeneration of the Nervous System" [26], include dystrophy of axons and scar formation. Cajal predicted that regeneration after injury was "likely", although much more difficult than the nerve growth that occurs during development, and while the regenerative capacity of the PNS is generally considered more ample than that of the CNS, the outcome of nerve damage is variable depending on location and extent.

Generally, damage close to the cell body initiates a strong regenerative reaction, but also correlates with less chance of survival and proper reinnervation. A range of changes in molecular expression follow in both glia and neurons (reviewed in [77], and [70]). Activated Schwann cells produce trophic factors that assist regenerating fibers on their path to reinnervate their peripheral targets [41], including nerve growth factor (NGF), glial cell-line derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (CNTF). De-differentiated Schwann cells also line up in formations known as bands of Büngner, providing axons with a growth-permissive substrate. The changes within the sensory neurons, however, differ between central and peripheral axotomy. For instance, damage to the peripheral segment of sensory neurons stimulates upregulation of regeneration-associated genes including GAP43, ATF-3, and c-Jun [25]. The proximal, centrally projecting portions of sensory neurons, on the other hand, do not elicit the same regenerative response. GAP43, for instance, is not upregulated in the DRG, and so there is a lesser drive for regrowth centrally. Key to regeneration is also the navigation of ECM constituents, such as laminin, integrin receptors to which are differentially expressed between injury to the central and peripheral axon branch [112].

Dorsal root injury also propagates changes to the CNS. Immediately following dorsal root injury, a process starts to enforce the boundary at the DRTZ. Central tissue projections start extending further into the dorsal root, where astrocytes hypertrophy and divide, producing various molecules inhibitory to regenerating axons, including myelin associated inhibitors, chondroitin sulfate proteoglycans (CSPGs), ephrins, and semaphorins [45]. Besides creating unpermissive environment, reactive astrocytes may also stabilize a sort of synapse formation with the recovering sensory axons [32], stalling them at the entry zone. As such, although a regenerative response of centrally damaged sensory axons may be potentiated by a lesion to their peripheral branch [88] - so called conditioning - without modifying the entry zone, these axons fail to re-enter the CNS [24].
**Avulsion injuries**

Avulsion injuries, which imply a sharp stretching of a nerve, may rupture or entirely sever the connection between the periphery and spinal cord. This type of injury often involves the brachial plexus, a collection of nerves at the shoulder level (spinal roots of C5-C8 and T1), and most commonly follows from trauma to the head and shoulder or shoulder dystocia during childbirth. Following such an injury, although motor neurons have some ability to extend axons through the reimplanted ventral root [15], and function may be restored by this method or nerve transfer [87], re-entry into the CNS of sensory afferents via the dorsal roots do not share this outlook. After an avulsion, there is a general degeneration of the gray matter of the spinal cord, with cell death visible in the ipsilateral gray matter just a few days post injury. This is accompanied by a loss of peptidergic and glutamatergic terminals, as well as a prolonged immune response, contributing to the initiation of neuropathic pain [6, 20, 22]. Although the latter may be alleviated by ventral root reimplantation or lesioning of the dorsal horn (DREZotomy) [103], the pain often persists, and sensory perception in the deafferented segment is never regained.

**Strategies to overcome impediments of regeneration**

Efforts to overcome the impediments to CNS regeneration have focused on various aspects, for example by blocking inhibitory molecules with neutralizing antibodies, degrading components of the glial scar using enzymes [10, 108] or chemicals [97], peripheral and central conditioning [88, 120], and enhancing regenerative capacity of axons by adding growth stimulating molecules [73] or providing permissive substrates to bridge the gap between the peripheral and central nervous tissue [12]. A number of studies have shown that while permissive substrates, such as autologous nerve grafts, attract ingrowth of both ascending and descending axons within the spinal cord, few if any of these exit the graft and re-enter the surrounding CNS tissue [89], highlighting the difference in permissiveness of the CNS and PNS environments. Neurotrophic factors, including molecules of diverse families, have been shown to exert growth promoting effects on particular subsets of sensory axons. Afferents have different repertoires of receptors, so that the populations that will respond depend on the combination of factors administered (as presented in an overview by Smith et al. [104]). For instance, while NGF will only target small, unmyelinated nociceptors, GDNF will influence multiple sensory subtypes. In the context of sensory regeneration, these have been targeted by overexpression in transplanted cells [101], delivery by injection [73], incorporation into diverse biomaterials [13], and by gene therapy in combination with nerve transfer [68].

Importantly, regeneration needs to not only reverse deficits, but avoid creating undesirable effects of treatment. Aberrant rewiring in the spinal cord may
for instance result in development of pain [31], and systemic administration of neurotrophic factors has presented side effects such as weight loss [75, 82].

**Cell therapy for nerve injury**

Regeneration of axons into the CNS thus necessitates a number of conditions: blocking of inhibitory molecules; modification of the scar formation; a permissive substrate; trophic support to surviving neurons; and to some extent an increased intrinsic drive to regrow.

Stem cells – self-renewing, multipotent constituents of the developing and adult body – in the context of nerve injury, may act on several of the aforementioned points [28, 43].

Depending on the source and stage of development, the fate of a stem cell is more or less restricted, and some populations are said to be "tissue-specific" stem cells, capable only of replenishing cells of a particular phenotype pertaining to a type of tissue. Thus, while embryonic stem cells (ESCs) are able to generate all cells of an organism, neural stem cells for instance are committed to create neurons or glia. This dogma is challenged, however, by reports of direct trans-differentiation between somatic lineages (Reviewed in [78] and [119]). The use of stem cells in treatment of diseases of the nervous system [94] has established a firm niche in research, presently used in a number of ongoing clinical trials. Conditions for which transplantation has proved efficacious in animal models include stroke, Parkinson’s disease, spinal cord injury, and retinal disorders. A conceivable benefit of using a cellular graft is the long-term integration with host tissue, avoiding the need to replace mechanical implants that may degrade over time. Further, with advents in induced pluripotency and transformation, stem cells can be prepared from a patient’s own tissue, obviating the need for immunosuppression to avoid rejection.

The mechanism by which stem cells may mediate repair of nerve injury are manifold. Primarily, the role of stem cells is a supportive one, providing damaged tissue with trophic factors, reducing inflammation, and creating permissive substrates for regrowth (reviewed in [43, 94]. Particular to glial and neuronal progenitors, these may act by remyelinating injured axons [100, 115] or providing replacement neurons that either incorporate into existing circuits [124], or create new relays [9].

In the adult rat, grafts of sensory neurons directly into the intact DRG proved able to enter the spinal cord via dorsal roots [73]. This capacity was apparently precluded by a dorsal root rhizotomy, explained by an induced gliosis at the DRTZ. However, transplantation of human embryonic sensory neurons to adult rat following rhizotomy showed that these could cross the DRTZ and connect with host circuitry [57, 64]. This, in combination with studies on neonatal dorsal root [14] indicates that certain populations of stem cells possess unique properties that circumvent the inhibitory CNS-PNS boundary.
Stem cell grafts may also by physical means provide regenerating axons access to the CNS. Studies involving olfactory ensheathing cells (OECs) have shown interaction with host astrocytes, creating a bridge for regenerating axons [66].

Challenges of stem cell therapy for nerve injury
Several caveats need to be considered when performing pre-clinical or clinical investigations using stem cells. Stem cells may, in brief: form tumors, remain undifferentiated, be heterogeneous, form inadequate connections, be very limited in supply, be difficult to track.

Purification, by magnetic sorting or certain culture methods, addresses several of these issues in that a more uniform, predictable, and mature cell fraction is used for in vivo applications. As further insurance, genetic suicide-triggers have been developed to be incorporated into stem cells prior to transplantation, allowing for purging of undifferentiated cells at a chosen moment.

Cells studied in this thesis

*Murine boundary cap neural crest stem cells*
Within a narrow window of neural development, the entry and exit points of sensory and motor axons in the spinal cord are created by a transient group of cells termed the boundary cap. Neural crest stem cells derived from the embryonic boundary cap (bNCSCs), have a broad adaptive capacity. In vitro, neurospheres create smooth muscle, glia, and neurons specified to a sensory lineage [52]. In vivo, not only are they able to form neurons and glia of the PNS [72], but can also adopt a central oligodendrocyte lineage [128]. In a study using ectopic expression of Runx1 specific to Sox10+ bNCSCs, transplanted cells were steered toward a non-peptidergic nociceptive phenotype, expressing GDNF family coreceptor RET and purinergic receptor P2X3 [4]. Neural crest stem cells are attractive for research as these can be found in hair follicles even after birth, and although differences between these and other origins of NCSCs are noted [99], there are nevertheless reports of multipotency also of this pool [102].

*Murine embryonic stem cell derived ventral spinal cord progenitors*
Wichterle et al. published a study in 2002 describing the generation and integration into the developing spinal cord of murine embryonic stem cell derived motor neurons. An ESC line was created from a transgenic mouse line where the reporter enhanced green fluorescent protein (EGFP) was driven by the promoter of motor neuron transcription factor Hb9. Being able to visually identify motor neuron progenitors meant that culture conditions could be optimized by comparing the yield of EGFP positive cells, as well as easy tracking
after transplantation. This source was used here both to generate motor neurons in the dorsal root ganglion cavity and as a comparison to other cell types in a dorsal root avulsion setting.

**Human fetal tissue derived neurospheres**

Stem/progenitor cells from neurogenic areas of the developing human - including the forebrain (hfNSPC), spinal cord (hscNSPC), and subventricular zone - can be isolated from embryonic tissue by dissociation and purification using mitogens that maintain a proliferative state [17, 40, 106]. These form spherical aggregates with a mixture of stem and progenitor cells, retaining the ability to differentiate into neurons and glia both in vitro and after transplantation to the CNS [55, 56], showing signs of adaptation to the microenvironment [114]. The fate of cells varies extensively between studies, but this may in part be explained by timing (avoiding the acute inflammatory phase), delivery form (neurospheres versus single cells), donor age (as well as passage number), and injury model. In addition to serving as potential replacement cells, these have demonstrated neuroprotective effects [34]. By one or several mechanisms, grafted human fetal derived stem/progenitor cells have also improved functional recovery from spinal cord injury in animal models [34, 63, 111]. Their potential in sensory regeneration, however, has not been explored.

**Human embryonic stem cell derived neural progenitors**

Embryonic stem cells allow for the generation of all cells of the organism, but make the differentiation process more lengthy and difficult. Different methods of generating neural cells from ESCs include aggregate formation (such as embryoid bodies; EBs), coculture with non-neuronal cells, and neural rosette selection. Neural stem cells are characterized by the ability to generate neurons and glia, including astrocytes and oligodendrocytes, and the use of these for nerve injuries, such as spinal cord injury, have been compared with tissue specific stem cells and induced pluripotent cells [92], showing capacity to remyelinate axons [83] and replace neurons [124]. The study of human neural stem/progenitor cells derived from embryonic stem cells (here hNPs) is highly relevant to the potential of clinical application, as advances in induced pluripotent stem cell (iPSC) technology means deriving neural progenitors will not be limited to the availability of embryonic material.

**Guidance of stem cell differentiation**

Cell fate is coordinated by guidance cues, gradients of trophic factors and mitogens, transcription factors, and cell to cell interactions. The aspects studied in this thesis are detailed below.
Runx1
Runt related transcription factor 1 (Runx1 or Aml1) specifies a subpopulation of nociceptors [18, 71] and C-mechanoreceptors [69], regulates their projections [58, 71, 122], is important for the development of certain motor neuron groups [105, 107], and is involved in definitive hematopoiesis. Ectopic expression in neural crest stem cells after transplantation increased survival and steered cells toward a nonpeptidergic sensory neuron phenotype [4]. Here, a tetracycline-inducible genetic expression system is employed for its ability to regulate expression even after transfection by the administration or withdrawal of tetracycline. Choosing a tet-off system means transcription is active in the absence of tetracycline, which obviates the need to administer anything to the animal after transplantation. This system can be expanded to include another gene regulated by the same or different tetracyclines for precise temporal and sequential control [61].

Neurotrophins
Neurotrophic factors play diverse roles in development, regulating both cell cycle and specification. Studied here are peptide mimetics of CNTF and GDNF. Acting on gp130-signaling, the former has a pro-survival effect on neurons and oligodendrocytes, and has been observed to support astrocytic differentiation of among others spinal cord progenitors [76] and pluripotent stem cells [59], while stimulating neurite extension in neurons [86]. There may be considerable inter-species differences, as CNTF contributes to a higher yield of neurons in human versus murine cultures [42]. GDNF is involved in differentiation of a range of neuronal sets [2, 35, 37, 96, 110], and via interaction with several pathways, it too stimulates axonal elongation [80].

Transplantation models
Dorsal root ganglion cavity
As established previously [4, 56, 57], removing the dorsal root ganglion and replacing residing neurons with stem cells allows for the study of survival and differentiation in a defined and accessible space, with a permissive environment, and minimal impact on the animals.

Dorsal root avulsion
Although avulsion injuries commonly involve both spinal roots, tearing of the dorsal roots from their entry zone to the spinal cord [20, 22] provides a platform to study the interaction of grafted cells with both the CNS and PNS at this unique interface, with a focus on sensory regeneration without significant detriment to motor function.
Aims
The aim of this thesis was to evaluate the usefulness of neural stem/progenitor cells in reconnecting the peripheral and central nervous system, providing insight on the integration and differentiation profiles of stem cells of different sources transplanted to the periphery. The specific objectives were to:

- Investigate the survival and integration of neural stem cells transplanted to the periphery in terms of migration and distribution.
- Influence the differentiation process of neural stem cells by internal and external modifications.
- Explore the potential of neural stem cells to assist regeneration of sensory axons.

Paper I
Steer the formation of projection neurons in human fetal forebrain and spinal cord stem/progenitor cells by forced expression of Runx1 and investigate their capacity to cross the DRTZ.

Paper II
Translate a modified in vitro protocol of murine motor neuron differentiation using nanoparticle-delivered neurotrophic factor mimetics of CNTF and GDNF to in vivo, and evaluate the maturation and functionality of grafted cells.

Paper III–IV
Investigate the migration and differentiation pattern of mouse embryonic stem cell derived ventral spinal cord progenitors and boundary cap neural crest cells in a mouse model of dorsal root avulsion.

Paper V
Assess the potential of human embryonic stem cell derived spinal cord progenitors to assist regeneration of sensory afferents in a dorsal root avulsion model.

Paper VI
Explore the potential of human fetal spinal cord derived neural stem cells to assist regeneration of sensory afferents in a dorsal root avulsion model, as well as the benefits of peptide mimetics of CNTF and GDNF in this setting.
Experimental procedures

Ethical permissions
All animal research was conducted with the approval of the Local Ethical Committee for Animal Experimentation, Uppsala, under the permits C298/11 and C178/14, and kept according to Swedish Legislation and EU Directives. The use of human abortive tissue took place with the informed consent of donor patients, as well as approval by the Regional Human Ethics Committee, Stockholm.

Cell culture
Boundary cap neural crest stem cells
First described by Hjerling-Leffler et al. [52], and adapted by Aldskogius et al. [4], neurospheres of bNCSCs were prepared as follows: Dorsal root ganglia (including boundary caps; BCs) from E11.5 embryos of heterozygous mice with $\beta$-actin promoter driven green fluorescent protein (GFP) were isolated, enzymatically dissociated, and plated in DMEM/F12 medium supplemented with B27, N2, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) on plastic dishes. Changing medium every other day, neurospheres were formed within two weeks, whereupon they were split 1:2 or 1:3.

Mouse embryonic stem cells
Murine embryonic stem cells carrying a GFP reporter under the Hb9 promoter (Hb9-GFP; "HBG3") were received from Kevin Eggan. These were propagated on gelatin-coated flasks for two days, when colonies were partially dissociated and transferred to low-adherence 6-well plates for formation of embryoid bodies (EBs). These then continued onto motor neuron differentiation and transplantation.

Motor neuron differentiation
Adaptations were made to a protocol presented by Wichterle et al [116], which was designed to enrich Hb9 expressing motor neuron progenitors in cultures of EBs. Retinoic acid (RA) and sonic hedgehog (Shh) (or agonists Ag1.3 or Purmorphamine) were used to caudalize and ventralize stem cells. After 6–7 days
in culture with these factors, EBs were either seeded on cover slips for in vitro differentiation assays (DA) or transplanted (Papers II and III). For in vitro DA, cells were plated at 50,000 cells/coverslip in medium termed ADFNB, consisting of Advanced DMEM/F12:Neurobasal (1:1), GlutaMAX, B27, N2, mercaptoethanol, Pen/Strep, CNTF (10 ng/ml), and GDNF (10 ng/ml). For some experiments, neurotrophins CNTF and GDNF were substituted with mimetics as detailed below.

**Nanoparticle delivery of trophic factor mimetics**
Peptide mimetics to CNTF ("Cintrofin") and GDNF ("Gliafin") were supplied by Professor Vladimir Berezin. These were loaded into nanoparticles composed of mesoporous silica created by Nanologica (www.nanologica.com). Total loading was determined using thermogravimetric analysis. Nanoparticles with loaded mimetics were applied both in culture (calculated to release a total amount equal to that of soluble factors), as well as in vivo as detailed in Papers II and VI.

**Voltage-sensitive fluorescent protein transfection**
Using the A033 program of the Amaxa Nucleofector, Hb9-GFP cells were transfected with a plasmid encoding voltage sensitive fluorescent protein 2.42 (VSFP2.42) [3] under a constitutive cytomegalovirus enhancer/chicken β-actin (CAG) promoter.

**Human fetal tissue derived neurospheres**
For papers I and VI, neurospheres derived from human first-trimester forebrain or spinal cord were used. These were prepared at the Division of Neurodegeneration, Department of Neurobiology, at Karolinska Institutet, and provided by Dr. Elisabeth Åkesson. Briefly, CNS tissue was separated from week 7-9 aborted fetal material, dissociated, and cultured in the presence of EGF, bFGF and CNTF to induce formation of neurospheres [55].

**Runx1 transfection**
Isoform b of RUNX1 (RUNX1b/AML1b), was introduced into a tetracycline-regulated system [60] by Dr. Christian Berens. Using the A033 program of the Amaxa Nucleofector, a 1:3 ratio of Runx1 (pBi-EYFP-Aml1B) and transactivator (pWHE120(B/D)HK64SL135-cTA2D-5) plasmids were used to transfect approximately 2x10⁶ cells. Transfection efficiency was evaluated by monitoring for EYFP expression in culture.

**Human embryonic stem cells derived spinal cord progenitors**
Primitive neuroepithelial cells were generated by culturing cells from a CAG-hrGFP hESC line on mouse embryonic fibroblasts (MEFs) as described else-
where [19]. Cells were plated in 1:1 DMEM/F12:Neurobasal with Glutamax, B27, N2, GSK3 inhibitor CHIR99021, TGF-β receptor inhibitor SB435142, ALK2 receptor inhibitor DMH1, and BMP, followed by the addition of RA and Purmorphamine. Resulting neuroepithelial cells were transferred to low adherence dishes and cultured in the presence of RA and Ag1.3. Cells formed spheres that expressed markers of ventral spinal cord progenitors, and these were either further differentiated in vitro or transplanted to the site of L3-L5 avulsions in mice (Paper V).

Differentiation assays
Glass coverslips were coated with poly-D-lysine or poly-L-ornithine followed by laminin. Cells were seeded as single cells or aggregates depending on the experiment. At the end of the assay, cells were fixed in their culture chambers with 4% paraformaldehyde (PFA) for 10 min. After washing with PBS, coverslips were used for immunohistochemistry (IHC).

Transplantation
Animals
Sprague-Dawley rats and nu/nu "nude" mice were the subjects of transplantation experiments. Nude, athymic mice cannot mount an adaptive immune response and so do not reject transplanted cells. Rats received daily subcutaneous injections of Cyclosporine A to avoid rejection.
All transplant procedures commenced with a partial laminectomy, which entails removing a portion of the vertebra between the spinal and lateral processes (see Fig 2, page 25).

Dorsal root ganglion cavity
Papers I and II
After a laminectomy of lumbar vertebrae L4-L5, just rostrally of the height of the iliac crest, the lateral processes were carefully removed until the dorsal root ganglia were visible. The meninges were carefully incised between the ganglion and the spinal cord roots. After separating the DRG from the ventral root running beneath, the roots proximal and distal to the ganglion were cut and the DRG removed. Neurospheres of hsc/hfNSPCs (Paper I), or Hb9-GFP EBs (Paper II), were placed in the resulting cavity.
Dorsal root avulsion

*Papers III–VI*

The laminectomies were performed over the T13-L1 vertebrae, under which the entry zones for dorsal roots L3-L5 are located in mice, expanded to include the L2 vertebra to reach the L6 dorsal root in rats [20]. After opening the dura with a needle and micro-scissors, the dorsal roots were teased apart and subsequently pulled with steady traction using forceps. Where indicated, after re-positioning the roots on the surface of the spinal cord, stem cells were placed on top. For the study in Paper VI, mesoporous silica nanoparticles loaded with Cintrofin and Gliafin in powder form were applied to the injury site using forceps.

Neuronal tracing

To assess whether regeneration of injured host axons had taken place, some of the studies used neuronal tracing. Cholera toxin B subunit (CTB) was diluted to a 1% solution in water, and after localizing the left sciatic nerve, an injection of 3-5 µl of working solution was made into the nerve using a fine glass needle. Animals were sacrificed three days later, and routine cryosectioning was performed. Uptake of CTB was visualized by immunohistochemistry, applying a goat anti-CTB antibody and appropriate secondary, performed at room temperature.

Tissue collection

Animals were sacrificed with an overdose of anesthetic, followed by transcardiac perfusion of saline and then 4% PFA in phosphate buffered saline (PBS) with picric acid. Dissected tissue was postfixed for another 4 hrs in fixative, and then kept in 15–20% sucrose overnight.

Analysis

Immunohistochemistry

Generally, cryosections on glass slides were incubated in blocking solution (1% BSA, 0.1% NaN₃, and 0.3% Triton X-100 in PBS) for 30–60 min. Primary antibody diluted blocking solution to working concentration was applied for 4 hours at room temperature or overnight at 4°C. After washing in PBS, secondary antibody diluted in blocking solution was applied for 1–2 hrs at room temperature. Where background interference was problematic, an additional blocking step using unlabeled antibody of the same species as that of the secondary antibody was applied for 30 min.
Figure 2. Overview of neuroanatomical context of experimental models used for transplantation. hESC = human embryonic stem cell mESC = murine embryonic stem cell hsc/hfNSPC = human spinal cord/forebrain neural stem/progenitor cell, bNCSC = boundary cap neural crest stem cell, CNS = central nervous system, PNS = peripheral nervous system, DRTZ = dorsal root transitional zone, DH = dorsal horn, VH = ventral horn, DRG = dorsal root ganglion.
Microscopy
Images were captured using an epifluorescence microscope (Nikon Eclipse E800) or a confocal system (Zeiss LSM 510 META or Zeiss LSM 700), unless otherwise specified in the included papers.

Image analysis
Pictures were combined using Photoshop or GIMP. Quantifications and neurite tracing were performed using ImageJ as well as "Process Length" (Protein Laboratory, Copenhagen, Denmark) [93].

Electrophysiology
Cultures of motor neurons (Paper II) were patch-clamped and recorded at the end of differentiation assays, continuously perfused with artificial cerebrospinal fluid (aCSF) and HEPES. Explants from experimental animals at two weeks and two months were perfused for 10 minutes in aCSF before being placed in a microincubator in an electrophysiology rig, where activity was induced by applying 10M carbachol. Changes in fluorescence intensity were recorded with a camera and custom-made software.

Behavioral analysis

nociceptive response to von frey filaments
To test for return of sensation, a maximum threshold withdrawal response frequency was obtained from experimental animals. A 5.5g filament was applied five consecutive times to the mid-plantar hind paw with 5 seconds in between, and a ratio of response to total stimulations was calculated.

Grip strength
A composite of strength and coordination, grip strength was investigated by letting subjects grip a grid of a force-measuring apparatus, gently pulling the mouse away from it, and measuring the force at the time of release.

Statistics
Software used to perform statistical computations were GraphPad Prism and Excel. Tests used to study differences in means between groups included unpaired t-tests and one- and two-way ANOVAs with appropriate post-hoc tests.
Results

Paper I

Runx1 is known to induce axonal outgrowth [122], and specify nociceptors in the dorsal root ganglion [18, 58]. Here, we transfected human fetal spinal cord and forebrain derived neural stem/progenitor cells to express Runx1, and transplanted these to the periphery after removing the dorsal root ganglion. While spinal cord derived cells remained clustered and inert, forebrain progenitors – both untreated and transfected – migrated along the dorsal root toward the spinal cord, and although re-entry into the CNS was not observed in any group, the Runx1 transfection did drive the extension of axons in spinal cord derived stem/progenitor cells (Figure 3). Transfected cells also showed more progressed differentiation judged by the appearance of specific markers vesicular glutamate transporter 1 and 2 (VGluT1, VGluT2), choline acetyl transferase (ChAT), and microtubule-associated protein 2 (MAP2). The proportion of neurons was generally higher in the forebrain derived cell groups, in line with previous findings [55, 114].

Figure 3. Runx1 transfected hscNSPCs (A) and hfNSPCs (B) transplants to the L4 DRG cavity labeled with human-specific Hsp27 (green) and β-III tubulin (red). Schematic depiction of axons extending from transplant into dorsal root toward the spinal cord (C). (Figure 5 of Paper I)
Paper II

To affect differentiation of stem cells with a method other than transfection of genetic material, we investigated the potential use of nanoparticles to deliver external factors. Here, we employed peptide mimetics of CNTF (Cintrofin) and GDNF (Gliafin) to assist the differentiation of murine ES cells into motor neurons. In culture, these molecules supported motor neuron generation as characterized by the expression of ChAT, Islet 1 (Isl1), and the Hb9 driven EGPF reporter, as well as the ability of patch clamping to elicit action potentials. When transplanted to the DRG cavity, Hb9-GFP cells expressed ChAT earlier and had more abundant arborizations when mimetics were applied (Figure 4). To test their functional properties, Hb9-GFP cells were transfected to express voltage sensitive fluorescent protein (VSFP2.42). Two months post transplantation, live grafts were mounted in an electrophysiology rig and their responses to carbachol stimulation were measured optically, providing evidence that cells both with and without nanoparticle delivered peptides were capable of producing action potentials. Fibers were seen traveling down the sciatic nerve into muscles, but connections were not investigated, although innervation of muscles by Hb9-GFP cells was previously shown after transplantation to developing chick by Wichterle et al. [116].

![Figure 4. Transplant of Hb9-GFP embryoid bodies with (A) and without (B) peptide mimetics Cintrofin and Gliafin (Figure 3 of Paper II).](image-url)
Paper III
As no stem cell used so far had projected fibers across the DRTZ, we studied the interface more closely by adopting a dorsal root avulsion model. To this end, Hb9-GFP ventral spinal cord progenitors and boundary cap neural crest stem cells were transplanted to avulsed dorsal roots in rats. These displayed different behaviors in their integration with host tissue. Hb9-GFP grafts were observed only outside the spinal cord. These were ChAT positive, and fibers labeled with this marker were found in the dorsal white matter. Numerous of these Hb9-GFP+ cells were also immunoreactive for calbindin-D28k – a calcium-binding protein expressed in various neuronal populations – as well as dendrite marker MAP2. Dense VGluT2 terminals, also immunoreactive for presynaptic proteins synaptophysin and neuroligin, were observed in the transplant area, and these may have come either from transplanted cells – indicating Hb9 interneurons [51, 126] – or from sensory axons [11, 62]. Grafted cells were also surrounded by CGRP and neurofilament 200kD (NF200, clone RT97) positive fibers, likely from regenerating sensory neurons.

Unlike the ESC derived spinal cord progenitors, boundary cap neural crest stem cells were localized both in the periphery and in the superficial dorsal horn. Interestingly, chains of bNCSCs in the periphery created band-like structures. Analysis of these showed close apposition with host Schwann cells. This is in keeping with the role of BC cells during development, where these are situated adjacent to astrocytes of the spinal cord and Schwann cells of the dorsal root [46]. The majority of bNCSCs outside the spinal cord were negative for nestin, while most observed in band formations expressed p75, a neurotrophin receptor found on Schwann cells. RT97+ fibers were observed in association with bNCSCs along the surface of the spinal cord, demonstrative of the preference of sensory axons during development and in cryocultures [46] to enter the CNS over these cells.

Paper IV
Boundary cap neural crest stem cells were further investigated in the dorsal root avulsion model. In this case transplantations were made to L3-L6 avulsions in rats, or L3-L5 avulsions in mice. Similar to the preceding study on bNCSC transplantation, those situated in the PNS had formed extensive band-like structures, and only those that had migrated into the spinal cord tissue were discernible single cells.

While nestin positive neuronal precursors and cells positive for Schwann cell marker p75 were only found on the outside, migratory neurons labeled with doublecortin (DCX) were only found within the dorsal horn. A small number expressed TrkB – a receptor for NT-3 and BDNF – though none were positive for TrkA or TrkC. Although TrkB is expressed at least at mRNA level in BC cells during development [36], these are reportedly only expressed in
postmitotic neurons, and not in migrating neural crest cells [38]. Taken together with the observation that BC cells normally contribute mostly to the TrkA population of the DRG [72], this indicates that the microenvironment has a significant influence on the fate of bNCSCs. Additionally, expression of ChAT in bNCSCs is not found in vitro [52], adding to this notion. Finally, the majority of grafted bNCSCs were immunoreactive for Sox2, a neural stem cell marker. Expression of Sox2 and c-Jun in grafted cells of the band-like structures is reminiscent of reactive Schwann cells (forming "bands of Büngner") that interact with regenerating axons [5].

**Paper V**

For clinical relevance, we sought to investigate the potential of human stem cells in a dorsal root avulsion model. As there is no access to human boundary cap neural crest stem cells, we opted for spinal cord progenitors, which were transplanted to L3-L5 dorsal root avulsions in mice. At three months, transplants expressed DCX (migrating neuronal precursors), human-specific glial fibrillary acidic protein (GFAP; astrocytes), and MAP2 (mature neurons), indicative of their commitment to the neuroglial lineage. Additionally, a small fraction expressed calbindin, and vesicular inhibitory amino acid transporter (VIAAT) was observed along GFP fibers, indicating generation of inhibitory neurons. Less than one percent were positive for tyrosine hydroxylase, and none for VGluT2. At five months, less than half as many DCX+ cells were present compared to three months, although levels of GFAP and MAP2 remained unchanged, indicating continuous maturation of neuronal cells. Even when hESCs are predifferentiated to neural stem cells, teratoma formation may still pose a risk in transplantation studies (review in [65]); however, we did not observe any such formations.

Several animals showed uptake of CTB in fibers of the injected sciatic nerve, terminating in the dorsal horns. CTB has traditionally been considered to be specifically transported by large diameter myelinated (Aβ) fibers; however, it has been observed to be transported by other populations (C and Aδ) after injury to peripheral nerves [109]. To distinguish non-myelinated fibers, IB4 was injected in conjunction with CTB, but none was transported to the dorsal horn. In corroboration, sensory fibers labeled with NF200 and CGRP were seen entering the transplant, but only NF200+ entered the dorsal horn.

In support of anatomical findings, the functional outcome of dorsal root avulsed animals also changed after transplantation of human spinal cord progenitors. Most salient was the change in grip strength. Five months post operation, sham animals (receiving only laminectomy), were able to grip with a normal tensile force. Dorsal root avulsion (DRA) animals receiving cellular grafts partially recovered this capacity, while DRA alone resulted in persistent
impairment. At this point, animals were subjected to transection of L3-L5 dorsal roots close to their ganglia, which resulted in a loss of grip strength. This phenomenon was observed also for paw withdrawal frequency.

Although it was not observed directly, it is possible that the reconnection between the PNS and CNS may have taken place not only within the dorsal horn, but somewhere in the periphery, as second order sensory neurons are capable of elongating dendrites into the periphery through a reimplanted dorsal root [16].

Paper VI

The finding that spinal cord progenitors derived from human ESCs could assist regeneration spurred us to test whether this was also true for spinal cord progenitors derived from fetal tissue. In addition, preliminary findings by Carl Trolle indicated that the mimetics of CNTF and GDNF used previously could also augment regeneration. Thus, human fetal spinal cord-derived neural stem/progenitor cells or mimetics alone, or in combination, were transplanted to L3–L5 dorsal root avulsion in nude mice.

Between grafts of cells alone, and cells with mimetics, proportions of cells expressing DCX, GFAP, MAP2, and Olig2 (oligodendrocyte precursors) did not differ significantly, indicating that the mimetics did not alter their fate in vivo. Interestingly, MAP2 was not observed in these cells when transplanted to the DRG cavity (Paper I). This suggests that the PNS milieu does not foster neuronal development, whilst access to the CNS in an avulsion setting provides cues for their differentiation. The mimetics did seem to have an effect on cell migration, however. While cells alone were found also on the contralateral side of the spinal cord, those treated with mimetics were confined to the ipsilateral side. The majority of cells found in the ipsilateral ventral horn or contralateral side were Olig2 immunoreactive. Taken together, the difference in migration, but similarity in differentiation, suggests that mimetics may not alter the fate of cells, but rather speed up their differentiation.

CTB profiles in the dorsal horn varied extensively between groups. While most prominent in the group receiving cells alone, tracing was seen in the mimetics group as well, although the latter did not reach significance. When the two treatments were combined, no tracing was detected in the dorsal horn, opposing the idea of synergistic effects of the two treatments (Figure 5). Virtually all CTB profiles in the dorsal horn colocalized with VGluT1, indicating that these were terminals of afferent axons. Both cells with and without mimetics extended a dense network of astrocytic processes into the spinal cord, possibly functioning as guides for ingrowing axons. At the site of avulsion, the presence of inhibitory proteoglycan neuro/glial antigen 2 (NG2) was most reduced in the group with cells alone. None of the human cells expressed this protein, which might otherwise "entrap" regenerating axons [39].
Figure 5. Ingrowth of sensory fibers detected by tracing with cholera toxin B subunit (CTB; white). Approximate limits of the dorsal horn are outlined. \( \text{DRA} = \text{dorsal root avulsion}; \) \( \text{hscNSPC} = \text{human fetal spinal cord-derived neural stem/progenitor cells}; \) \( \text{mim} = \text{mimetics}. \)

Altogether, this stem cell population seems to share some permissive properties of spinal cord progenitors derived from human ESCs (hNPs, Paper V), warranting further search for the mechanism at hand.
Discussion

Cell therapy for neurological diseases, including peripheral nerve injury, relies on stem cells providing trophic factors, permissive substrates, and a source of replacement cells. Here we have investigated a combination of cell types, modifications, and transplantation models to gain some insight on the practicalities of these approaches and their potential therapeutic benefit.

Survival and integration of neural stem cells
In the works presented, grafted cells of different origins survived when placed in the periphery or at the interface with the central nervous system. Some showed greater migratory behavior, including bNCSCs (Papers III&IV), which is in keeping with other studies of this cell type [127]. Access to the CNS in the DRA model seemed to greatly influence migration of human fetal spinal cord-derived neural stem/progenitor cells (hf/hscNPSCs), where they migrated throughout the cord and expressed DCX and Olig2 (Paper VI), whereas in the DRG cavity model these were rather inert (Paper I). Their migration also seemed to be lessened when exposed to mimetics of neurotrophic factors CNTF and GDNF. One possibility is that these matured faster than without mimetics, and so lost their migratory properties earlier on. GDNF also functions as a chemoattractant to both enteric neural cells [123] and motor neuron axons [33], which may explain the preference of transplanted cells to stay in the vicinity of the mimetic loaded particles. Grafts derived from embryonic stem cells pose the greatest risk of tumorigenesis, as pluripotent cells may remain in the culture. This can be addressed either preemptively – by sorting and thus purifying the culture – or by introducing a suicide construct that can be triggered after transplantation to specifically ablate undifferentiated cells. No tumor formation was observed when transplanting human embryonic stem cell derived spinal cord progenitors (Paper V), indicating proper pre-differentiation may be sufficient. Human fetal spinal cord and forebrain derived progenitors did not form any teratomas, probably due to developmental fate restriction. As regeneration was observed for both fetal and embryonic stem cell derived spinal cord progenitors, both of these appear to supply sufficient and safe enough cells to be suited to clinical application.

Differentiation of stem cells in vivo
A wide set of factors influence the survival and final fate of transplanted stem cells, including the local environment, internal cues, inflammation processes,
and more. These elements influence the process of translating findings from in vitro to in vivo, and ultimately from animal models to clinic. Cell therapy thus requires control and predictability of cell grafts. To direct cell fate we introduced both internal and external factors. Transfection allows driving or silencing gene expression, ectopic expression, and production of recombinant proteins otherwise not found endogenously. Depending on the vector, considerations need to be made with regards to the safety, efficiency, and specificity of the effect on the cell. Here we used a two-component Tet-system of plasmids to ectopically express Runx1 in human fetal forebrain and spinal cord-derived neural stem/progenitor cells. The transfection was transient, except in a few instances, serving as a reminder that even plasmids that usually exist transiently in transfected cells may in rare occurrences integrate randomly in the genome. For many purposes, transitory expression is enough to steer cells toward a certain fate. Ectopic expression of Runx1 in spinal cord and forebrain progenitors did not produce any cells of nociceptive phenotype, indicating that it is not enough to derail the cells from their inherent lineage. It did, however, induce axonal growth from spinal cord progenitors and result in expression of glutamatergic neurons. These could conceivably be used as projecting relay neurons between sensory neurons and the spinal cord, provided these cross the DRTZ. Other examples of neuronal relays have been studied experimentally, implying neuronal networks can be reshaped.

External factors such as neurotrophins do not manipulate gene expression directly, but rather activate different signaling pathways, and so do not pose the same risk of deleterious changes to the genome. On the other hand, the delivery of these factors needs to be correctly dosed, delivered locally, and over a sustained period. Examples of side effects of systemic application include weight loss induced by CNTF [75] and BDNF [82]. Here, mimetics to CNTF and GDNF were delivered via mesoporous nanoparticles, effectively enhancing motor neuron ChAT production and neurite extension, as was shown in vitro [44, 129]. The release of factors was sustained during several weeks, and no side effects were noted. This supports the addition of mesoporous nanoparticles to the list of potential biomaterials that could be used to deliver trophic factors to cellular grafts.

The differentiation profile of human fetal spinal cord-derived neural stem/progenitor cells was not affected by addition of these neurotrophic factors (Paper VI). This is likely a result of the advanced stage of differentiation of the cells present in the neurospheres, at which point these factors probably play a greater role in survival than fate determination. Although CNTF has been reported to increase astrocyte yield [8, 48], through LIF signaling, this also serves to maintain a proliferating stem/progenitor state [55], so it is unclear what role it plays in the differentiation of these cells in the in vivo context. GDNF is involved in neuronal differentiation of different populations, as well as an important factor for motor neuron survival. However, no ChAT was observed among the few neurons in the mimetics treated transplants, with several
possible explanations: differentiation of hscNSPCs at this stage is not sensitive to these factors; the concentration of factors is not adequate; or the instructive cues from the CNS overruled those of the mimetics.

Where fetal spinal cord derived cells were not modified, discrepancies in cell fate were likely due to differences in cues from the microenvironment. For instance, MAP2 was detected when untreated hscNSPCs were transplanted to a dorsal root avulsion (Paper VI), but not into a DRG cavity (Paper I). On the other hand, the proportion of GFAP+ cells was substantially lower in DRG cavity transplants, while β-III tubulin was detected in approximately 20% of cells. This suggests the PNS environment was more conducive of neuronal differentiation, but was not sufficient to mature the cells to the point of expressing MAP2, despite longer survival time.

It thus seems that a calculated balance must be made between the source of stem cell, predifferentiation, stage of transplantation, and subsequent treatment. Generally, the microenvironment should not be underestimated in its role of instructing stem cell fate, so generating specific cell types requires precise control.

Replacing lost cells

Of the cell types transplanted to a dorsal root avulsion model (Papers III–VI), different neuronal phenotypes were generated. While embryoid bodies from murine ES cells created Chat+/Hb9+ presumptive motor neurons, there were also Hb9-/Calbindin+ cells detected, indicating interneurons. Neither of these were found in the dorsal horn, however. On the other hand, individual cells from bNCSC transplants migrated into the superficial spinal cord and expressed both pan-neuronal markers MAP2, HuC/D, and DCX, in addition to calbindin, ChAT, and RET. In addition to having potential to supply the spinal cord with neurons, the presence of ChAT is relevant in the context of pain, which is modulated by cholinergic interneurons in the dorsal horn [74]. Within the peripheral compartment, bNCSCs expressed both TrkB and GFAP in addition to p75, indicating they may produce both Schwann cells and neurons of the DRG. Stem/progenitors derived from human fetal spinal cord also generated neurons within the gray matter, visualized with MAP2, and displayed migration throughout the spinal cord after dorsal root avulsion, expressing both migratory neuron marker DCX and oligodendrocyte marker Olig2 (Paper VI). This is in keeping with previous work indicating transplantation of spinal cord-derived neurospheres gives rise to neurons and oligodendrocytes in the injured spinal cord [114]. Although there are methods available to direct differentiation to oligodendroctye precursors, which successfully remyelinate after transplantation [100], these may not provide the same growth permissive environment as observed in the avulsion setting. As forebrain derived stem/progenitors have been observed to generate higher yields of neu-
rons, their application in the context of avulsion-induced cell loss [22] is also relevant. The spinal cord progenitors transplanted in Paper V created a number of neurons, but these were never found within the dorsal horn. Clinically, avulsion injuries involve both spinal roots, leading to cell loss in both the ventral and dorsal horns of the spinal cord. Here we have shown that murine ES cells can be guided toward a motor neuron fate, and that these possess several qualities of spinal neurons that might be integrated in the ventral horn [81] replacing lost cells [49] and forming functional connections with muscles preventing atrophy [121]. These findings have also been noted for human motor neuron progenitors transplanted to the injured spinal cord, which were in addition able to support endogenous cells [95]. This line of transplantation work then becomes highly relevant for developing treatment strategies for motor neuron diseases, such as amyotrophic lateral sclerosis (ALS) [47].

Assisting regeneration

Finally, the question that needs utmost attention is the efficacy of cell therapy on the investigated injury model. Do the cells provide any benefit in terms of function or other aspects of life quality? Here at least two stem cell populations have some evident capacity to assist regeneration. The observed ingrowth of sensory fibers into the avulsed dorsal horn (Papers V and VI) suggests a common mechanism for these two spinal cord progenitor populations. One aspect that may contribute to the permissiveness of the cell grafts is their production of trophic factors, which for spinal cord progenitors has been shown in vitro, even after differentiation [118]. Another is their production of extracellular matrix molecules. Human fetal spinal cord-derived neural stem/progenitor cells transplanted to the entry zone of avulsed dorsal roots did not produce NG2, for instance, a proteoglycan known to inhibit axonal regeneration [125]. Interestingly, despite providing a permissive substrate for regenerating axons, no CTB tracing in the dorsal horn was observed following transplants of boundary cap neural crest stem cells (unpublished data), which participate in establishing the DRTZ during development. This suggests that some other factor is needed to finalize the ingrowth. Following re-entry into the spinal cord, there is no guarantee of functional connections being made, or that they are made with adequate targets. The partial restoration of grip strength and sensitivity to mechanical stimulation (Paper V) implies successful reconnection with the spinal cord. Standard treatment for avulsion injuries, such as to the brachial plexus, consists of reimplanting ventral roots and trying to treat the commonly persistent neuropathic pain. Sensory regeneration, however, has no effective remedy. A number of studies have made recent progress using nerve grafts and gene therapy [53, 68], and application of neurotrophins [85, 113], and the work presented here contributes to indicating stem cell transplantation [66] as a valuable alternative. Noteworthy is the ap-
parent regeneration promoted by neurotrophic factor mimetics Cintrofin and Gliafin in the avulsion model. Though other factors have permitted re-entry of sensory axons into the CNS following dorsal root crush or rhizotomy, this particular combination of factors has not been used in an avulsion setting. The slow release of these factors from nanoparticles is a particularly attractive alternative to cell transplantation as it allows controlled, local, and safe delivery. In addition to being attractants of axons [33], GDNF has also been found to reduce gliosis [30, 54], which may explain why sensory axons were able to enter the spinal cord. Emgård et al. [34] found that hscNSPCs were neuroprotective when injected into the contused spinal cord, and Schizas et al. [98] recently showed neuroprotective effects of bNCSCs when cocultured with spinal cord slices, which provides another aspect of their potentials to assist regeneration of host tissue.
Conclusions

From the days of Aguayo and colleagues’ inquiries of the reasons for limited CNS regeneration [1, 27, 89], a long line of experiments have been undertaken to reestablish damaged neuronal circuits. Our aim was to investigate the potential of neural stem cells of different sources to reconnect the peripheral and central nervous system. The initiative for this came when human fetal sensory neurons were observed to traverse the DRTZ and synapse in the spinal cord, a phenomenon that is not seen following damage to the adult sensory system. The main findings summarized in this thesis are as follow:

- Runx1 induces axonal outgrowth from spinal cord derived neural stem/progenitors, but does not initiate neurogenesis nor switch the fate of forebrain or spinal cord derived cells to a sensory phenotype.

- Hb9-GFP cells can generate a substantial number of motor neurons, are functional after transplantation to the periphery, and their differentiation is enhanced by nanoparticle delivered peptide mimetics of CNTF and GDNF.

- Boundary cap neural crest stem cells form a permissive substrate for regenerating sensory axons in formations similar to bands of Bungner, although it is unclear whether they assist ingrowth into the spinal cord after dorsal root avulsion. A fraction is capable of migrating into the spinal cord and generating neurons.

- Human neural stem/progenitor cells derived from fetal spinal cord or embryonic stem cells can assist ingrowth of sensory fibers into the dorsal horn following dorsal root avulsion. Grafting human embryonic stem cell derived neural progenitors improves grasping function and restores nociceptive reflexes.

- Human fetal spinal cord derived neural stem/progenitors do not support ingrowth when combined with peptide mimetics of CNTF and GDNF, although these factors alone may allow regeneration of sensory axons into the spinal cord.

Thus, neural stem cells can be used to reconnect the peripheral and central nervous system by providing a permissive bridging substrate for sensory axons, and others may be used to replace lost neurons in the spinal cord and create relay neurons in the periphery.
Future

*Human sensory neurons*
Attempts to isolate neural crest stem cells from human fetal dorsal root ganglia was unsuccessful in our experience, possibly due to development stage being too late for BC isolation. There are at present a few papers indicating successful derivation of neural crest stem cells from human embryonic and induced pluripotent stem cells [67] as well as generation of peripheral sensory neurons [84]. Of interest would be to study whether these could be differentiated after transplantation to the PNS and successfully enter the CNS, providing a possible source of relay neurons and allowing for the study of the mechanisms that contribute to their ingrowth.

*Permissive ingrowth mechanism*
A continued inquiry of the molecules expressed by human embryonic or fetal stem cell derived grafts is warranted to determine how they assist regenerating axons. One might study: the expression of integrins and laminins; the production of enzymes that break down inhibitory molecules; their trophic factor secretion.

*Pain*
There are currently a small number of studies that have presented models of avulsion-induced neuropathic pain [23, 50, 91, 117] and attempts to treat these pharmacologically [21, 29]. This is a condition worth investigating in all transplantation studies, and especially relevant for avulsion studies, where this is part of the clinical picture [7, 79]. Minocycline was shown to reverse behavioral changes related to avulsion induced pain by reducing microglial activation in a rat model [21]. As preliminary data indicates that bNCSCs are neuroprotective of spinal cord neurons, and suppress microglial activation (Schizas et al. 2015), there is reason to believe engraftment of these cells may also be effective.

*Spinal root avulsion*
Also clinically relevant is injury to both ventral and dorsal roots, as avulsion injuries cause traction of whole spinal roots. This will make it possible to study the effects of spinal cord neural stem cells on the survival and regeneration of ventral horn neurons.
**Neurotrophins**

The regenerative effect of peptide mimetics Cintrofin and Gliafin need to be delineated by studying them separately and checking for a dose-response effect.

**Clinical sources of stem cells**

The limited availability, ethical concerns, and need for immunosuppression of the patient make neural stem cells difficult to apply in clinical settings. To test the regenerative capacity of induced pluripotent stem cells, or expanded adult neural stem cells, in the avulsion model is highly relevant.
Svensk sammanfattning

Nervsystemet med sin enorma komplexitet och livsviktiga funktion är vår kanske mest känsliga vävnad. Beroende på plats och utbredning kan skador på nerver ha förödande konsekvenser för patienten, som kan förlova rörelsefunktion, känsla, och utveckla smärtor och svårigheter i både kognitiva och fysiologiska processer. Nervsystemet är uppdelat i perifera och centrala delen. Medan hjärna och ryggmärg tillhör den centrala, består den perifera av alla nerv- och stödceller (neuron och glia) i resten av kroppen, och fungerar såväd som en koppling mellan vår innre och yttre värld. Skador till den perifera delen, specifikt på sensoriska neuron, har olika utfall om skadan sker nära eller långt från cellkroppen, som befinner sig i en klump med flera andra nervcellskroppar i det så kallade dorsalrotsgangliet. Sensoriska nervcellers axon (nervtrådar) kan delas i en perifer och en central gren, där den första går ut till känselorgan och den senare in genom dorsalroten till ryggmärgen. Skador på den centrala grenen präglas av låg regenerativ kapacitet, samt att centrala nervsystemet är ökänt för att vara ogästvänligt för återbildande nervtrådar. Syftet med den här avhandlingen var att utforska rollen och potentialen av neuronala stamceller att återskapa kopplingen mellan perifera och centrala nervsystemet genom att antingen skapa nya nervceller i periferin som kan projicera axon in i ryggmärgen, eller stödja befintliga, skadade nervceller i deras regenerering. Stamceller finns både i den utvecklande och vuxna organismen, och de som använts här har olika ursprung och utvecklingspotential. Både humana och musstamceller har framtagits från det allra tidigaste stadiet – embryonala stamceller – och mer utvecklade vävnaderna – neuronala stamceller från begynnande ryggmärg och hjärnorna.

Humana stam/progenitorceller från fosters ryggmärg och hjärna modifierades genetiskt för att uttrycka ett överskott av proteinet Runx1, som har kända roller i att specificera sensoriska neuron och förlänga deras axon. Efter transplantation till hålighetens som kvarstår efter att ha tagit bort dorsalrotsgangliet så projicerede de förändrade ryggmärgsstamceller långa nervtrådar mot ryggmärgen. Både ryggmärg- och hjärnstamceller påvisade mer utmognad – differerande – när Runx1 överproducerades. Däremot sågs inga axon korsa inträdeszon en i ryggmärgen.

Vi letade också efter andra sätt att påverka stamcellers utmognad utan att modifiera deras genetik, och använde oss därför av externa faktorer. Tillväxtfaktorer – eller neurotrofiska faktorer – har diverse funktioner i nervcellers utveckling och överlevnad. Men för att kunna tillämpa dessa i transplantation krävs
kontrollerad och lokal utsändning, då systemisk applicering kan ha sidoeffekter. För att öka utdifferentiering av motornervceller från embryonala musstamceller använde vi oss av nanopartiklar som var packade med syntetiska tillväxtfaktorer. Stammcellerna förändrades så att de uttryckte ett grönt fluorescerande protein i samband med den interna processen av motoneurondifferentiering. Därför kunde cellerna dels späras efter transplantation, och dels utvärderas för förekomst av motoneuron. Experimenten påvisade att substanserna var verksamma i att generera funktionella motoneuron, samt att de kunde levereras på ett kontrollerat sätt efter transplantation.

Eftersom vi fortfarande var intresserade av sensorisk regenerering flyttade vi oss närmare ryggmärgen in en modell av så kallad dorsalrotsavulsion. Det innebär att rötterna som bär sensoriska trådar ryckts loss från ryggmärgen, och simulerar det som kan ske av våldsam trauma, som t.ex. vid en bilolycka. Placerar man tillbaka rötterna vid inträdeszonen sker ingen återväxt in i ryggmärgen, och därför tillsatte vi stammceller för att undersöka dels om de själva hade kapacitet att skapa kopplingar, och dels om de kunde bistå existerande nervceller i deras återväxt. Neuronala stammceller från mus och människa med ursprung i embryonala stammceller eller fostervävnad uppvisade skillnader i migration och differentiering. Medan celler med ursprung i embryonala stammceller inte migrerade in i ryggmärgen sågs celler med ursprung i embryonal vävnad både utanför och innanför ryggmärgen, med huvudsaklig differentiering till glia respektive neuron. De två humana celltyperna påvisade dessutom en kapacitet att bistå återväxt av sensoriska nervtrådar in i ryggmärgen. Det sågs som upptag av spårsubstans i ryggmärgen, samt förbättringar av vissa motoriska och sensoriska funktioner. Applicering av tidigare beskrivna syntetiska tillväxtfaktorer hade viss effekt, men inte jämförbart med cell transplantation.

Vi har genom dessa försök fortsatt arbetet mot att sammankoppla perifera och centrala nervsystemet efter skada. Medan teknologiska framgångar gör det möjligt att styra mognadsprocessen av transplanterade stammceller så verkar ett mer realistiskt hopp ligga i stammcellers förmåga att understödja de sensoriska nervcellernas egen regenerering och återväxt av axon in i ryggmärgen.
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