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Cellular and axonal plasticity in the lesioned spinal cord of adult zebrafish

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**Abstract**

Zebrafish, in contrast to mammals, are capable of functional regeneration after complete transection of the spinal cord. In this system I asked:

(1) Which spinal cell types regenerate in the lesioned spinal cord? (2) To what extent do the dopaminergic and 5-HT systems regenerate and (3) do dopaminergic axons from the brain influence cellular regeneration in the spinal cord?

(1) Lost motor neurons are replaced by newly born motor neurons that mature and are integrated into the spinal circuitry after a spinal lesion in adult zebrafish. Using immunohistochemical and transgenic markers in combination with BrdU labeling, we showed that also 5-HT, parvalbuminergic, Pax2+ and Vsx1+ cells are newly born after lesion. Thus, my work shows that diverse cell types are newly generated in the lesioned spinal cord of adult zebrafish.

(2) After spinal cord lesion, zebrafish completely recover locomotion within six weeks. Previous work suggested that axonal regeneration is crucial for functional recovery. Here I analyzed changes in the density of 5-HT and dopaminergic axon terminals in the lesioned spinal cord during recovery. Rostral to the lesion site, I observed die-back and sprouting of dopaminergic axons within two weeks post-lesion. Caudal of the lesion, axons are lost indicating Wallerian degeneration. At six weeks post-lesion I tested functional recovery with a behavioral swim test. In recovered fish, a third of the axonal density was restored just caudal of the lesion site, but not at far caudal levels. In contrast, in fish that had non-recovered, only few axons had bridged the lesion site. Thus dopaminergic axon regrowth correlates with functional recovery. Re-transection of the spinal cord in recovered animals abolished re-gained swimming capability, suggesting that behavioral recovery critically depends on axons that crossed the spinal lesion site and not on an intraspinal circuit. 5-HT axon terminals are of both intra- and supraspinal origin. The overall time course of changes in axon terminal density during recovery is similar to that of dopaminergic axon terminals and also correlates with functional recovery. Overall, the organization of the spinal dopaminergic and 5-HT systems, consisting of neuronal somata in the spinal cord and descending axons, differs significantly from their unlesioned organization. I observe sprouting rostral to the lesion site and limited innervation of the caudal spinal cord, as axons do not regrow into the far distal spinal cord.

(3) We further hypothesized that signals released by descending axons are involved in cellular regeneration around the lesion site. Dopaminergic axons of supraspinal origin sprout rostral, but are almost completely absent caudal to the lesion site at two weeks post-lesion. Moreover, we observe that expression of the dopamine receptor drd4a is only increased rostral to the lesion site in the ventricular zone of progenitor cells, including olig2 expressing motor neuron progenitor cells. Correlated with these rostro-caudal differences, numbers of regenerating motor neurons are almost two-fold higher rostral than caudal of the lesion site. To functionally test whether dopamine is involved in motor neuron regeneration, we ablated tyrosine hydroxylase positive, mostly dopaminergic axons by injecting the toxin 6-hydroxydopamine. This treatment significantly reduced motor neuron numbers only rostral to the lesion site. As a gain-of-function experiment, we injected the dopamine agonist NPA after spinal lesion, which increased motor neuron numbers only rostral to the lesion site at two weeks post-lesion. These results suggest that dopamine released by descending axons, augments the generation of motor neurons in the lesioned spinal cord of adult zebrafish. In summary, during spinal cord regeneration I observe generation of various cell types and plastic changes of descending axonal projections. Dopamine released by descending axons is able to increase motor neuron regeneration, showing for the first time that signals from descending axons influence cellular regeneration in the spinal cord.
Statement of original contribution

The work in this thesis has been performed by the candidate, Veronika Kuscha, unless specifically stated otherwise.

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Table of Contents

GENERAL INTRODUCTION – SPINAL CORD INJURY AND THE CENTRAL
PATTERN GENERATOR................................................................................................. 9

1. PLASTICITY OF THE DOPAMINERGIC AND SEROTONERGIC SYSTEMS
IN THE REGENERATING SPINAL CORD................................................................. 16

1.1 INTRODUCTION................................................................................................................. 16
  1.1.1 Regeneration of supraspinal pathways after spinal lesion............................ 16
  1.1.2 Anatomical and functional description of the dopaminergic and
the 5-HT+ systems reveals similarities among different species ............ 19

1.2 RESULTS .......................................................................................................................... 32
  1.2.1 Spinal dopamine is exclusively derived from the brain............................ 32
  1.2.2 Lesion induces quantitative changes in TH1+ axons
in the spinal cord.............................................................................................................. 36
  1.2.3 TH1+ axon regrowth correlates with recovery of
swimming capability........................................................................................................ 46
  1.2.4 Relesion abolishes functional recovery and TH1+ axons caudal
to the lesion site.............................................................................................................. 46
  1.2.5 Spinal 5-HT+ fibers are of supra- and intraspinal origin...................... 46
  1.2.6 TH1+ or 5-HT+ varicosities located on cell somata are sparse
and appear equally distributed on different cell types ......................... 47
  1.2.7 Lesion induces quantitative changes in 5-HT axons
in the spinal cord.............................................................................................................. 51
  1.2.8 Number of 5-HT+ axons caudal to the lesion site correlates with
recovery of swimming capability.............................................................................. 55
  1.2.9 The contribution of spinal neurons to spinal innervation
increases after a lesion............................................................................................... 57
  1.2.10 Proximity of axotomy to neuronal somata might enhance axonal
regrowth into the spinal cord.................................................................................... 57
  1.2.11 Lesion induces generation of 5-HT+ cells........................................... 58
  1.2.12 Newly born 5-HT+ cells possibly originate from a p3-like zone........ 61
  1.2.13 The shh antagonist cyclopamine reduces the number
of 5-HT+ cells at 6 wpl............................................................................................ 63
  1.2.14 Numbers of newly generated 5-HT+ neurons caudal
to the lesion site correlate with recovery of swimming capability
and axon regrowth.................................................................................................... 64
  1.2.15 Verification of automated quantification method...................................... 66

1.3 DISCUSSION..................................................................................................................... 67
  1.3.1 Plasticity in the spinal cord after transection
and subsequent recovery....................................................................................... 67
  1.3.2 Correlation of axonal regeneration with behavior.................................... 69
  1.3.3 Lesion-induced generation of 5-HT+ cells.......................................... 70
  1.3.4 5-HT+ neurons in far caudal spinal cord do not react
to the spinal lesion.................................................................................................... 71
  1.3.5 TH+ or 5-HT+ varicosities in the spinal cord...................................... 71
  1.3.6 Limitation of automated quantification method...................................... 72
2. CELL TYPES IN THE SPINAL CORD OF ADULT ZEBRAFISH

2.1 INTRODUCTION

2.1.1 Relation of location and function of neurons in embryonic zebrafish spinal cord

2.1.2 Neurogenesis in the embryonic spinal cord

2.1.3 Neurogenesis in adult zebrafish after spinal lesion

2.2 RESULTS

2.2.1 Lesion-induced death and generation of motor neurons

2.2.2 Expression of pMN transcription factors is retained in adult zebrafish and is increased after a spinal lesion

2.2.3 shh is upregulated in a distinct class of proliferating ependymo-radial glial cells at the ventral midline of the spinal cord

2.2.4 Expression patterns of shh receptor patched suggests lesion-induced activity of the pathway in motor neuron progenitor cells

2.2.5 shh agonist does not increase number of newly-generated motor neurons

2.2.6 Tg(vsx1:GFP) cells are newly generated after lesion

2.2.7 Tg(vsx1:GFP) cells are distinct from HB9 or islet-1/2 motor neurons

2.2.8 Rostro-caudal difference in Tg(vsx1:GFP) cell numbers

2.2.9 Tg(vsx1:GFP) cells are different from pax2 interneurons

2.2.10 Tg(vsx1:GFP) cells emerge from a p2-like zone

2.2.11 Pax2 cells are newly generated but do not increase in number after lesion

2.2.12 Tg(Pax2:GFP) interneurons and parvalbumin-interneurons form distinct neuronal cell populations in the spinal cord of adult zebrafish

2.2.13 Dorsal parvalbumin cells are not newly generated and do not die after lesion

2.2.14 GABA cells are not newly generated at two weeks post-lesion

2.2.15 GAD67:GFP cells were identified in the ventral and dorsal horns in adult zebrafish spinal cord

2.2.16 Tg(Olig2:GFP) cells are newly born after spinal lesion

2.2.17 A large transcriptional regulatory region of growth associated protein GAP43 is required to drive GFP expression after spinal lesion

2.2.18 Various cell types upregulate Tg(GAP43:GFP) transgene

2.3 DISCUSSION

2.3.1 Time courses of various mature and undifferentiated cell markers during recovery elucidate changes mainly in ventral but not in dorsal cell populations

2.3.2 Various cell types are newly generated but not in an uniform pattern

2.3.3 What are the progenitor cells for these newly generated cells?

2.3.4 Rostro-caudal differences in cell numbers of V2 interneurons and motor neurons

2.3.5 Differences between recovered and non-recovered fish

2.3.6 Regeneration is not a mere recapitulation of development

2.3.7 Future functional characterization of cell types
3. SUPRASPINAL DESCENDING DOPAMINERGIC AXONS CONTROL MOTOR NEURON REGENERATION IN THE LESIONED SPINAL CORD OF ADULT ZEBRAFISH ............................................................... 152

3.1 INTRODUCTION ........................................................................................................ 152
3.2 RESULTS .................................................................................................................... 153
  3.2.1 TH1+ axon numbers and drd4a receptor expression are increased in the rostral lesioned spinal cord ............................................................ 155
  3.2.2 Almost twice as many motor neurons are newly-generated rostral than caudal to a spinal lesion ................................................................. 156
  3.2.3 Ablation of TH1+ axons decreases the number of newly-generated motor neurons rostral, but not caudal to a spinal lesion ....................... 158
  3.2.4 6-OHDA does not increase numbers of V1 interneurons rostral to the lesion site ..................................................................................... 162
3.3 DISCUSSION ............................................................................................................ 164
4. MATERIALS AND METHODS ............................................................................... 168
  4.1 MATERIALS ............................................................................................................. 168
    4.1.1 Primary Antibodies ............................................................................................. 168
    4.1.2 Secondary Antibodies ....................................................................................... 170
    4.1.3 Chemicals and Products .................................................................................... 171
    4.1.4 Enzymes ............................................................................................................. 172
    4.1.5 Bacterial strains and Bacterial media ................................................................. 172
    4.1.6 Buffers and Medias ............................................................................................ 173
    4.1.7 Kits ...................................................................................................................... 173
    4.1.8 Equipment .......................................................................................................... 174
    4.1.9 Plasmids for probe making ............................................................................... 174
    4.1.10 Paraformaldehyde solution ............................................................................. 175
    4.1.11 Transgenic fish lines ........................................................................................ 175
  4.2 METHODS .............................................................................................................. 175
    4.2.1 Fish maintainance ............................................................................................... 175
    4.2.2 Lesion and injections ........................................................................................... 175
    4.2.3 Tissue preparation of adult zebrafish ................................................................. 176
    4.2.4 Tissue preparation of embryonic zebrafish ...................................................... 177
    4.2.5 Immunohistochchemical labeling in vibrating blade microtome sections .......... 177
    4.2.6 Immunohistochemical stainings in whole mount embryos .............................. 178
    4.2.7 In situ hybridization on 50µm in vibrating blade microtome sections ............. 179
    4.2.8 Whole mount in situ hybridization .................................................................... 180
    4.2.9 TUNEL staining in embryos .............................................................................. 180
    4.2.10 TUNEL staining in 50 µm vibrating blade microtome sections ...................... 181
    4.2.11 Image acquisition and figure plates ................................................................. 182
    4.2.12 Fiber quantification ......................................................................................... 182
    4.2.13 Stereological counts of cells ........................................................................... 183
    4.2.14 Profile counts in spinal cord sections ............................................................... 183
    4.2.15 Statistical analysis ............................................................................................ 183
    4.2.16 Transformation of plasmid DNA and DNA isolation ..................................... 183
    4.2.17 Colony-PCR ................................................................. 184
    4.2.18 DNA extraction ............................................................................................... 184
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.19</td>
<td>Restriction enzyme digestion and agarose gel electrophoresis</td>
<td>184</td>
</tr>
<tr>
<td>4.2.20</td>
<td>DNA precipitation</td>
<td>185</td>
</tr>
<tr>
<td>4.2.21</td>
<td><em>In vitro</em> transcription</td>
<td>186</td>
</tr>
<tr>
<td>4.2.22</td>
<td>Forced swim test</td>
<td>186</td>
</tr>
</tbody>
</table>

**SUMMARY** | 188

**LIST OF ABBREVIATIONS** | 190

**LIST OF FIGURES** | 191

**BIBLIOGRAPHY** | 193
General Introduction – Spinal cord injury and the central pattern generator

The spinal cord is the main control center for movement in vertebrates. Traumatic spinal cord injuries can be caused by different events such as motor vehicle accidents or sport’s accidents (adjusted from Ho et al., 2007). Non-traumatic spinal injury can result from amyotrophic lateral sclerosis, vascular diseases, tumors, spinal stenosis or developmental malformation such as spina bifida (for review, see e.g. Ho et al., 2007; Kuzma-Kozakiewicz and Kwiecinski, 2010). The consequences are severe, as patients permanently lose motor function and sensation below the lesion site (e.g. for review, see Schwab, 2002). Long-term medical issues after spinal injury such as osteoporosis or infections are additional health risks for the patients (e.g. Chiodo et al., 2007; Fattal et al., 2011). In addition, long-term medical health care is involved with high costs, creating an socio-economic challenge for society. Spinal cord injury initiates several processes in the spinal cord. They are categorized into primary and secondary injury. Primary injury is the direct mechanical destruction of the tissue. Secondary injury comprises ischemia and electrolyte imbalance, which results in oxidative stress and excitotoxicity. These insults in turn induce necrotic and apoptotic cell death. Spinal injury also causes a general inflammatory reaction, which can lead to an extension of the injury site and further tissue destruction (e.g. for review, see Schwab, 2002; Kwon et al., 2004). However, inflammation-related responses also release factors that protect cells from cell death: thus, inflammation is a “dual-edged-sword” after spinal injury, as effects can be neuro-toxic as well as neuro-protective (e.g. Kwon et al., 2004).

Nowadays, many therapeutic inventions increase life expectancy as well as quality of life quality for patients (e.g. Carlson and Gorden, 2002; Kwon et al., 2005). However, many promising neuro-protective and neuro-regenerative treatments fail to be successful in clinical studies and a cure is still lacking (e.g. Kwon et al., 2010).
General Introduction

Why is the mammalian spinal cord not capable of overcoming spinal injury?

Axonal regrowth is highly impaired after spinal injury by glial scar formation and inhibitory myelin components (for review, see e.g. Yiu and He, 2006). Moreover, axons in the mammalian CNS have poor intrinsic capacity to regrow after injury (for review, see Sun and He, 2010a).

In addition to the lack of axonal regrowth, neurogenesis in the mammalian adult spinal cord has not been reported. Interestingly, Meletis et al. (2008) report the existence of radial glial cells with stem cell characteristics within the spinal cord. However, these stem cells can only give rise to glia and astrocytes, but not to neurons (Meletis et al., 2008). Remarkably, when these cells are exposed to a suitable environment, they become pluripotent and are successful in producing neurons (Shihabuddin et al., 2000). This suggests that these cells possess the intrinsic capability to produce neurons when provided with suitable external cues. Thus, it is worthwhile to study spinal cord regeneration in an animal model that is able to regenerate after a lesion, such as the zebrafish (for review, see Becker and Becker, 2008), because knowledge of processes and signaling involved in successful regeneration might help us to understand the lack of regeneration in the mammalian spinal cord. I have analyzed two main processes in spinal cord regeneration in zebrafish – regrowth of transected axons and neurogenesis to replace lost neurons. Previous work has shown that zebrafish, in contrast to mammals, are able to regrow axons over the lesion site and recover functionally (Kirsche, 1951; Becker et al., 1997; van Raamsdonk et al., 1998b; Becker et al., 2004b). We could also show that motor neurons are newly generated after a spinal lesion (Reimer et al., 2008). The ability of zebrafish to regenerate after a spinal lesion provides an excellent model to study factors involved in regenerative success. It is worthwhile to note that the central nervous system (CNS) of adult zebrafish is a mature, differentiated system comparable to mammals: like mammalian CNS, the CNS of zebrafish shows high degree of myelination (Wullimann et al., 1996). Also, within the uninjured spinal cord, neurogenesis does not occur.

Moreover, regeneration in zebrafish is not a mere recapitulation of development but a highly regulated and specific process: after complete spinal transection,
descending tracts originating from about 20 brain nuclei, as well as ascending tracts to the brain, and intraspinal ascending and descending axons are severed (Becker et al., 1997; Becker et al., 1998; Becker et al., 2005). If regeneration was a mere recapitulation of development, one would expect that all systems regenerate equally. However, during regeneration, only a subset of descending neurons shows regenerative capacity and regrow their axons. The ascending ones do not regenerate at all. Also other tissue in the zebrafish is able to regenerate after injury and shows features different from development: for instance, during heart regeneration in zebrafish, transcription factors different from those during heart development are activated (Raya et al., 2003). These results reveal that regeneration is not a mere recapitulation of development.

The zebrafish is an excellent model organism: its genome is fully sequenced, which facilitates knock-down of genes of interests and other manipulations. Also, the availability of various transgenic reporter lines and mutants, and the cost-effective maintenance compared to mammals make the zebrafish a useful system to study regeneration.

To gain further knowledge about successful spinal cord regeneration and subsequent recovery of locomotion, I addressed the following questions in three results chapters of my thesis:

1. To what extent do the dopaminergic and serotonergic (5-HT) systems regenerate?
2. Which spinal cell types regenerate in the lesioned spinal cord?
3. Do dopaminergic axons from the brain influence cellular regeneration in the spinal cord?

Why are these questions important to study?

Catecholamines and 5-HT are both important modulators of the central pattern generator for locomotion (CPG, for review, see e.g. Grillner, 2006). The central pattern generator is a neuronal control structure that is able to generate rhythmic burst pattern (for review, see Grillner and Jessell, 2009; Fetcho and McLean, 2010; Garcia-Campmany et al., 2010; Mulloney and Smarandache,
2010). Rhythmic burst patterns are required for walking, flying and swimming (for review, see Marder and Bucher, 2001). During locomotion, rhythmic burst patterns are important for coordinated alternation in left-right or flexor-extensor activation. CPGs also control the respiratory and the circulatory systems (Coote, 1982).

The precise performance of motor tasks requires a complex interaction of supra- and intraspinal neuronal circuits. However, the actual translation of neuronal activity into a defined muscle response is controlled within the spinal cord. The CPG is the local neuronal circuit within the spinal cord that controls the output of motor neurons and the resulting motor response. The CPG itself contains the circuitry necessary to produce simple locomotion patterns, however, descending systems are required to initiate and modulate movement (for review, see Grillner and Jessell, 2009).

Several studies in various species contributed to the knowledge that movement is generated by a central generator: in the 80s, experiments in dogfish revealed principles about locomotion (Grillner, 1975). Spinalized dogfish exhibit swimming movements caudal of the lesion site, suggesting that movement is controlled by CPG. Measurements of muscle activity showed that activity alters between the left and right body site and that the CPG is equally distributed along the segments. When more rostral segments are activated earlier than more caudal segments, forward swimming is initiated, whereas backward swimming is achieved by when more caudal segments are activated first. These data strongly suggested that swimming is generated by a central generator that is responsible for the alternating activity of the two body sites and the phase lag of activity between the consecutive body segments along the rostro-caudal axis, resulting in an undulating rostro-caudal wave of muscle activity (Grillner, 1974; Grillner et al., 1976).

One of the most detailed studied CPG is the one for swimming in lamprey. In spinal cord preparations of lamprey, activation of glutaminergic NMDA-receptors induces fictive locomotion (Sigvardt et al., 1985). The basic components of the CPG to generate a rhythmic pattern are motor neurons, inhibitory interneurons and excitatory interneurons. On each side of the animal,
all spinal neurons of the CPG are activated by excitatory glutaminergic interneurons. Axons of glycinergic inhibitory interneurons cross the midline and ensure by mutual inhibition that when one side is activated, the neurons of the contralateral side are silent (Grillner et al., 1995). The CPG can be modulated by amines, such as 5-HT. 5-hydroxytryptamine – a precursor of 5-HT – reduces the amplitude of afterhyperpolarisation in lamprey spinal motor neurons and influences NMDA-oscillations, possibly by blocking calcium dependant potassium channels (Wallen et al., 1989b). Blocking 5-HT uptake and subsequent increase of endogenous 5-HT leads to increase of intensity and duration of ventral root bursts, and decrease of locomotion frequency (Harris-Warrick and Cohen, 1985; Christenson et al., 1989).

Because of the importance of monoaminergic signals for locomotion, we hypothesized that after a spinal lesion, functional recovery is associated with successful regeneration of the dopaminergic and 5-HT systems. To test this hypothesis, I quantified anatomical changes in the dopaminergic and 5-HT systems and functional recovery to assess correlations between axon regrowth and swimming capability.

Not only axonal regrowth, but also cellular regeneration in the damaged spinal cord may contribute to functional recovery. However, little is known as to the extent of regeneration of different cell types. We have shown that zebrafish regenerate motor neurons after a spinal lesion (Reimer et al., 2008). To understand how locomotor function is recovered after a spinal lesion, knowledge about neuronal circuits within the spinal cord is required. Thus, we identified a need to elucidate the cellular anatomy in the adult zebrafish spinal cord. The results of this project might form the basis for future studies on neuronal cell types in the adult zebrafish spinal cord, their contribution to locomotion and their repair.
Which signals are important to allow neurogenesis in the injured adult spinal cord?

During development, signaling pathways and molecules involved in specification of different cell types have been studied broadly. Little is known about signaling pathways that might promote regeneration after spinal injury or in motor neuron diseases. We could show that sonic hedgehog is involved in motor neuron regeneration (Reimer et al., 2009), but which other signals might promote motor neuron regeneration? We hypothesized that supraspinal signals might influence motor neuron regeneration: a striking asymmetry between the rostral and caudal lesioned spinal cord of adult zebrafish prompted us to formulate this hypothesis:

At 2 weeks post-lesion, when motor neuron regeneration peaks, dopaminergic axons are increased in number rostral to the lesion site, but have not regenerated into the caudal spinal cord. Likewise the drd4a dopamine receptor is upregulated in spinal progenitor cells only rostral to the lesion site. Consistent with these differences, there are twice as many newly generated motor neurons rostral than caudal to the lesion site. Thus, we addressed the question of whether supraspinal dopaminergic axons might be involved in motor neuron generation. Dopamine was already shown to be involved in neurogenesis in mammalian brain (Hoglinger et al., 2004), thereby raising the possibility that it might also be involved in motor neuron regeneration in the lesioned zebrafish spinal cord.

Might findings about signal pathways involved in motor neuron regeneration be applicable to mammalian systems? Interestingly, Meletis et al. (2008) report the existence of ependymo-radial glial cells in mammalian spinal cord. These cells renew themselves in unlesioned spinal cord and can give rise to glia and astrocytes, but not to neurons in the lesioned spinal cord (Meletis et al., 2008; Barnabe-Heider et al., 2010). Remarkably, mammalian radial glia cells and ependymo-radial glial cells in zebrafish resemble each other in their morphology. Ependymo-radial glial cells in zebrafish are stem cell like progenitors for motor neurons after spinal lesion (Reimer et al., 2008). Thus,
understanding of the signals that induce motor neuron regeneration in the zebrafish arising from ependymo-radial glial cells might help to induce motor neuron regeneration pharmalogically in human lesioned spinal cord and in motor neuron diseases.

My observations show a significant degree of plasticity in dopaminergic and 5-HT innervation of the spinal cord that occurs concomitant with functional recovery. These findings emphasize the role of axon regrowth beyond the lesion site for recovery to occur. Interestingly, innervation of the caudal spinal cord close to the lesion site is reduced compared to unlesioned control animals and the far caudal spinal cord is almost completely devoid of reinnervation. Thus, during regeneration, a significant amount of plasticity occurs, showing that regeneration does not simply restore the system by re-activating developmental programs.

Moreover, my findings in cellular regeneration show that not simply all cells are equally newly generated after lesion: I observed as a general pattern that transcription factors that are expressed in the ventral portion of the developing spinal cord are upregulated in the lesioned spinal cord. In contrast, markers that are expressed in the dorsal portion of embryonic spinal cord are mainly absent during regeneration. Most ventrally located cells are preferentially newly generated after spinal lesion, whereas dorsal cell populations are less or not responsive following spinal lesion.

Is there a correlation between axonal and cellular regeneration?
We can show that the descending dopaminergic system from the brain is involved in motor neuron regeneration in the lesioned spinal cord. This knowledge might help to promote regeneration in the mammalian spinal cord in future.
1. Plasticity of the dopaminergic and serotonergic systems in the regenerating spinal cord

1.1 Introduction

After a spinal lesion, adult zebrafish are able to regrow axons over the lesion site (Becker et al., 1997). Axonal re-growth is correlated with the ability to regain swimming ability, as Becker et al. showed with two different experiments: when a piece of Teflon tape was inserted into the lesion site to prevent axons from regrowing beyond the lesion, fish failed to recover functionally; also blocking the recognition molecule L1.1 after lesion with a morpholino-based approach impaired axonal regeneration. Swimming ability was reduced in L1 morpholino treated fish six weeks after spinal transection: when free swimming was tested, the total distance moved was significantly reduced in L1 morpholino treated fish compared to control fish (e.g. Becker et al., 2004a).

Catecholamine and serotonin (5-HT) are both important modulators of the central pattern generator (see below and for review, see Grillner, 2006). We hypothesized that functional recovery is associated with successful regeneration of these systems. To test this hypothesis, we quantified anatomical changes in catecholaminergic and serotonergic (5-HT) systems.

1.1.1 Regeneration of supraspinal pathways after spinal lesion

In contrast to mammals, several adult anamniotes are able to regrow axons beyond the lesion site after a spinal transection. Due to axonal regeneration, Zottoli (1994) and Becker (2004) observed functional recovery in adult goldfish and zebrafish, respectively. Larval and adult lampreys also regenerate descending projections and regain swimming ability after a spinal cord transection (McClellan, 1990; Davis and McClellan, 1993; McClellan, 1994; Zhang et al., 2002).

In goldfish, the tegmental nucleus medial longitudinal fasciculus (nFLM) sends descending projections to the spinal cord. Swimming activity requires input of this nucleus (Kobayashi et al., 2009). After a hemisection, descending
projections of the nFLM cross the lesion site. Beyond the lesion site, these axons re-innervate ipsilateral calcitonin gene-related peptide (CGRP) positive motor neurons and CGRP negative interneurons (Takeda et al., 2007). In zebrafish, axons descend from different nuclei in the brain to the spinal cord. After lesion, these axons re-grow and form synapses with cells in the spinal cord (Becker et al., 1997; Becker et al., 1998; Becker and Becker, 2001; Becker et al., 2005).

Within tetrapods, solely urodele amphibians regenerate completely in adulthood after spinal injury (Chernoff et al., 2003). For example, studies in spinal transected salamanders revealed differences in neuronal plasticity for recovery of swimming or walking, respectively (Chevallier et al., 2004). Moreover, upregulation of the transcriptin factor Fgf2 goes along with functional recovery in salamanders (Moftah et al., 2008). Also, changes in cholinergic modulation of the motor neuron excitability differs in spinal-transected salamanders compared to unlesioned animals. This might explain the spontaneous recovery of salamanders after a spinal transection (Chevallier et al., 2006, 2008). In other tetrapods, such as *Xenopus laevis* (Gibbs and Szaro, 2006) and chicken (Hasan et al., 1993; Steeves et al., 1994; Keirstead et al., 1995), regeneration of descending axons is limited to early developmental stages and only occurs in tadpoles and embryos, respectively.

After injury in the CNS of mammals, axonal regeneration is impaired. Many publications provided evidence that inhibitory factors associated with CNS myelin and proteoglycans in the context of astrogial scar formation strongly contribute to the failure of axonal regeneration (for review, see Yiu and He, 2006). The glia-derived molecules myelin associated glycoprotein (MAG), Nogo and Oligodendrocyte myelin glycoprotein (OMgp) have been considered as key players in preventing regeneration of descending tracts in the CNS. Thus, these factors offer potential for therapeutic intervention. To investigate the lack of axonal regrowth, and to approach possible improvement, several studies broadly investigated these molecules, their receptors or downstream signaling (for review, see Hunt et al., 2002; Schwab, 2004; Giger et al., 2008; Cao et al., 2010).
However, recent publications have put into question the extent to which these factors really inhibit axonal regeneration. Studies in four different Nogo-mutant mice showed inconsistent data in the degree of corticospinal tract regeneration (Simonen et al., 2003; for review, see Woolf, 2003; Zheng et al., 2003). To reconcile the conflicting results of these studies, Lee et al. (2009) generated a mouse mutant that is deficient in all known Nogo isoforms. They did not observe any enhancement in corticospinal tract regeneration (Lee et al., 2009). Thus, they concluded that improvement of axonal regeneration would require knockout of other inhibitory molecules. Accordingly, they created mice mutant for Nogo, MAG and OMgp. In triple mutants with a complete spinal cord transection, Lee et al. observed sprouting of 5-HT+ axons of the raphespinal tract. However, the axons did not grow across the lesion site. After a dorsal hemisection, axons of the corticospinal tract neither sprouted nor regrew beyond the lesion site. Thus, the authors conclude that Nogo, MAG and OMgp are not key players in inhibiting axonal regeneration (Lee et al., 2010). A study of another group came to the opposite conclusions: Cafferty et al. (2010) provided data indicating that knock-out of all three glia derived inhibitors Nogo, MAG and OMgp leads to increased sprouting above the lesion site and improved regeneration of axons of the corticospinal as well as the raphespinal tract, compared to wildtype or single mutants of each protein, respectively. Thereby, they propose a synergistic effect of these three proteins. These opposing observations led to further discussions within the scientific community (Nature Medicine, Community corner 2010, http://www.nature.com/ezproxy.webfeat.lib.ed.ac.uk/nm/journal/v16/n8/full/nm0810-860.html, accessed on 24/12/2010). In addition to the myelin-derived inhibitors, also the glial scar is mainly considered to be an inhibitory factor. The glia scar forms after lesion and consists of astrocytes and extracellular matrix (Hirsch and Bähr, 1999; Qiu et al., 2000; Schwab et al., 2001). Spinal lesioned mice deficient in GFAP or vimentin – two major components of reactive astrocytes – showed axonal sprouting. Furthermore, rebuilding of neuronal circuits and subsequent functional recovery was observed in these mice (Menet et al., 2003). On the other hand, Renault-Mihara et al. (2008) reviewed several studies showing a beneficial
1. Plasticity of the dopaminergic and serotonergic systems

effect of astrocytes after spinal injury. Migrating reactive astrocytes seem to prevent further expansion of the lesion center and control the inflammatory response (Renault-Mihara et al., 2008).

Not only environmental factors, such as myelin derived inhibitors and the glial scar, but also intrinsic properties of the neurons account for the lack of axonal regeneration in the CNS: several studies have shown that successful axonal regeneration requires a combination of injury signals generated by the axons as well as the ability of the injured neuron to react appropriately to these signals (for review, see Sun and He, 2010b).

To overcome inhibition of axonal regeneration in mammals, a lot of effort has been taken: administration of nerve growth promoting factors, neutralization of inhibitory factors, transplantation of grafts into the lesion site, electrophysiological stimulation or bridging of the lesion site have shown partial success in improving repair (for review, see Fouad et al., 2001; Schwab, 2002; Deumens et al., 2005; Webb et al., 2010).

Altogether, these studies have revealed that our understanding of the beneficial or inhibitory factors of axonal regeneration is still limited. Therefore, it is important to study species that are able to regenerate axons and recover after lesion. This thesis presents further insights into the processes that are involved in regeneration, in order to develop medical applications in the future.

1.1.2 Anatomical and functional description of the dopaminergic and the 5-HT+ systems reveals similarities among different species

We hypothesized that 5-HT+ and catecholaminergic descending systems are restored after a spinal transection in adult zebrafish. My project aimed to quantify anatomical changes of these systems after a spinal lesion. I performed labeling for 5-HT and tyrosine hydroxylase. Tyrosine hydroxylase serves as a general marker for catecholamines, as it is the rate-limiting enzyme in the synthesis of the catecholamines dopamine, adrenaline and noradrenaline. In the following, I will review differences and similarities in the anatomy of the dopaminergic and the 5-HT+ systems between different species to show that the zebrafish is a representative model to use for anatomical studies. I will also give
1. Plasticity of the dopaminergic and serotonergic systems

an overview about the known functions of dopamine and 5-HT in order to illustrate their importance in CNS and, particularly, for movement.

Supraspinal dopaminergic projections originate in different brain regions in various animal species but are evolutionary conserved to some extent

McLean and Fetcho (2004) postulated that in zebrafish embryos, dopaminergic projections originate putatively in the nucleus posterior tubercule in the ventral diencephalon and noradrenergic projections descend from the locus coeruleus in the rombencephalon (McLean and Fetcho, 2004). A more recent study confirmed and extended these results by using genetic tools (Kastenhuber et al., 2010). They provided data that catecholaminergic projections to the spinal cord are mainly dopaminergic and that only a minor contribution is noradrenergic. Also in amphibians, the posterior tubercle nucleus and the locus coeruleus are the source of supraspinal catecholaminergic innervation of spinal cord (Sanchez-Camacho et al., 2001). These findings strongly suggest that the nucleus posterior tubercule as supraspinal catecholaminergic origin is conserved in early vertebrates (Barreiro-Iglesias et al., 2008).

In larvae of sea lamprey, descending dopaminergic projections to the spinal cord originate from the hypothalamus in the diencephalon and from the caudal rhombencephalon. In more detail, in the diencephalon, cerebrospinal fluid-contacting (CSF-c) neurons in the mammilary nucleus and the paratubercular nucleus send dopaminergic projections to the spinal cord (Barreiro-Iglesias et al., 2008). The paratubercular nucleus in lamprey was discussed to be homologous to the nucleus posterior tubercule (Abalo et al., 2005; Barreiro-Iglesias et al., 2008). Also, the paratubercular nucleus in lamprey has been suggested to be equivalent the mammalian substantia nigra in regard to cellular composition and striatal projections (Baumgarten, 1972; Pombal et al., 1997).

In rats, dopaminergic projections that descend to the spinal cord, originate in the so-called A11 area within the dorsal and posterior hypothalamus in the diencephalon (Hokfelt et al., 1979; Skagerberg and Lindvall, 1985) and substantia nigra in the mesencephalon (Bjorklund and Skagerberg, 1979; Commissiong et al., 1979). In contrast to rats, zebrafish do not possess
1. Plasticity of the dopaminergic and serotonergic systems

dopaminergic cells in the mesencephalon (Holzschuh et al., 2001; Kaslin and Panula, 2001). However, comparative studies suggest that the dopaminergic cells in the ventral diencephalon in teleosts gave rise to the mesencephalic dopaminergic cells in mammals by migration or caudal expansion (Smeets and Gonzalez, 2000). Thus, these two cell populations might be closely related in teleosts and mammals. Investigation into the genetic regulation during development of these two different cell populations elucidated supporting data for this hypothesis: the regulatory protein Ortho-9 homeodomain protein (Otp) during development is necessary for the development of A11 dopaminergic neurons in the diencephalon in mice as well as for the development of dopaminergic neurons in the posterior tuberculum of zebrafish (Ryu et al., 2007). In mice, diencephalospinal projections originate in a different area than A11 (Qu et al., 2006) and in rabbits, diencephalospinal projections originate from two other areas in the hypothalamus in addition to the A11 area (Blessing and Chalmers, 1979).

In homology to rats, non-human primate dopaminergic projections descend from the A11 area in the hypothalamus to the spinal cord (Barraud et al., 2010). Anatomical comparison elucidate the existence of human hypothalamic A11 area, however with no discussion about a putative descending projection (Kitahama et al., 1998).

In summary, dopaminergic supraspinal projections are partly conserved between different animal species. They differ in the exact area of origin within the hypothalamus. Also, in some species, dopaminergic supraspinal projections exclusively originate in the hypothalamus, whereas other species harbor additional brain areas that give rise to dopaminergic spinal projections. Nevertheless, they share a common feature, which is a dopaminergic diencephalospinal projection. Yet, many of these studies have not shown directly dopamine expressing cells or axons but use the absence of exclusively adrenaline or noradrenaline-synthesizing enzymes or their metabolic products in combination with TH-immunoreactivity as criteria to characterize a population as putatively dopaminergic. Therefore, conclusions are to be taken carefully. Taken together, zebrafish present a suitable model to study the
1. Plasticity of the dopaminergic and serotonergic systems

descending dopaminergic system in the context of spinal lesion and subsequent functional recovery.

**The functions of dopamine are versatile**

Dopamine modulates various functions within the brain, such as voluntary movement, sleep, mood, motivation and learning (Kobayashi and Sano, 2000; Schultz, 2007; Blythe et al., 2009; Beeler et al., 2010). Misregulation or lack of dopamine in the brain can cause diseases, such as Parkinson’s or Schizophrenia and is also involved in addictive behavior (Hirsch et al., 1988; Dawson et al., 2010; Lodge and Grace, 2010; Rommelfanger and Wichmann, 2010). Defects in the dopaminergic diencephalospinal projection can perturb regulation of pain (Fleetwood-Walker et al., 1988) or cause the disease restless-legs-syndrome (Clemens et al., 2006). Various studies elucidated that this projection can modulate the CPG involved in movement: in snails and leech, dopamine is able to produce fictive motor movement in isolated CNS preparations in vitro (Tsyganov and Sakharov, 2000; Matsunaga et al., 2004; Puhl and Mesce, 2008). When isolated spinal cords of rats are exposed to dopamine, a slow motor-rhythm could be measured in the ventral roots (Barrere et al., 2004). In spinal cord preparations of mouse, bath-application of dopamine increased the excitability of motorneurons. This happens by reduction of the first spike latency and the afterhyperpolarization. The authors of this study concluded “although the net effect of DA onto motorneurons is excitatory, this effect is most likely a result of a complex interaction between network, synaptic and intrinsic properties” (cited from Han et al., 2007).

Intravenous infusion of dopamine-receptor agonists to spinal cord transected cats decreased monosynaptic motor output (Carp and Anderson, 1982). “Monosynaptic output” means that a motor reflex depends on a monosynaptic transmission, thus only one sensory neuron and one motor neuron contribute to the reflex arc (Purves et al., 2001). In case of polysynaptic reflex, one or several interneurons are interposed between the motor neuron and the sensory neuron (Eltorai, 2003). Here, the monosynaptic output was decreased by increased latency or increased time to peak (Carp and Anderson, 1982). Also in mice,
administration of dopamine agonists depresses monosynaptic reflexes such as the “stretch” reflex. In parallel to the depression of this monosynaptic reflex, some polysynaptic reflexes were facilitated (Clemens and Hochman, 2004).

In lamprey, dopamine regulates the cycle rate of fictive swimming in a concentration-dependent manner: bath-application of dopamine at a concentration of 10 µM or above reduced the rhythm of glutamate-induced fictive swimming. Low concentrations of dopamine (0.1-1µM) showed an opposite effect, accelerating the swimming rhythm frequency (McPherson and Kemnitz, 1994). In zebrafish larvae, the dopamine receptor agonist clozapine blocks movement completely or causes hypoactivity in a dose-dependent manner (Boehmler et al., 2007).

Hence, the netto effect of dopamine in the spinal cord depends on the respective neuronal circuit that is modulated (Barbeau and Rossignol, 1991; Rekling et al., 2000; Lapointe et al., 2009). In summary, monoamines show a wide range of distinct modulatory activities in the spinal cord. The heterogeneity of receptors for dopamine distributed differently on neurons in the ventral spinal cord might explain the diversity of resulting netto effects of dopamine (Han et al., 2007).

The neurotransmitter 5-HT and the 5-HT+ raphe-spinal projection is evolutionarily conserved

The neurotransmitter 5-HT is expressed in different animal species and is phylogenetically conserved in the cnidarian Rennilla koellikeri. Fossil cnidarinas could be dated back to 580 millions years ago (http://en.wikipedia.org/wiki/Cnidaria, accessed on 25/04/2011). Therefore, 5-HT might be one of the most ancient neurotransmitters identified so far (Umbriaco et al., 1990).

In most mammalian brains, the 5-HT system consists of cell somata that are exclusively found in the superior and inferior raphe nuclei in the hindbrain and some areas of the reticular formation, and a complex axonal system innervating almost all other areas of the CNS. More specifically, axonal projections from the superior raphe nucleus ascend to the striatum, nucleus accumbens, globus pallidus, limbic structures (such as the amygdala and hippocampus), thalamus,
1. Plasticity of the dopaminergic and serotonergic systems

hypothalamus, and cerebral isocortex. Axons originating from the inferior raphe nucleus descend and innervate the brainstem and spinal cord (Dahlstrom and Fuxe, 1964; Lidov and Molliver, 1982). Among mammals, only monotremes harbor an additional cluster of 5-HT\textsuperscript{+} cells elsewhere than in the brain stem, namely in the periventricular organ of the hypothalamus (Manger et al., 2002).

In non-mammalian vertebrates, several additional clusters of 5-HT\textsuperscript{+} cells have been identified in the brain, such as in the caudal mesencephalic tegmentum and in the medulla oblongata of lizards (Wolters et al., 1985), in the periventricular organ of the hypothalamus and in the pretectal nucleus of the crocodile Crocodylus niloticus (Rodrigues et al., 2008) and in the central grey matter of the midbrain in close proximity of the nucleus of the trochlear nerve of the viper, Vipera apsides (Challet et al., 1991).

In early stages in zebrafish development, anterograde-labeling experiments indicated that 5-HT\textsuperscript{+} fibers from the inferior raphe nucleus project not only caudally into the spinal cord but also rostrally to various brain regions. However, in adult zebrafish, tracing experiments suggest that 5-HT\textsuperscript{+} neurons in the inferior raphe exclusively send descending projections to the spinal cord. Embryonic rostral projections of the raphe nucleus have been retracted (Lillesaar et al., 2009).

In addition to the raphe nucleus, zebrafish display several other areas in the ventrolateral hindbrain and in the diencephalon populated with 5-HT\textsuperscript{+} cell somata (Lillesaar et al., 2009).

Despite the differences in which specific brain areas possess 5-HT\textsuperscript{+} cells, animals ranging from sharks to primates (Azmitia and Gannon, 1986), share a conserved feature – that is long axonal projections descending to the spinal cord originating from the inferior raphe, revealing a remarkable evolutionary stability.

Spinal cord harbors 5-HT\textsuperscript{+} cells and fibers

McLean et al. (2004) have described the 5-HT system in the spinal cord of developing zebrafish. We expected to find a similar anatomy in the spinal cord of adult zebrafish.
1. Plasticity of the dopaminergic and serotonergic systems

In the embryonic spinal cord of zebrafish, 5-HT$^+$ fiber tracts run along the dorsal and the ventral aspect of the lateral margin. In addition to the supraspinal origin, 5-HT$^+$ fibers in the ventral horn can also arise from 5-HT$^+$ cells located within the spinal cord. These intraspinal 5-HT$^+$ cells are found ventrally and medially close to the central canal of the spinal cord and are scattered along the entire length of spinal cord. Their cell bodies are oval or round in shape. They possess unipolar processes that run ventro-laterally and then dorsally into the motor column for 1-2 muscular segments (McLean and Fetcho, 2004). These features distinguish them from the ventromedial interneurons which have unipolar branches descending for at least 9 segments (Hale et al., 2001). In the adult zebrafish, Van Raamsdonk et al. (1996) have identified 5-HT$^+$ cells in the ventromedial part of the spinal cord. In adult goldfish, two different types of 5-HT$^+$ cells were described within the spinal cord: 5-HT$^+$ neurons with ovoid to spindle-shaped cell somata and several fine branches, and round or oval mast cells devoid of any processes (Takeda et al., 2008). Also in adult chick and turtle, 5-HT$^+$ cells were found in the ventral part of the spinal cord (Okado et al., 1992). Lamprey posses intraspinal 5-HT$^+$ neurons along the midline and ventral to central canal (Harris-Warrick et al., 1985).

5-HT$^+$ axons in zebrafish share a common feature with those in mammals and other vertebrates: spinal 5-HT$^+$ axons originate both from intraspinal neurons and supraspinal neurons in the raphe nucleus. Therefore, we consider zebrafish as a suitable model for studying regeneration of the 5-HT system.

In my project, I have described the dynamics of the numbers of 5-HT$^+$ cells and additionally the axonal density of 5-HT$^+$ fibers during recovery.

Functions of 5-HT in the CNS are versatile

The role of 5-HT in the CNS is manifold. 5-HT is important for behavior such as aggression and anxiety, and physiological processes such as perception, sexual behavior, sleep-wake-cycle, appetite, and respiratory and vascular function (Jacobs and Azmitia, 1992; Okado et al., 1992). Moreover, 5-HT plays a crucial role during development and affects plasticity in the CNS (Jacobs and Azmitia, 1992; Daubert and Condron, 2010; Reisol et al., 2010). For instance, descending
1. Plasticity of the dopaminergic and serotonergic systems

5-HT\textsuperscript{+} projections influence the expression of glycine receptors on motor neurons during development in rat spinal cord (Sadlaoud et al., 2010). 5-HT is involved in proliferation, regulation of neurite growth (Fricker et al., 2005), synapse formation (Jones et al., 2009; Ferreira et al., 2010) and differentiation (Gaspar et al., 2003; Allain et al., 2010). For example, in the adult rat brain, 5-HT is able to regulate the sonic hedgehog (shh) signaling pathway which is involved in proliferation (Rajendran et al., 2009).

Impaired regulation of 5-HT transmission can result in diseases, such as depression, schizophrenia and eating disorders (Kaye, 2008; Hayes and Greenshaw, 2011).

My project focuses on the anatomical plasticity of the raphespinal and the intraspinal 5-HT system in the context of spinal lesion and subsequent functional recovery.

5-HT modulates locomotor network neurons

Severe spinal cord injuries result in paralysis caused by the destruction of several different axonal tracts. Not only the transection of axonal tracts involved in directing and coordinating movement (such as the corticospinal and the reticulospinal tract) but also the destruction of the raphespinal tract that modulates neurons of the locomotor network contributes to loss of motor function (Carlsson et al., 1963; Rekling et al., 2000; Perrier and Delgado-Lezama, 2005; Jordan et al., 2008). For example, selective lesioning of the dorsal funiculus in cat inverses an inhibitory modulation by afferents onto motorneurons present before lesion, into an activating modulation after lesioning. Administration of a 5-HT receptor agonist restores the lesion-induced effect (Aggelopoulos et al., 1996).

The excitability of spinal motor neurons is influenced by two main mechanisms, an ionotopic and a modulatory one: the ionotopic mechanism is initiated by binding of excitatory or inhibitory neurotransmitters to an ionotopic receptor. The ionotopic receptor is responsible for a direct gating of the ion channel. The binding of the neurotransmitter induces a fast change of conformation of the
1. Plasticity of the dopaminergic and serotonergic systems

receptor so that the ion channel opens within milliseconds. Thus, ion in- and outflow within the cell is altered. This in turn results in depolarization or hyperpolarization of the neuron, thus in exciting or inhibiting the neuron to fire an action potential (Kandel et al., 2000 for review, see; Powers and Binder, 2001). In contrast, the modulatory mechanism relies on the binding of neurotransmitters to G-protein coupled receptors that activate intracellular signaling cascades and can recruit second messengers. Thus, different channels such as voltage-gated channels, resting channels and ligand-gated channels can be influenced. Therefore, the electrophysiological properties of cells can be changed, such as the resting potential, the action potential duration or repetitive firing patterns. In contrast to the ionotropic mechanism, these changes happen slowly in a range of seconds or minutes. The exact modification depends on the particular receptor subtypes expressed by the neuron (Kandel et al., 2000, for review see; Heckman et al., 2009).

A basic feature in excitability of motor neuron is the so-called bistability of motor neurons. This means that a brief depolarising pulse can initiate prolonged, self-sustained bursting activity of a motor neurons which eventually can be terminated by a short hyperpolarising pulse (Schwindt and Crill, 1980a; Schwindt and Crill, 1980b; Hounsgaard et al., 1984; Crone et al., 1988).

In decerebrated cats, motor neurons loose this property. Interestingly, administration of a 5-HT precursor can reinitiate this motor neuron property, which for example is important for a functional tonic stretch reflex. It was suggested that plateau potential – which is a is a stable membrane potential more depolarized than the resting membrane potential – play a crucial role in depolarisation of the motor neurons and that 5-HT recruits this plateau potentials by influencing Ca2+ -current influx (Hounsgaard et al., 1988). Having a plateau potential is a feature of motor neurones which is evolutionary conserved among various vertebrates, suggesting a crucial role for motor function (Perrier and Hounsgaard, 2000; Alaburda et al., 2002).

More recent studies confirm and explore the role of 5-HT for plateau potentials. For instance, electric stimulation of the raphe nucleus in spinal cord-brain preparations of turtle provokes the release of 5-HT. In consequence, a plateau
1. Plasticity of the dopaminergic and serotonergic systems

potential in spinal motor neurons is facilitated and the excitability of the motor neurons is increased (Perrier and Delgado-Lezama, 2005).

In addition to facilitating a plateau potential, 5-HT has been shown to reduce afterhyperpolarisation. In neotatal cats, administration of 5-HT results in a decrease of hyperpolarisation on motorneurons. This effect is mediated by metabotropic receptors and results in a decrease of Ca$^{2+}$ influx and increase excitibility of the motor neurons (Bayliss et al., 1995). In lamprey, the reduction of the afterhyperpolarisation in interneurons mediated by 5-HT results in an increase of burst intensity and duration which in sum causes a decrease of the swimming rate (Wallen et al., 1989a).

Endogenous serotonin decreases the frequency of NMDA induced locomotor rhythms in fictive swimming of adult zebrafish spinal cord preparations by increasing mid-cycle inhibitory postsynaptic potentials and delaying the subsequent onset of hyperpolarization (Gabriel et al., 2009). This is in contrast to embryonic zebrafish, where 5-HT does not influence the frequency of the swimming itself but decreases the quiescent period between periods of swimming (Brustein et al., 2003). In lamprey and tadpole, locomotor frequency is reduced by the activation of 5-HT receptors (Grillner and Jessell, 2009). Moreover, serotonin has been shown to directly modulate firing patterns of rat motor neurons (Kjaerulff and Kiehn, 2001).

Inhibition of 5-HT synthesis with p-chlorophenylalanine (PCPA) in neonatal mice resulted in asymmetric posture indicating a lack of interlimb coordination. Excitability of motor neurons was decreased. Also, extension and flexion at knee, angle and hip were changed (Pflieger et al., 2002). Depletion of 5-HT with the neurotoxin 5,7-dihydroxytryptamine after intercollicular decerebration in adult rats weakened muscle tension (Sakai et al., 2000). In neonatal rat spinal cord preparation, 5-HT induced fictive locomotion (Cazalets et al., 1992). To treat depression in human, administration of serotonin-uptake inhibitors can cause spontaneous involuntary muscle twitching or impair muscle coordination (Lane and Baldwin, 1997).

In summary, 5-HT modulates motor networks by influencing various parameters, such as facilitation of plateau potentials and modulation of
afterhyperpolarization as well as NMDA receptor channels (Bayliss et al., 1995; for review, see Sillar et al., 1998; Perrier and Hounsgaard, 2000; Schmidt and Jordan, 2000; Alaburda et al., 2002).

5-HT plays an important role in regeneration

Different studies have stressed the importance of 5-HT for regaining locomotor ability after a lesion (Murray et al., 2010). In one study, several months after complete transection of rat spinal cord at S2 sacral level, the mRNA expression of constitutively active 5-HT receptors was upregulated within the spinal cord. These receptors do not necessarily require the binding of ligand to initiate signal pathways. They spontaneously change their conformation, thereby activating coupled G-proteins and downstream signaling. In this study, constitutively 5-HT receptors were capable of initiating downstream signaling pathways even when the raphespinal tract was destroyed and 5-HT innervation caudal to the lesion site was not yet restored. To study whether the upregulation of constitutively active receptors would be beneficial for locomotor recovery, a selective lesion was performed. This lesion destroyed all descending 5-HT fibres but left enough axons of the propriospinal tract intact. Therefore, rats were still able to initiate voluntary movement of their hindlimbs. Within three weeks post-injury, they showed recovery in hindlimb locomotion and were able to walk with almost normal weight support. When the constitutively active 5-HT receptors were blocked with a specific antagonist, recovery of hindlimb body support was impaired, therefore showing the importance of these receptors for recovery. However, the upregulation of the constitutively 5-HT receptors also lead to detrimental muscle spasm (Murray et al., 2010).

In another study, one week after complete spinal transection in rats, exogenous 5-HT was administered to compensate for the loss of intraspinal 5-HT input. Locomotor ability was assessed with various behavioral tests, such as measuring the hindlimb body weight support (BWS) on full weight-bearing bipedal treadmill. One week after injury, combined treatment of electrical stimulation (ES) and administration of 5-HT agonists, resulted in a BWS of 50%
compared to unlesioned controls. Injured animals solely treated with ES reached a BWS of just 10%. Additional training starting at one-week post injury, in combination with previous ES stimulation and 5-HT agonists, further improved the locomotor ability. After full combination of pharmalogical and ES intervention and training, rats managed to perform very similar to unlesioned controls and reached almost 100% of BWS (Courtine et al., 2009).

Using another approach, transplantation of 5-HT⁺ embryonic neurons of the raphe nucleus into chronically lesioned rats significantly improved motor recovery and axon regrowth over the lesion site. Administration of a 5-HT reuptake inhibitor enhanced these effects (Feraboli-Lohnherr et al., 1997; Ribotta et al., 2000). The term chronic lesion refers not to the acute phase after spinal injury but to weeks and months after injury. By then, glial and extracellular scar formation has permanently changed the tissue and thereby the environment of the axons. On the other hand, grafts containing no source of 5-HT at all also had a positive effect on regeneration (Privat et al., 1989; Moreno-Flores et al., 2006), questioning whether the grafts containing 5-HT⁺ cells were beneficial not due to the 5-HT but rather to the positive effects of the transplanted tissue.

Other studies question the beneficial role of 5-HT for regeneration: after a thoracic spinal cord (T11) compression-induced injury, Hashimoto and Fukuda (1991) selectively destroyed the 5-HT⁺ cells completely with 5,7–dihydroxytryptophane. In a parallel group of injured animals, they blocked the enzyme tryptophane hydroxylase. This enzyme is required for the synthesis of 5-HT. The blocking of the enzyme left the 5-HT⁺ cells intact. They observed a difference in behavioural read-out between these two groups. The group with the completely destroyed 5-HT⁺ cells showed less recovery from motor and sensory deficits within 14 days after lesion compared to injured but untreated animals. In contrast, the group in which only the 5-HT synthesizing enzyme was blocked performed as well as the injured untreated control group. Therefore, the authors hypothesized that, 5-HT⁺ cells might have other functions in addition to 5-HT transmission. These additional functions might be beneficial for recovery rather than 5-HT transmission. The exact nature of these
substances or the exact functions of these cells yet remains to be elucidated (Hashimoto and Fukuda, 1991).

Even though there is a controversy about the function of 5-HT for the regeneration process itself, the studies discussed above strongly suggest that 5-HT is important for a fully functional motor circuit. All these studies indicate that 5-HT is crucial for regaining motor function after spinal lesion, however the whole process of overcoming inhibition of regeneration and initiating functional recovery is too complex to be explained by one single factor.

Regeneration of the 5-HT and the dopaminergic systems after spinal lesion

In addition to the line of evidence indicating a role in regaining locomotor function after spinal injury, other studies point out that the 5-HT and the dopaminergic systems are able to recover after lesion under certain circumstances.

Recent work has shown that in zebrafish, axotomized neurons in the inferior raphe and the periventricular nucleus of the posterior tuberculum, were able to grow axons beyond the lesion site into the distal spinal cord (Becker et al., 1997). Also, van Raamsdonk et. al (1998) showed reinnervation of the distal spinal cord with 5-HT immunoreactive and TH immunoreactive fibers after lesion (van Raamsdonk et al., 1998b). As mentioned earlier (see citations above), the inferior raphe gives rise to descending 5-HT+ axons projecting to the spinal cord. In rats with partial thoracic spinal cord injury, Nogo-A antibody treatment improved axon regrowth of the raphe-spinal tract. The density of 5-HT+ varicosities on spinal motor neurons was increased (Mullner et al., 2008). When olfactory ensheathing cells were transplanted into the transected rat spinal cord, overall 5-HT+ axonal density was not changed caudal to the lesion site. Interestingly, the number of 5-HT+ varicosities in close proximity to somatic motor neurons and motor associated cells close to the central canal was increased. This might be partially the reason for previously observed improvement in treadmill stepping after a lesion (Kubasak et al., 2008; Takeoka et al., 2009). Interestingly, in chick spinal cord motor neurons are also not
1. Plasticity of the dopaminergic and serotonergic systems

equally innervated by 5-HT fibers but certain subpopulation show a higher density of innervating 5-fibres (Okado et al., 1988). In mice, grafts of stem cells transplanted caudal to the transection site relieved the inhibitory effect of the glial scar and resulted in growth of 5-HT+ axons across the lesion site with concomitant improvement of locomotor ability (Boido et al., 2009). In goldfish, neurogenesis occurs following hemisection and the number of 5-HT+ neurons was increased at three weeks post-lesion (Takeda et al., 2008). However, axon numbers and a correlation with behavioral recovery was not studied.

Taken together, these studies prompted us to hypothesize that in zebrafish, 5-HT+ and dopaminergic axons regenerate after a spinal lesion and 5-HT+ neurons in the spinal cord are newly generated. In addition, 5-HT plays a crucial role in locomotion. Thus, it is worthwhile to study the regeneration of the entire spinal 5-HT and dopaminergic system in zebrafish in a quantitative way in order to reveal adaptive or non-adaptive plasticity of the system.

1.2 Results

1.2.1 Spinal dopamine is exclusively derived from the brain

To localize a dopamine signal in the adult spinal cord, we analyzed expression of tyrosine hydroxylase 1 (TH1) and tyrosine hydroxylase 2 (TH2), the rate limiting enzymes for dopamine synthesis. Immunohistochemistry with a TH1 specific antibody (Chen et al., 2009) showed a network of TH1+ terminals in the spinal cord at a midthoracic level. No TH1+ somata were found in the spinal cord. Double immunohistochemistry with a dopamine antibody revealed that in unlesioned control fish, more than 80% and in recovered fish at 6 wpl caudal to the lesion site, more than 70% of the TH1+ axons were dopaminergic, indicating that TH1+ axons in the spinal cord are mainly dopaminergic (Fig. 1.1A,B,C). The remaining TH1+ axons could be adrenergic or noradrenergic. 

*In situ* hybridization for TH2 indicated absence of TH2 expressing cells in the spinal cord. Positively labeled cell bodies were found in the posterior periventricular preoptic nucleus, (Fig. 1.2) which projects to the spinal cord
(Becker et al., 1997). In agreement with a minor contribution of TH2+ axons to the dopaminergic innervation of the spinal cord, in unlesioned control fish 10.4 ± 3.19% (n = 2 animals) dopaminergic fibers were TH1-. In recovered fish at 6 wpl, 2.3% ± 1.51% (n = 1) of spinal dopaminergic fibers were TH1-.

To localize the source of TH1+ axons in the brain, we retrogradely traced the descending projection from a midthoracic level in combination with TH1 immunohistochemistry (Fig. 1.1D). This showed double-labeled cells in the periventricular nucleus of the posterior tuberculum in the diencephalon, similar to what has been reported for the embryonic spinal cord (McLean and Fetcho, 2004; Kastenhuber et al., 2010). Overall, this suggests that the spinal cord contains dopaminergic axons that originate from TH1+ neurons in the brain. No spinal intrinsic source of dopamine was detected.
1. Plasticity of the dopaminergic and serotonergic systems

Fig. 1.1: TH1+ axons in the spinal cord are mostly dopaminergic and originate in the brain. Double-labeling of TH1 and dopamine in spinal cross sections at a mid-thoracic level in unlesioned fish (A) and in recovered fish at 6 wpl (B) reveals overlap (arrows). Levels of sectioning are depicted in Fig. 1.3. An axon labeled only by the TH1 antibody (arrowhead), the central canal (asterisk) and the Mauthner axons (M-ax) are indicated. C: Quantification of TH1+ axons double-labeled with dopamine antibody. D: In horizontal cross-sections of the brain, a cell (arrow) in the periventricular nucleus of the posterior tuberculum is double-labeled by TH1 immunohistochemistry and retrograde tracing from a mid-thoracic level of the spinal cord with RDA. Scale bars: A = 20 µm; B = 5 µm and D = 20 µm.

(Fig 1.1D was generously provided by Dr. Thomas Becker.)
Fig. 1.2: TH2+ neurons are found in the brain, but not the spinal cord. 

A: In horizontal brains sections, strongly labeled cells (arrowheads) are found in the posterior periventricular preoptic nucleus (PvPp) by in situ hybridization. 

B: No TH2+ cells were detected in spinal cross sections (asterisk = central canal). 

C: To illustrate the localization of the PvPp within the brain, a brain of a one-month old zebrafish is shown. Line D indicates the level of transection for the PvPp. 

D: Transverse sections showing th2 expression in the diencephalon within the PvPp indicated by the vertical line D in image C. 

D': High magnification of the PvPp. Abbreviations: habenula (ha), optic tectum (TeO), optic tract (OT), the posterior periventricular preoptic nucleus (PvPp)(Filippi et al., 2010)

Scale bars: A = 15 µm, B = 20 µm, C and D = 100 µm.

(This figure was generously provided by Dr. Antón Barreiro-Iglesias.)
1. Plasticity of the dopaminergic and serotonergic systems

1.2.2 Lesion induces quantitative changes in TH1⁺ axons in the spinal cord

I examined the time course of changes in TH1⁺ axon numbers rostral (within 750 µm), caudal (within 750 µm) and far caudal (3500 µm) to the position of a midthoracic lesion site (Fig. 1.3A) at 1, 2, 6 and 13 wpl. I chose these time points because Wallerian degeneration in the caudal spinal cord is advanced at 1 wpl and axon regrowth beyond the lesion site starts at around 2 wpl. Functional recovery plateaus at 6 wpl (Becker et al., 2004a). I chose the 13 wpl time point to detect any late changes in innervation. For the late time points (6 wpl and 13 wpl), when recovery can be assessed, only animals that showed recovery in a forced swim test (see Material and Methods) were included in the anatomical analysis.

To determine whether axotomized neurons retract or sprout after lesion, I quantified numbers of axonal protrusions rostral to the lesion site.

Rostral to the lesion site, the number of TH1⁺ axonal profiles showed a tendency towards decrease at 1 wpl compared to unlesioned control fish (Fig. 1.3D). This decrease might indicate lesion-induced die-back of axons. However, when I analyzed the difference with ANOVA followed by the post-test Dunnett’s multiple comparison test, the decrease was not statistically significant (unlesioned: 673.2 ± 52.70 profiles/section, n = 7; 1 wpl: 391.4 ± 82.43 profiles/section, n = 5, n.s.). When I analyzed only numbers of axonal profiles at one week post-lesion compared to unlesioned fish using Mann-Whitney-U, the decrease was significant (p = 0.0101).

Thereafter, I observed a continuous increase (Fig. 1.3B,C,H): at 6 wpl, the number of TH1⁺ axonal profiles has reached 180% of the number of unlesioned control fish (ANOVA, p = 0.0002, Dunnett’s multiple comparison test, unlesioned: 673.2 ± 52.70 profiles/section, n = 7; 6 wpl: 1204.6 ± 145.36 profiles/section, n = 6 profiles/section; n = 6; p < 0.05). At 13 wpl, the last time point analyzed, number of TH1⁺ axonal profiles have reached 230% of numbers in unlesioned fish (ANOVA, p = 0.0002, Dunnett’s multiple comparison test, unlesioned: 673.2 ± 52.70 profiles/section, n = 7; 13 wpl: 1571.9 ± 368.55 profiles/section, n = 4, p < 0.01).
This time course is consistent with initial die-back and later sprouting of axons or increased TH1 expression in axons not previously detectable by immunohistochemistry.

To determine the extent of lesioned TH1+ axons that are able to cross the lesion site and innervate the caudal part of the spinal cord, I analyzed the number of axonal profiles caudal to the lesion site (Fig. 1.3D,I). At 1 wpl, hardly any axonal profiles were left caudal to the lesion site, indicating Wallerian degeneration (ANOVA, p < 0.0001, post test Tukey’s multiple comparison test, unlesioned: 673.2 ± 52.70 profiles/section, n = 7; 1 wpl: 0.9 ± 0.61 profiles/section, n = 5, p < 0.001). At 6 wpl, numbers of axonal profiles had reached 44% of the numbers in unlesioned fish, but still differed significantly from unlesioned fish (ANOVA, p<0.0001, post test Tukey’s multiple comparison test, unlesioned: 673.2 ± 52.70 profiles/section, n = 7; 2 wpl: 24.8 ± 10.32 profiles/section, n = 6, p < 0.001; 6 wpl: 296.3 ± 80.57 profiles/section, n = 6, p < 0.01). Also, there was a significant increase in the number of axonal protrusions from 1 wpl to 6 wpl (ANOVA, p < 0.0001, post test Tukey’s multiple comparison test, 1 wpl: 0.9 ± 0.61 profiles/section, n = 5; 6 wpl: 296.3 ± 80.57 profiles/section, n = 6, P < 0.05).

At 13 wpl, the number of TH1+ axonal profiles was half in comparison to 6 wpl, however this reduction was not significant (6 wpl: 296.3 ± 80.57 profiles/section; 13 wpl: 149.3 ± 124.34 profiles/section). In contrast to 6 wpl, the number of TH1 axonal profiles was not any more significantly increased compared to 1 wpl.

These results indicate that innervation of the caudal spinal cord was partly restored and that regeneration peaks at 6 wpl. About half of the axons that had crossed the lesion site at 6 wpl were lost at 13 wpl. As this decrease was not significant, it could mean that numbers of TH1+ axonal profiles plateau at 6 wpl. Another possibility is that only some of the protrusions connect to the neural network caudal of the lesion site and remaining processes degenerate. It might be that only a few axons are required caudally to release dopamine and are capable of modulating the propagating rostro-caudal wave of excitation in the spinal network during swimming (Grillner and Wallen, 2007) without the need to do this at every level of the spinal cord.
To investigate how far TH1+ axons would grow into the spinal cord after crossing the lesion site, I analyzed numbers of axonal profiles far caudal to the lesion site, at the level of the dorsal fin, corresponding to the 13th vertebra, ~ 3.5 mm caudal to the lesion site (Fig. 1.3E,F,K). At 6 wpl, when I first observed axons bridging the lesion, hardly any axons were detectable at the far caudal level (ANOVA, $p < 0.0001$, post test Tukey’s multiple comparison test, unlesioned: $484.6 \pm 86.01$, $n = 4$; 6 wpl: $3.3 \pm 3.33$ profiles/section, $n = 6$, $p < 0.001$). Even 8 weeks later, at 13 wpl, hardly any axons (13 wpl: $6.5 \pm 2.77$ profiles/section $n = 4$, $p < 0.001$) had grown into the far caudal spinal cord.

In summary, I observed sprouting rostral to the lesion site, partial reinnervation of the caudal spinal cord and hardly any reinnervation of the far caudal spinal cord.
1. Plasticity of the dopaminergic and serotonergic systems
1. Plasticity of the dopaminergic and serotonergic systems
Fig. 1.3: Spinal lesion induces plasticity of TH1+ axons. Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. Levels of sectioning are depicted in A. A schematic of a cross section is depicted in G, B: TH1+ axons, but no cells are labeled in the unlesioned spinal cord. This section serves as control for C and D. C,H: Numbers of TH1+ profiles increase continuously rostral to the lesion site for at least 13 weeks post-lesion. D,I: Caudal to the lesion site all axons are lost and axon regrowth is observed at 6 weeks post-lesion. E,F,K: Axon regrowth to a level 3.5 mm caudal to the lesion site is rarely (arrowhead) observed. *P < 0.05, **P < 0.01, ***P < 0.001; Scale bars = 25 µm.
1.2.3 **TH1⁺ axon regrowth correlates with recovery of swimming capability**

As previously reported, regenerative success in spinal lesioned fish is variable and anatomical regeneration of axons beyond the lesion site correlates with regenerative success (Becker et al., 1997; Becker et al., 2004a). I wanted to determine whether plastic changes of TH1⁺ axons also correlate with recovery of function. To assess recovery of swimming capability we used a previously established test of forced swimming against a water flow (Fig. 1.4A) (Reimer et al., 2009). In a set of 37 fish, I found that 17 fish were unable to hold their position in the water flow for more than 30 seconds. These fish were classified as non-recovered. Another 17 fish held their position for an hour, at which time the test was terminated. These fish were classified as recovered. Three individuals that showed intermediate swim times (9, 13 and 50 minutes) were also classified as recovered (Fig. 1.4B).

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**Fig. 1.4:** Recovery of swimming capability is variable and is abolished by re-transsection of the spinal cord. **A:** A schematic representation of the flow-through tank used to test swim performance is shown. **B:** Fish that were unable to hold their position against a constant water flow for more than 30 seconds were classified as non-recovered. **C:** Recovered fish lose their regained swimming capability after re-lesion.
I hypothesized a correlation between good performance in the swim test and axonal regeneration.

I did not observe a significant difference between numbers of axonal protrusion comparing recovered and non-recovered fish rostral to the lesion site (recovered: 1204.6 ± 145.36 profiles/section, n = 6; non-recovered: 1011.4 ± 115.82 profiles/section, n = 6, p > 0.05). Thus, sprouting does not seem to differ between recovered and non-recovered fish and thus does not account for differences in swimming capability (Fig. 1.5A,B).

Therefore, I investigated whether differences in the number of TH1+ axonal profiles caudal to the lesion site would correlate with differences in swimming capability (Fig. 1.5C,D). I observed significantly higher numbers of axonal profiles in recovered than in non-recovered fish caudal to the lesion site (recovered: 296.3 ± 80.57 profiles/section, n = 6; non-recovered: 39.7 ± 32.31 profiles/section, n = 6, p < 0.05, Mann-Whitney-U). This 7.5 fold difference for TH1+ axons caudal to the lesion site is consistent with the importance of axon regrowth beyond the lesion site, found in other studies (e.g. Becker et al., 2004a).

Calculation of correlation of TH+ number of axonal profiles and endurance in swim test reveals a tendency that both variables increase with each other. (Spearman r, 0.5371, p = 0.0750, 12 pairs). An r-value between 0 and 1 means that the two variables tend to increase together. However, the high p-value does not allow to draw the conclusion that this correlation is real. Larger n-numbers are required to fully answer this question.
Fig. 1.5: Recovery of swimming capability correlates with regrowth of TH1\(^+\) axons beyond the lesion site. Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. Levels of sectioning are depicted in Fig. 1.3. 

**A,B:** Rostral to the lesion site numbers of TH1\(^+\) profiles did not differ between recovered and non-recovered fish. 

**C,D:** Significantly fewer TH1\(^+\) axons regenerated beyond the spinal transection site in non-recovered fish than in those showing recovery of swimming capability (*P < 0.05). 

**E:** Schematic overview of a spinal cross section. 

Scale bar = 25 µm.
1. Plasticity of the dopaminergic and serotonergic systems

1.2.4 Relesion abolishes functional recovery and TH1\(^+\) axons caudal to the lesion site

To determine whether recovery and TH1\(^+\) innervation of the caudal spinal cord depends on continuity of the spinal cord I relesioned the spinal cord in the same position in three recovered animals that held their position in the water flow for 60 minutes. All three animals completely lost their swimming capability (Fig. 1.4C) and TH1\(^+\) axons were almost completely absent caudal to the lesion site (0.4 ± 0.43 profiles/section, \(n = 3\)) 1 week after relesion. Thus functional recovery is unlikely to be due to plasticity of spinal circuitry caudal to the lesion site alone and TH1\(^+\) reinnervation of the caudal spinal cord after a lesion is most likely derived from descending axons.

1.2.5 Spinal 5-HT\(^+\) fibers are of supra- and intraspinal origin

5-HT\(^+\) axons were detectable both in the ventral and the dorsal horn. Monopolar oval shaped 5-HT\(^+\) cells with a diameter between 7 and 8 µm were localized exclusively ventrolateral of the central canal. Their axons might contribute to axon numbers in the spinal cord (Fig. 1.6A).

Using retrograde tracing from the midthoracic level in combination with 5-HT immunohistochemistry we have found 1 double-labeled cell in the raphe region of the brainstem in 1 out of 5 animals (Fig. 1.6B), confirming previous reports (Lillesaar et al., 2009). Thus 5-HT\(^+\) axons in the spinal cord are of supraspinal origin and are derived from local 5-HT\(^+\) cells.
1. Plasticity of the dopaminergic and serotonergic systems

Fig. 1.6: Spinal 5-HT⁺ axons originate in the spinal cord and brainstem. **A**: 5-HT immunohistochemistry on spinal cross sections (dorsal is up) reveals labeled somata (arrowheads) in the ventral spinal cord. The central canal is indicated by dots. **B**: A horizontal section through the brainstem is shown; rostral is right. Retrograde tracing from the spinal cord reveals 5-HT immunoreactive somata of neurons in the inferior raphe that project to the spinal cord. A double-labeled cell (arrowhead) is shown in higher magnification on the right. Scale bars: **A** = 15 µm; **B** = 20 µm and 5 µm for high magnification. (Figure 1.6**B** was generated in collaboration with Dr. Antón Barreiro-Iglesias.)

1.2.6 TH1⁺ or 5-HT⁺ varicosities located on cell somata are sparse and appear equally distributed on different cell types

I analyzed possible differences in innervation patterns of ChAT⁺ motor neurons, 5-HT⁺ neurons and parvalbumin⁺ neurons by TH1⁺ or 5-HT⁺ fibers. Differences in innervation pattern would enable further investigations in regard to re-innervation after lesion. Axons might either re-innervate their original target cells in a guided manner or re-innervate the spinal cord in a random manner, or both, depending on the respective cell type. For example, van Raamsdonk et al. reported that three months after spinal transection in zebrafish, the synapse density on motor neurons was restored but the morphology of synapses had changed after spinal transection (van Raamsdonk et al., 1998a). In leech, Duan et al. (2005) described the precise restoration of synaptic connections after axotomy (Duan et al., 2005).
1. Plasticity of the dopaminergic and serotonergic systems

No preferential concentration of axonal profiles around specific cell types was detectable, as determined with double-labeling combined of axon profiles (TH1/5-HT) and cell markers (5-HT/ChAT/parvalbumin), thus TH1/5-HT (Fig. 1.7A), TH1/ChAT (Fig. 1.7B), 5-HT/parvalbumin (Fig. 1.8A) and 5-HT/ChAT (Fig. 1.8B).
Fig. 1.7: Few TH1+ varicosities are in close proximity to 5-HT+ interneurons or ChAT+ motorneurons.

A: Double-labeling of 5-HT (green) and TH1 (red) in the ventral horn of an unlesioned spinal cross section. 
B: Double-labeling of ChAT (green) and TH1 (red) in the ventral horn of an unlesioned spinal cross section. Arrows indicate cell somata that are not innervated by TH1+ fibers. Arrowheads point out TH1+ varicosities in close proximity to cell somata.

Scale bars = 5 µm.
1. Plasticity of the dopaminergic and serotonergic systems

Fig. 1.8: Few 5-HT⁺ varicosities are in close proximity to parvalbumin⁺ interneurons or ChAT⁺ motorneurons.

**A**: Double-labeling of 5-HT (green) and parvalbumin (red) in the dorsal horn of an unlesioned spinal cross section (red).

**B**: Double-labeling of 5-HT (green) and ChAT (red) in the ventral horn of an unlesioned spinal cross section. Arrows indicate cell somata that are not innervated by 5-HT⁺ fibers. Arrowheads point out 5-HT⁺ varicosities in close proximity to cell somata. Scale bars are

A = 5 µm,
B = 15 µm and 5 µm for high magnification.
1.2.7 Lesion induces quantitative changes in 5-HT$^+$ axons in the spinal cord

To investigate if the described time course of axonal regeneration is unique for TH1$^+$ axons or if these observations also apply to another aminergic system, we established a time course for 5-HT axon profile densities after a spinal lesion in the same animals used for TH1 immunohistochemistry using the same locations and time course. The pattern was very similar to that observed for TH1.

Rostral to the lesion site, numbers of axonal profiles were non-significantly reduced at 1 wpl compared to unlesioned fish (Fig. 1.9A) when I performed an ANOVA over the complete time course (unlesioned: 679.1 ± 126.32 profiles/section, n = 7; 1 wpl: 505.8 ± 36.95 profiles/section, n = 5; P > 0.05). Thereafter, numbers increased until they were 80% higher than in unlesioned animals at 6 wpl (ANOVA, p = 0.0092, post test Tukey’s multiple comparison test, unlesioned: 679.1 ± 126.32 profiles/section, n = 7, 6 wpl: 1224.1 ± 105.04 profiles/section, n = 6, p < 0.05). Numbers plateaued at 13 wpl (1048.2 ± 247.04 profiles/section, n = 4). This suggests considerable axonal sprouting (Fig. 1.9 A,B,G) as a result of the lesion.

Caudal to the lesion site, numbers of 5-HT$^+$ profiles were strongly and significantly reduced at 1 wpl compared to unlesioned control animals (ANOVA, p < 0.0001, post test Tukey’s multiple comparison test, unlesioned: 679.1 ± 126.32 profiles/section, n=7; 1 wpl: 35.0 ± 5.62 profiles/section, p < 0.001, n = 5). As opposed to TH1$^+$ axons, some axons remained at 1 wpl, suggesting that these originate from local 5-HT$^+$ neurons, whereas descending axons had undergone Wallerian degeneration at this time point. Numbers of axonal profiles significantly increased thereafter and peaked at 6 wpl (unlesioned: 679.1 ± 126.32 profiles/section, n = 7; 2 wpl: 103.0 ± 17.41 profiles/section, n = 6; 6 wpl: 383.7 ± 71.91 profiles/section, n = 6, n.s.). Although numbers of axonal profiles at 6 wpl reached only 58% of the numbers in unlesioned animals, this difference was not statistically significant.

The increase in axon profile numbers between 1 wpl and 6 wpl was statistically significant (ANOVA, p < 0.0001, post test Tukey’s multiple comparison test, 1wpl: 35.0 ± 5.62 profiles/section, n = 5; 6 wpl: 383.7 ± 71.91 profiles/section,
1. Plasticity of the dopaminergic and serotonergic systems

n = 6; p < 0.05). At 13 wpl (132.1 ± 19.42 profiles/section, n = 3), there was a non-significant reduction in profile numbers, compared to 6 wpl (Fig. 1.9C,H). Thus axon numbers gradually increased again caudal to a lesion site, suggesting regrowth of descending axons or sprouting of resident 5-HT⁺ cells.

Far caudal to the lesion site, I also observed a significant reduction in axon profile numbers at 1 wpl (ANOVA, p < 0.0001, post test Tukey’s multiple comparison test, unlesioned: 659.9 ± 110.05 profiles/section, n = 3; 1 wpl: 32.5 ± 7.86 profiles/section, n = 5, p < 0.001). Although numbers of axons were slightly increased at 2 wpl (67.8 ± 5.31 profiles/section, n = 3) and 6 wpl (91.3 ± 14.23 profiles/section, n = 6), none of these changes was statistically significant. Even at 13 wpl (64.2 ± 17.85 profiles/section, n = 3) no significant increase in profile number was observed (Fig. 1.9D,E,I). This is consistent with descending 5-HT⁺ axons not regrowing to far caudal levels and no compensatory sprouting of intraspinal 5-HT⁺ cells.

In summary, I observed increase of 5-HT⁺ axonal profiles rostral to the lesion site, a reduction in the caudal spinal cord and almost complete loss in the far caudal spinal cord. Therefore, 5-HT⁺ axons and TH¹⁺ axons react very similarly in response to a spinal lesion.
1. Plasticity of the dopaminergic and serotonergic systems

A. Unlesioned

B. 6 weeks post lesion

C. 13 weeks post lesion

Caudal

D. Unlesioned, far caudal

E. 6 weeks post lesion

F. 13 weeks post lesion

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central canal

grey matter

white matter

Mauthner axons
1. Plasticity of the dopaminergic and serotonergic systems

**Graph A**

- 5-HT+ profiles/section (rostral)
- **n=7** no lesion
- **n=5** 1 week
- **n=6** 2 weeks
- **n=6** 6 weeks
- **n=4** 13 weeks

**Graph B**

- 5-HT+ profiles/section (caudal)
- **n=7** no lesion
- **n=5** 1 week
- **n=6** 2 weeks
- **n=6** 6 weeks
- **n=4** 13 weeks

**Graph C**

- 5-HT+ profiles/section (far caudal)
- **n=3** no lesion
- **n=5** 1 week
- **n=3** 2 weeks
- **n=6** 6 weeks
- **n=3** 13 weeks
1. Plasticity of the dopaminergic and serotonergic systems

Fig. 1.9: Spinal lesion induces plasticity of 5-HT⁺ axons. Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. Levels of sectioning are depicted in Fig. 1.3 A: 5-HT⁺ axons and cells are labeled in the unlesioned spinal cord. This section serves as control for B and C. F: Schematic of a spinal cross section. B, G: Numbers of 5-HT⁺ profiles increase continuously rostral to the lesion site and plateau at 6 weeks post-lesion. C, H: Caudal to the lesion site most axons are lost and significant axon regrowth is observed at 6 weeks post-lesion. D, E: Numbers of axonal profiles remain low at a level 3.5 mm caudal to the lesion site for at least 13 weeks post-lesion. ***P < 0.001, **P < 0.01, *P < 0.05; Scale bar = 25 µm.

1.2.8 Number of 5-HT⁺ axons caudal to the lesion site correlates with recovery of swimming capability

Rostral to the lesion site there was no significant difference between recovered (1224.1 ± 105.04 profiles/section, n = 6) and non-recovered fish (966.8 ± 70.95 profiles/section, n = 6, P > 0.05, Mann-Whitney Test) in the number of 5-HT⁺ axon profiles at 6 wpl (Fig. 1.10A,C). In contrast, caudal to the lesion site recovered fish (383.7 ± 71.91 profiles/section, n = 6) had significantly more axonal profiles than non-recovered ones (88.0 ± 24.12 profiles/section, n = 6; P < 0.01; Mann-Whitney-U, Fig. 1.10B,D). This suggests that regrowth of 5-HT⁺ axons beyond a spinal lesion is associated with recovery of swimming capability. I also found that numbers of 5-HT axonal profiles caudal to the lesion site of individual fish correlate with the endurance in the swim test (Spearman r 0.6112, p = 0.0372, α = 0.05, 12 pairs tested). A r-value between 0-1 means that the two variables tend to increase together. A small p-value indicates that the correlation is real and not due to random sampling.

Numbers of axonal terminals for TH1 and 5-HT caudal to the lesion site in the same fish, respectively, correlate significantly (Spearman r 0.6699, two tailed, p = 0.0202, 12 pairs tested, α = 0.05). In contrast, numbers of axonal terminals for TH1 and 5-HT rostral to the lesion site in the same fish do not correlate.
Fig. 1.10: Recovery of swimming capability correlates with regrowth of descending 5-HT⁺ axons beyond the lesion site. Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. Axial levels of sectioning are depicted in Fig. 1.3 A,C. Rostral to the lesion site numbers of 5-HT⁺ profiles do not differ between recovered and non-recovered fish. B,D: Significantly fewer 5-HT⁺ axons are present caudal to the spinal transection site in non-recovered fish than in those showing recovery of swimming capability (**P < 0.01). Relesion of recovered fish strongly reduces the number of axons caudal to the lesion site, indicating that axonal profiles are mainly derived from descending axons.

Scale bar = 25 µm.
1. Plasticity of the dopaminergic and serotonergic systems

1.2.9 The contribution of spinal neurons to spinal innervation increases after a lesion

By comparing axon profile numbers at 1 wpl (~35 profiles/section) with the unlesioned situation (~679 profiles/section) I estimated that local 5-HT cells make a minor contribution (~5%) to spinal 5-HT innervation in unlesioned animals. After re-lesion at 6 wpl and analysis 1 week after relesion, 122.1 ± 32.95 profiles/section (n = 3) profiles were observed (Fig. 1.10C,D). This was a substantial reduction compared to ~384 profiles/section observed at 6 wpl, indicating loss of descending axons that had regenerated over the lesion site. Moreover, this indicated an absolute increase in the number of spinal-derived 5-HT axons after regeneration (1 wpl vs. relesioned: P < 0.036). This also implies that the contribution of intraspinal axons was increased in the regenerated situation to ~32%. Increased innervation by spinal 5-HT+ neurons may be due to lesion-induced generation of 5-HT+ neurons.

1.2.10 Proximity of axotomy to neuronal somata might enhance axonal regrowth into the spinal cord

So far, I observed that axotomized TH1+ or 5-HT+ axons bridge the lesion site but mainly fail to grow further into the far caudal spinal cord. Nevertheless, functional recovery occurs. I hypothesized that the distance between lesion in the spinal cord and the 5-HT+ cell somata in the raphe nucleus or the TH1+ cell somata in the posterior tuberculum would influence how far the axons would grow into spinal cord once they crossed the lesion site. In mammals, for some neuronal subtypes, the elongation of axons after transection depends on the distance between neuronal cell somata and site of axotomy (Fernandes et al., 1999). Previous work in zebrafish has shown that some brain nuclei react with enhanced regenerative capacity towards a proximal compared to a distal lesion (Becker et al., 1998). Also, axotomy induces upregulation of expression of growth-related molecules such as growth associated protein 43 (GAP43) and L1.1 in the neuronal somata (Becker et al., 1998; Becker et al., 2004a). Therefore, I hypothesized that the capability of TH1+ or 5-HT+ neurons to regenerate their axons is higher when the lesion is
performed more rostrally than in the above-described experiments. Instead of performing a lesion at the midthoracic level (~3.5 mm caudal to the brainstem/spinal cord transition) as for the experiments described before, I lesioned at the brainstem/spinal cord transition. Within a pilot study, I reduced the distance between axotomy and somata. As for lesion at the midthoracic level, I quantified numbers of axonal protrusions 3.5 mm caudal to the lesion site, thereby keeping the analyzed distance of putative axonal elongation constant.

Preliminary results showed that 3.5 mm far caudal of the respective lesion site, numbers of 5-HT$^+$ axonal protrusions at 6 weeks after a lesion between brain stem and spinal cord reached 40% (n = 1) of unlesioned control fish. In contrast, numbers of axonal protrusions at 6 weeks after a lesion at a midthoracic level was only 14% (n = 6) of numbers in unlesioned fish.

Numbers of TH1$^+$ axonal protrusions at 6 weeks after a lesion between brain stem and spinal cord reached 14% (n = 1) of unlesioned control fish. Number of axonal protrusions at 6 weeks after a lesion at midthoracic level was 0% (n = 6) of numbers in unlesioned fish 3.5 mm caudal of the lesion site. This observation is consistent with the hypothesis that a distal lesion may not be sufficient to trigger a full regenerative response in axotomized TH1 neurons. More experiments to increase n-numbers are required to confirm these initial observations.

1.2.11 Lesion induces generation of 5-HT$^+$ cells

We previously reported proliferation in the lesioned spinal cord and found that diverse cell types are newly generated after lesion (Reimer et al., 2008, Kuscha et al., in preparation and see Chapter 2). Also, Takeda et al. (2008) described after a frontal hemisection of the left side of the spinal cord 500 µm caudal to the first spinal nerve an increase of numbers of 5-HT$^+$ cells in goldfish and newly generated 5-HT$^+$ cells (Takeda et al., 2008). Therefore, I analyzed numbers of 5-HT$^+$ cells after a spinal lesion. Numbers of 5-HT$^+$ cells did not differ significantly between the rostral and caudal levels at any time points and are therefore presented together.
1. Plasticity of the dopaminergic and serotonergic systems

Absolute numbers of 5-HT+ cells increased steadily after a lesion up to 6 wpl:
At 2 wpl, 5-HT+ cell numbers increased significantly by 3.9 fold around the lesion site compared to unlesioned control fish (ANOVA, p < 0.0001, post test Tukey's multiple comparison test, unlesioned: 93.1 ± 14.17 cells/1500 µm spinal cord, n = 18; 2 wpl: 363.4 ± 30.41 cells/1500 µm spinal cord, n = 19, p < 0.001) and by 5.1 fold at 6wpl. (ANOVA, p < 0.0001, post test Tukey's multiple comparison test, unlesioned: 93.1 ± 14.17 cells/1500 µm spinal cord, n = 18; 6 wpl: 471.2 ± 80.57 cells/1500 µm spinal cord, n = 10, p < 0.001). At 13 wpl, cell numbers were not different any more from unlesioned fish and were only half of the number observed at 6 wpl (ANOVA, p < 0.0001, post test Tukey's multiple comparison test, 6 wpl: 471.2 ± 80.57 cells/1500 µm spinal cord, n = 10, 13 wpl: 217.8 ± 16.70 cells/1500 µm spinal cord, p < 0.01; Fig. 1.11A,B). This suggests an overproduction of 5-HT+ cells in the lesioned spinal cord that is later pruned back.

To directly show that 5-HT cells were newly born after spinal lesion, I injected fish with with the proliferation marker 5-bromo-2-deoxyuridine (BrdU). At 2 wpl, I observed 74.8 ± 21.10 (n = 8) BrdU/5-HT+ cells. At 6 wpl, I observed 209.8 ± 26.93 (n = 8) BrdU/5-HT+ cells (ANOVA, p = 0.002, post test Tukey's multiple comparison test, p < 0.01; Fig. 1.11C,D). No BrdU/5-HT+ cells were present in the unlesioned spinal cord (n = 3).

Not all of those 5-HT+ cells in lesioned animals that exceeded the number in unlesioned fish, and were therefore presumed newly generated, were double labeled with BrdU. This was expected, because BrdU is available for only two hours after injection and our BrdU injection scheme also labels only ~25% of newly generated Tg(HB9:GFP)+ neurons after a lesion (Reimer et al., 2008).
Fig. 1.11: 5-HT+ cells are newly generated in the vicinity of a spinal lesion. Complete spinal cross sections are shown; rostral is up; central canal is outlined by a dotted line. 

A: Arrows depict 5-HT+ neurons in the unlesioned and 6 weeks post-lesion spinal cord. 

B: Numbers of 5-HT+ neurons rise until 6 weeks post-lesion and are reduced again at 13 weeks post-lesion. 

C: BrdU-labeled 5-HT+ cells are detectable at 2 weeks post-lesion (arrow; higher magnification is show in small panels on the right). 

D: A sizable proportion of 5-HT+ cells are double-labeled with BrdU at different time points post-lesion. 

Scale bar in A = 20 μm, scale bar in C for high magnifications = 10 μm.
1. Plasticity of the dopaminergic and serotonergic systems

1.2.12 Newly born 5-HT⁺ cells possibly originate from a p3-like zone

In unlesioned animals, we never observed 5-HT⁺ cells contacting the central canal. At 6 and 13 wpl, 5-HT cells with contact to the central canal were frequent. Out of 8 animals, we could not identify any 5-HT/BrdU⁺ positive cells contacting the central canal. Nevertheless, we assume that cells with central canal contact are newly generated as we never observe them in unlesioned fish. In earlier studies, we reported that a pMN-like and p2-like zone are reestablished around the spinal central canal during spinal regeneration and give rise to HB9⁺ motor neurons and vsx1⁺ interneurons, respectively (Reimer et al., 2009, Kuscha et al., in preparation, and see Chapter 2).

In order to identify a zone from where the newly born 5-HT⁺ cells likely originate, we performed labeling for 5-HT in Tg(olig2:egfp)⁺ or Tg(shha:gfp)⁺ transgenic fish at 13 wpl. Out of 19 analyzed 5-HT⁺ cells with central canal contact, all located either more ventral than Tg(olig2:egfp)⁺ ependymo-radial glia cells (n = 12) or their position appeared to overlap with the Tg(olig2:egfp)⁺ zone but 5-HT⁺ cells were themselves Tg(olig2:egfp)⁻ (n = 5 cells). None of the 5-HT⁺ cells contacted the central canal dorsal to the Tg(olig2:egfp)⁺ zone (Fig. 1.12A).

Out of 5 analyzed 5-HT⁺ cells with central canal contact, three located within the or Tg(shha:gfp)⁺ zone but were themselves Tg(shha:gfp)⁻. The other two cells were adjacent to the Tg(shha:gfp)⁺ zone (Fig. 1.12B). However, the area and the labeling intensity of the Tg(shha:gfp)⁺ zone was strongly reduced compared to 2 wpl, when upregulation of expression of shh peaks (Reimer et al., 2009). Also, mature 5-HT⁺ cells might have lost Tg(olig2:egfp)⁺ or Tg(shha:gfp)⁺ expression. Thus, lineage tracing would be required to completely elucidate the origin of the newly generated 5-HT⁺ cells. However, we can conclude that the Tg(olig2:egfp)⁺ zone forms the most dorsal border of the zone where 5-HT⁺ cells contact the central canal and that putative newly generated 5-HT⁺ cells likely emerge from a p3-like zone.
Fig. 1.12: 5-HT\(^+\) cells that contact the central canal in a ventral position are found in the lesioned spinal cord. Cross sections of the zone ventral of the central canal at 13 weeks post-lesion are shown; dorsal is up. Position adjacent to the central canal is depicted in phase-contrast images underlying double-labeling in bottom row. **A:** Central canal-contacting 5-HT\(^+\) cells are located close to *Tg(shh:GFP)*\(^+\) ependymo-radial glial cells. **B:** Central canal-contacting 5-HT\(^+\) cells are located in ventral to ventro-lateral zones of *Tg(Olig2:GFP)*\(^+\) ependymo-radial glial cells. Dots outline the central canal. Scale bar = 10 µm.
1.2.13 The shh antagonist cyclopamine reduces the number of 5-HT\textsuperscript{+} cells at 6 wpl

I hypothesized that shh might be involved in lesion-induced generation of 5-HT\textsuperscript{+} neurons, because this cell type is generated in the ventral spinal cord, close to the source of shh (see chapter 2). Shh is strongly upregulated after lesion and the shh antagonist cyclopamine effectively reduces the number of newly generated motor neurons. Moreover, after cyclopamine injection, mRNA expression of genes downstream of the shh-pathway was reduced compared to vehicle injected lesioned fish at 2 wpl (Reimer et al., 2009).

Thus, I decided to use the same injection scheme and concentration of cyclopamine used to study motor neuron regeneration to study the effect on 5-HT\textsuperscript{+} neurons. Analysis took place at 6 wpl, when number of newly generated 5-HT\textsuperscript{+} neurons peaks. Numbers of 5-HT\textsuperscript{+} cells were significantly reduced compared to fish injected with tomatidine, a related control substance (tomatidine: 10.8 ± 0.86 cell profiles/50 µm, n = 8; cyclopamine: 8.3 ± 1.00 cell profiles/50 µm; Mann-Whitney-U, one tailed, p = 0.0406). This suggests that shh promotes the generation of 5-HT\textsuperscript{+} cells in the lesioned spinal cord. Future experiments might include analysis at 2 wpl to establish a time course. Moreover, more repetition of injections might enhance the observed effect. Also, the effect of shh agonists, or fgf agonists and antagonists on the generation of 5-HT\textsuperscript{+} cells might be investigated.

![](image)

**Fig. 1.13**: Cyclopamine treatment impairs regeneration of 5-HT\textsuperscript{+} neurons. Quantification of numbers of 5-HT\textsuperscript{+} profiles in control (tomatidine, left bar) and cyclopamine (right bar) treated fish at six weeks post-lesion.
1.2.14 Numbers of newly generated 5-HT+ neurons caudal to the lesion site correlate with recovery of swimming capability and axon regrowth.

To determine whether numbers of 5-HT+ cells correlate with recovery of swimming capability, I compared numbers of 5-HT+ cells between recovered and non-recovered fish at 6 wpl, when cell numbers peak and regenerative success can be assessed.

I found that rostral to the lesion site, numbers of 5-HT+ cells did not differ significantly between recovered and non-recovered fish (Mann-Whitney test, recovered: 365.0 ± 73.59 cell profiles/750 µm, n = 6; not recovered: 292.6 ± 63.14 cell profiles/750 µm, n = 6, p > 0.05; Fig. 1.14A,B). In contrast, caudal to the lesion site, fewer 5-HT+ cells present in not recovered fish (Mann Whitney test, recovered: 253.7 ± 28.32 cell profiles/750 µm, n = 6; non-recovered: 111.1 ± 18.59 cell profiles/750 µm, n = 6; p = 0.0198; Fig. 1.14C,D). This observation is consistent with descending axons, present in much lower numbers in non-recovered fish caudal to the lesion site, stimulating lesion-induced generation of 5-HT+ cells or preventing death of these cells.
Fig. 1.14: Numbers of 5-HT* cells caudal to the lesion site correlate with recovery of swimming function at 6 weeks post-lesion. **A,B:** Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. Axial levels of sectioning are depicted in Fig. 1.3. Arrowheads indicate 5-HT* cells. **C,D:** Cell numbers caudal, but not rostral to the transection site are significantly less in non-recovered fish (*P < 0.05). Scale bar = 50 µm.
1. Plasticity of the dopaminergic and serotonergic systems

1.2.15 Verification of automated quantification method

I quantified axonal profiles in the preceding charts (Fig. 1.3, 1.5, 1.9, 1.10) according to Grider et al (2006) and see Material and Methods. The principle steps of analysis are portrayed in Figure 1.15A-D. To investigate whether TH1+ axonal profiles form synapses in the ventral or dorsal horns, I performed double-labeling of tyrosine hydroxylase and the synaptic protein synaptophysin, and double-labeling of 5-HT and synaptic vesicle protein 2 (SV2). I applied deconvolution of high power confocal image stacks (see Material and Methods) to investigate colocalization of fiber profiles. I found that only few of the TH1+ protrusions colocalized with synaptophysin and that only few of the 5-HT+ axonal profiles colocalized with SV2. This might indicate that TH1 and 5-HT act through volume transmission (Fuxe et al., 2010).

Fig. 1.15: Principles of the quantification method and characterization of the axonal profiles are shown. Complete spinal cross sections are shown; dorsal is up; central canal is outlined by a dotted line. A: Labeling of tyrosine hydroxylase (TH1) positive axonal profiles. The box depicts an area that is shown in high magnification in B, B' and B''. C: Labeling of 5-HT+ axonal profiles. The box depicts an area that is shown in high magnification in D, D' and D''. B and D were transformed with "FJ Hessian" filter of the Feature J plugin (not shown). B', D' Hessian images were transformed into a binary image with a set threshold of 140. B'', D'' Particle analysis of the binary images in B' and D'. E: Double-labeling of TH1 (red) and synaptophysin (green). Some TH1+ protrusions colocalize with synaptophysin (arrow). F: Double-labeling of 5-HT (red) and synaptic vesicle protein (SV2, green). Some TH1+ protrusions colocalize with SV2 (arrow). Scale bars B = 20 µm, D = 20, B = 20 µm, F = 5 µm.
1. Plasticity of the dopaminergic and serotonergic systems

1.3 Discussion

Catecholamines and 5-HT are important modulators of the spinal CPG for movement. However, the quantitative anatomical changes in catecholaminergic and 5-HT systems have not yet been analyzed with regard to functional recovery from a spinal lesion in fish. Here I show that, in fish that fully regain swimming ability after spinal lesion, descending tyrosine hydroxylase\(^+\) (mainly dopaminergic) and 5-HT\(^+\) axons undergo significant sprouting rostral to the spinal transection site. Dopaminergic and 5-HT\(^+\) axons cross the lesion site and reinnervate the spinal cord caudal to the lesion site but do not grow into the far caudal spinal cord. While dopaminergic axons are exclusively descending, 5-HT\(^+\) interneurons were present in the unlesioned spinal cord and additional 5-HT\(^+\) neurons were generated both rostrally and caudally in close proximity to the lesion site. Re-transection of the spinal cord abolished functional recovery. Thus, functional recovery requires supraspinal input. My observations show a significant degree of plasticity in dopaminergic and 5-HT innervation of the spinal cord concomitant with functional recovery. These findings further emphasize the role of axon regrowth beyond the lesion site for recovery to occur.

1.3.1 Plasticity in the spinal cord after transection and subsequent recovery

My findings suggest that functional recovery after a complete spinal lesion in adult zebrafish does not coincide with a complete restoration of the original neural network. In fact, I found that the neuronal network is significantly changed after recovery from a spinal lesion, yet this does not translate into different functional outputs in our test of swimming capability. One major change after a lesion is limited reinnervation of the caudal spinal cord and almost complete lack of innervation of the distal cord at 6 wpl when functional recovery peaks. My results in this study are consistent with earlier published work showing that apparently functionally recovered fish do not restore their original state (Becker et al., 1997). In that study only a third of the supraspinal neurons with descending axons regrow spinal axons after spinal
lesion. This is in line with my observations that 5-HT\(^+\) or TH\(^+\) fiber density in the caudal spinal cord is only partially restored. Within the TPa, the origin of TH1 descending axons, 5.6 cells were traced back from level 2 and 2.9 traced back from level 3 in unlesioned animals. After spinal lesion, only about 17\% of the TPa neurons regrow their axons to a far caudal level after a lesion at a midthoracic level. Within these animals, only 0.3 cells were traced by retrograde labeling. These results are in line with my observation regarding innervation of the caudal and the distal spinal cord. I found 1 animal out of 6 animals (16.6\%) with axons innervating the far caudal spinal cord.

How are fish able to recover motor function despite limited 5-HT and TH1 innervation of the distal cord? A possible conclusion would be that TH1\(^+\) and 5-HT\(^+\) innervation is not necessary for functional recovery. It might be that bridging of the lesion site by TH1\(^+\) and 5-HT\(^+\) axons just goes along with regrowth of other axons that manage to grow further into the distal cord. In this scenario, TH1\(^+\) and 5-HT\(^+\) innervation of the caudal cord would be an indicator for successful regeneration rather than being the reason for successful regeneration.

Another possible explanation is that 5-HT and dopamine act by volume transmission (Fuxe et al., 2010). In this case, bridging of the lesion site would be enough to reinnervate the caudal spinal cord and release transmitter beyond the lesion site. More distal neurons might then be reached by diffusion of transmitter.

In a recent study, we measured total distance moved and found it to be correlated with forced swimming (Reimer et al., 2009). We cannot exclude that swimming capability beyond our detection method might still be affected by the lack of distal innervation. Further experiments should include broader behavioral tests. One possibility would be using software by Ethovision. Anichtchik et al. (2004) have used this program to detect behavioral differences after injections with the TH1 ablating toxin 6-hydroxydopamine and were able to measure a decrease in velocity and an increase of the turn angle (Anichtchik et al., 2004).
1. Plasticity of the dopaminergic and serotonergic systems

Also other animal models show plasticity after spinal injury: when functional recovery was robust after a spinal injury, size and number of synapses of regenerated giant reticulospinal (RS) neurons had decreased compared to control animals in lamprey after a spinal injury (Oliphint et al., 2010). Parker et al. (2009) observed higher excitability and changes in synaptic structure such as reduction of the synaptic cleft and an increase in synaptic vesicle pools below the lesion site in recovered lampreys (Cooke and Parker, 2009). In contrast, after hemisection in goldfish, the density of boutons originating from descending projections of the nFLM, reaches a similar level on motor- and interneurons compared to before lesion (Takeda et al., 2007). We observe hyperinnervation of the rostral spinal cord, suggestive of sprouting. Fouad and Tse (2008) review the importance of spinal plasticity, particular sprouting, for promoting functional recovery also in mammals (Fouad and Tse, 2008). However, in our model, sprouting does not seem to account for functional recovery.

Thus, these models also suggest complex changes and spinal plasticity facilitating functional recovery following spinal injury. In summary, we conclude that spinal plasticity is crucial for functional recovery after spinal transection in zebrafish.

1.3.2 Correlation of axonal regeneration with behavior

Recovery of swimming was concomitant with the reappearance of TH\(^+\) and 5-HT innervation of the caudal spinal cord close to the lesion site. In the case of 5-HT, numbers of axonal profiles and swimming endurance significantly correlate. After hemisection in rat, Saruhashi et al. report a correlation between regrowing from the unlesioned contralateral side into the caudal half of the ipsilateral lesioned site and functional recovery. Four weeks after injury, the numbers of 5-HT\(^+\) terminals have reached 20\% of control animals. Number of terminals or intensity of 5-HT immunoreactivity correlated with motor scores (Saruhashi et al., 1997).
1. Plasticity of the dopaminergic and serotonergic systems

### 1.3.3 Lesion-induced generation of 5-HT+ cells

After spinal lesion, I observed a continuous increase in numbers of 5-HT+ cells up to 6 wpl. Remarkably, I find higher numbers of 5-HT+ cells caudally in recovered fish in contrast to non-recovered fish. Also, we find partial re-innervation of the caudal spinal cord in recovered fish but not in non-recovered fish. These results suggest a correlation between axonal regeneration and cellular regeneration. Possibly, supraspinal input is involved in proliferation of cells caudal to the lesion site or in protecting cells from cell death.

In agreement with higher numbers of 5-HT+ cells after lesion, I find an increase of intraspinal fiber contribution to overall fiber density in recovered fish.

At 13 wpl, numbers of 5-HT cells have decreased compared to 6 wpl and reach levels comparable to unlesioned fish. This might be explained by malregeneration after 6 wpl. Another possibility is plasticity in the spinal cord after 6 wpl, reducing over compensatory effects during recovery back to levels in the unlesioned situation.

**Are there similarities between developmental 5-HT neurogenesis and adult neuronal regeneration?**

Specific signaling pathways and transcription factors that play a role during embryonic generation of 5-HT neurons might also be upregulated after spinal lesions. We showed in a recent study that in addition to shh, components of the fibroblast growth factor (fgf) or retinoic acid signaling pathways are upregulated after lesion (Reimer et al., 2009). Here, I show that treatment with the shh antagonist cyclopamine reduces number of 5-HT+ neurons. This is in agreement with studies in development, showing that the shh antagonist cyclopamine is able to reduce the number of monoaminergic neurons in the raphe nucleus (Wen et al., 2008). Fgf is another putative candidate for initiating generation of 5-HT+ cells after lesion. Fgf and shh signaling pathways regulate the development of 5-HT cells in the raphe nucleus in zebrafish (Teraoka et al., 2004). A transcription factor which might be involved in generation of the newly 5-HT+ cell is Fox2a. During development, Fox2a induces the switch from...
visceral motor neurons to 5-HT+ neurons (Norton et al., 2005). Other putative candidates for regulation of adult 5-HT neuron regeneration are the transcription factors Gata2, Gata3, Mash1, Pet1, Lmx1b, Nkx2.2, and Phox2b. These transcription factors are involved in specification and differentiation of 5-HT+ neurons in the mouse embryo (Alenina et al., 2006; Jacob et al., 2009). Specifically GATA 3, a transcription factor consisting of two zinc fingers, has been shown to be essential of development of 5-HT neurons in raphe nuclei in the brain during development in mice (van Doorninck et al., 1999). Also in zebrafish, GATA 3 is important for the differentiation of a subpopulation of neurons in the spinal cord and brain nuclei such as the raphe system (Neave et al., 1995).

A recent study has shown that electric activity evoked by calcium spikes influences generation of 5-HT cells in the brain. Decrease of Ca2+ spikes increases numbers of cells positive for tryptophane hydroxylase – the rate-limiting enzyme in the synthesis of 5-HT (Demarque and Spitzer, 2010).

1.3.4 5-HT+ neurons in far caudal spinal cord do not react to the spinal lesion

In contrast to the region in close proximity to the lesion site, 5-HT+ cells are not newly generated in the far caudal spinal cord nor do they decrease in number. At 1 wpl, numbers of axonal profiles decrease drastically, suggesting that the intraspinal input is contributing much more to number of axonal profiles than the intraspinal cells. Thereafter, I did not observe any changes in number of 5-HT+ axonal profiles. Thus, we can draw two conclusions. First, regenerating 5-HT+ axons cross the lesion site but do not grow any further into the distal spinal cord. Secondly, resident 5-HT+ cells far caudal to the lesion site do not react to the lesion, neither with proliferation nor with sprouting.

1.3.5 TH+ or 5-HT+ varicosities in the spinal cord

As we rarely observed contacts between 5-HT and TH protrusions and cell somata, and only little colocalization with the synaptic markers SV2 and synaptophysin, we hypothesize that 5-HT and dopamine might act through
Volume transmission within the spinal cord of zebrafish. This is in line with studies performed in lamprey, where 5-HT+ varicosities were never observed to form synapses with any neuronal structure (Christensen et al., 1990). Ridet et al. (1992) found both synaptic and asynaptic dopaminergic innervation in the spinal cord in rats (Carp and Anderson, 1982; Ridet et al., 1992). Several studies focusing on the brain suggest that dopamine and 5-HT mainly act via volume transmission (Fuxe et al., 2010). Miguel and Trueta (2005) reviewed the mechanism of secretion of 5-HT from extrasynaptic axonal and somatodendritic release sites: these extrasynaptic structures contain dense core vesicles filled with 5-HT in close proximity to the plasma membrane. No synaptic structures are present. Electric stimulation mobilizes these vesicles, which then fuse with the plasma membrane and release 5-HT by exocytosis (De-Miguel and Trueta, 2005). Regarding dopamine, Garris et al. have shown that dopaminergic synapses posses structural features that allow dopamine efflux out of the synaptic cleft and thereby enable extrasynaptic transmission (Garris et al., 1994). Thus, even TH1 sites positive for synaptophysin might not only form direct synaptic contact but might act through volume transmission on more distant cells.

1.3.6 Limitation of automated quantification method

To quantify axonal profile density, we used the method of Grider et al. (see Material and Methods). To confirm the numbers I received by this automated method, I performed in addition manual counts. I took about 30 randomly chosen section from different experiments and with different protrusion density. A z-stack of 22.5 µm thickness was transformed into a 8-bit image. I manually counted the protrusion in each image within a square of 25 x 25 µm. Protrusions connected by thin fiber but clearly distinguishable by intensity and shape from the connecting fiber were counted as individual events. Rarely, we observed long axons running through the section. Those were counted as one single event. Comparing the manual counts with the results received from the automated methods indicated an average divergence of 6.3% for TH1 and 13.4 % for 5-HT. This divergence reveals certain limitation of the automated method:
1. Plasticity of the dopaminergic and serotonergic systems

I aimed to find one ideal threshold that will present the pattern observed by eye into a binary black-white image. As stated in the original publication, “altering the intensity threshold will change the intensity at which a pixel is determined as ‘positive’ labeling” (Grider et al., 2006).

Within the original image, I observed a wide range of different intensities. When I transform the image into a binary image, I set a threshold to decide which intensity is scored as an event and thereby appears as black protrusion and which intensity is not valued as an event and thereby disappears. Mostly, the set threshold will match the observation by eye: thus, a connecting fiber between protrusion will not be an event and disappear (white). The protrusions are mostly more intense than the connecting fibers and will stay as a black dot and be quantified individually. However, due to variable intensity within a section as well as between different sections, I cannot avoid errors in the quantification: occasionally, a fiber connecting several protrusions has a high enough intensity to be valued as an event and will then appear as a black shape in the binary image. In these examples, several protrusions will not be counted individually but merge into a continuous shape and will be counted as single event. On the other hand, some axons running through the sections will show so a weak signal intensity that the threshold will split them into several events.

However, despite these disadvantages, a constant set threshold avoids biased counting by the observer. It also allows quantifying sections with high axonal density that would be impossible with manual counts. I am aware of the limitations of this method. Therefore, I do not state that I quantify absolute numbers of axonal profiles. However, I receive a valuable approximation of axonal density at different time points after lesion and at different positions within the spinal cord. This approximation allows me to give a more valid statement of differences in axonal profile numbers than a pure statement given by subjective impression by eye. Future quantification will be improved by introducing a threshold for particle size. Then, huge particles, which are likely to be merged protrusions, would be assigned a higher value.
Cell types in the spinal cord of adult zebrafish

2.1 Introduction

During development in vertebrates, various cell types have been described in their marker expression, distribution in the spinal cord, developmental generation and partly also their function (e.g. Tanabe and Jessell, 1996 and see below). However, little is known about the anatomy of adult zebrafish spinal cord. To understand how locomotor function is recovered after a spinal lesion, knowledge about neuronal circuits within the spinal cord is required. Thus, we identified a need to elucidate the cellular anatomy in the adult zebrafish spinal cord. The results of this project might be the basis for future studies on neuronal cell types in the adult zebrafish spinal cord, their contribution to locomotion and their repair.

Previous work has shown that after spinal lesion in zebrafish, lost motor neurons are replaced and newly generated neurons are integrated into the spinal circuitry (Reimer et al., 2008). However, a functional neuronal network for movement requires not only motor neurons but also interneurons and modulating factors (Grillner, 2003, 2006). Moreover, interneurons might also play a role in the motor neuron disease ALS: a recent study presented data indicating the changes in burst patterns of interneurons might contribute to excitotoxicity (Pasinelli and Brown, 2006; Jiang et al., 2009). Excitotoxicity is one of the mechanisms considered as the reason for motor neuron degeneration in ALS (for review, see Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006). Therefore, it is crucial to gain knowledge of other cell types in addition to motor neurons.

2.1.1 Relation of location and function of neurons in embryonic zebrafish spinal cord

In zebrafish larvae, McLean et al. (2007) revealed a systematic relation between location of neurons within the spinal cord and swimming behavior. When dorsally located motor neurons are recruited, the swimming frequency is fast. When more ventrally located motor neuron are recruited, swimming frequency
2. Cell types in the spinal cord of adult zebrafish

decreases. Excitatory interneurons show a similar pattern: multipolar commissural descending interneurons (MCoDs) which fire during slowest swimming frequency, are located ventrally (McLean et al., 2007a). The more dorsally located Circumferential descending interneurons (CiD) show activity within a wider range of swimming frequency than the MCoDs. Within the CiDs, the more dorsal located neurons are recruited at higher swimming frequencies than the more ventrally located ones.

In contrast, activation of inhibitory interneurons at different frequencies occurs in an inverted pattern compared to excitatory interneurons: inhibitory interneurons in the dorsal spinal cord fire during slow movement and the ones in the ventral spinal cord during fast movement. In summary, spinal premotor and motor neurons reveal a systematic topographic map in regard to dorso-ventral location and swimming speed within one cell type and across different cell types.

In addition, Mclean and Fetcho (2009) find evidence that the spatio-temporal development of primary and secondary motor neurons as well as excitatory CiDs and MCoDs interneurons along the dorsal-ventral axis correlates with the appearance of distinct swimming behaviors: the differentiation of the neuronal network from dorsal to ventral translates into patterns in body movements that are used for swimming speed from fast to slow in larvae. In detail, during early development in zebrafish embryo, swimming is achieved through simultaneous movements of the head and tail. Zebrafish larvae use the same pattern for fast movement. Tail displacement with hardly any head movement results in slow swimming in larvae (McLean and Fetcho, 2009).

Interestingly, in adult zebrafish, this relationship between recruitment and swimming frequency does not seem to apply (personal communication, Jessica Ausborn, Göttingen, 2011).

Neurons in the dorsal spinal cord play a crucial role in somatosensory integration (for review, see Helms and Johnson, 2003): in lamprey, touch and pressure evoke a response of dorsal primary neurons (Christenson et al., 1988). In embryonic zebrafish spinal cord, the somata of Rohon-Beard neurons are located in the dorsal third of the spinal cord. They send both ascending and
2. Cell types in the spinal cord of adult zebrafish

descending axons into the dorsolateral fascicle (Metcalfe et al., 1990). Douglas et al. (2008) suggest that Rohon-Beard neurons along with trigeminal neurons initiate escape behavior (Douglass et al., 2008).
These studies emphasize the importance of knowledge of location of various cell types within the spinal cord to understand their function in movement. Thus, a description of the spatial distribution of cell types in the adult zebrafish spinal cord is crucial for further functional studies.

2.1.2 Neurogenesis in the embryonic spinal cord

Is regeneration simply a reactivation of developmental processes? The following paragraph will touch upon neurogenesis in the developing spinal cord to set a starting point for subsequent comparison with my own results.

Sonic hedgehog (shh) plays a crucial role in the specification of neuronal subtypes in the vertebrate neural tube (Dessaud et al., 2008). The floorplate and the notochord secrete sonic hedgehog. Shh then diffuses through the tissue and a ventro-dorsal gradient is established. Depending on the concentration of shh and the responding cells, a complex regulatory network of transcription factors is formed. Distinct combinations of transcription factors give rise to specific cell subtypes along the dorso-ventral axis in a strictly positionally ordered manner. The premotor neuron zone (pMN zone) expresses olig2/pax6/nkx6.1 and gives rise to motor neurons. In chick spinal cord, nkx6.1 initiates the expression of the homeoprotein MNR2, which in turn activates downstream transcription factors such as HB9 (Tanabe et al., 1998; Briscoe et al., 2000). The adjacent, more dorsally positioned p2 zone is also defined by pax6/nkx6.1 expression but is devoid of Olig2 and gives rise to chox10+ V2 interneurons (Briscoe et al., 2000). Nkx6.1 knock-out mice display a dorso-ventral shift: they mainly lack motor neurons and V2 interneurons but show an excess in generation of V1 interneurons (Sander et al., 2000). Ericson et al. (1997) have studied how loss of pax6 – caused by a point mutation in the pax6 gene that depletes pax6 expression completely – affects the identity of progenitors cells and cell fate: Depending on the exact position along the dorso-ventral axis, lack of pax6 has different consequences: within the dorsal area of the ventral part of the
neuronal tube, lack of pax6 depletes V1 interneurons, however, the identity of progenitor cells remains unaltered, within a more ventral region, the loss of pax6 decreases the number of somatic motor neurons (Ericson et al., 1997). In zebrafish, knock-down of olig2 with antisense morpholino oligonucleotides strongly reduces the number of generated primary motor neurons, whereas overexpression of olig2 results in an increase in motor neuron numbers (Park et al., 2002). Lu et al. (2002) provided data that Olig2 mutant mice are devoid of motor neurons in the spinal cord. At the same time, V2 interneurons are produced in excess and their progenitor domain increases ventrally into the pMN zone. Interestingly, overexpression of olig2 showed the opposite effect, in other words, a drastic decrease in V2 interneurons (Lu et al., 2002). Figure 2.0 shows a schematic of the transcription factor domains set up during development. I studied the expression pattern of the shown transcription factors in the lesioned spinal cord and showed that motor neurons and vsx1:GFP interneurons emerge from different progenitor zones in the regenerating adult spinal cord.

Fig. 2.0: Schematic presentation of transcription factor domains set up in the developing vertebrate neural tube by a ventrodorsal gradient of Shh (modified after Vallstedt and Kullander, 2007). The p2 zone gives rise to V2 interneurons. The pMN zone gives rise to motor neurons (MN).
2. Cell types in the spinal cord of adult zebrafish

During development, the dorsal roof plate releases bone morphogenetic proteins (BMPs) (Lee and Jessell, 1999). BMP signaling initiates the expression of transcription factors that define distinct dorsal cell subpopulations such as the basic helix-loop-helix transcription factors neurogenin and Mash 1, and the homeobox transcription factors pax7 and pax2 (for review, see Helms and Johnson, 2003). Mash 1 and neurogenin 1 provide a code that is required for the cellular diversity and numbers of neurons within each different subpopulation in the dorsal horn (Helms et al., 2005). Pax3 and pax7 have been shown to restrict ventral spinal cord cell fate (Mansouri and Gruss, 1998). Also Wnt signaling is required for pattern formation in the dorsal spinal cord (Bonner et al., 2008).

We hypothesized that during regeneration, shh is upregulated. In my project, I looked at the expression of the transcription factor pax6, nkx6.1 and Tg(olig2:GFP) transgene expression after spinal lesion compared to unlesioned fish. I also studied the mRNA expression of patched1, a downstream gene and receptor of shh.

2.1.3 Neurogenesis in adult zebrafish after spinal lesion

In a recent publication, we showed that lesion triggers proliferation around the central canal. We have shown that lost motor neurons are replaced by newly born motor neurons which are integrated into the spinal network (Reimer et al., 2008). However, only 7.6% of all BrdU+ cells in the zone of proliferation around the central canal are Tg(HB9:GFP)+ at 2 wpl. Hence, we concluded that other cell types in addition to Tg(HB9:GFP)+ cells are newly generated after lesion. In my project, I contributed to the findings on motor neuron regeneration and investigated several other cell types to find out whether they are newly born after a lesion. Here, I present evidence that various other cell types are newly generated after a lesion. I used different cell type markers that have been described broadly during development. I investigated cell types according their ventro-dorsal distribution within in the spinal cord.
In summary, I observed plastic changes during recovery after spinal lesion, showing that some cell types significantly increased in number during recovery, whereas others were unaffected by spinal lesion or temporarily decreased.

2.2 Results

2.2.1 Lesion-induced death and generation of motor neurons

To study motor neurons in the adult spinal cord, I used the transgenic fish line \( Tg(HB9:GFP) \) (Flanagan-Steet et al., 2005; Feldner et al., 2007) and immunohistochemical labeling for choline acetyltransferase (ChAT). Development of motor neurons requires the transcription factor HB9 (Jessell, 2000). The enzyme cholin acetyltransferase (ChAT) synthesizes the transmitter acetylcholine. Spinal motor neurons release acetylcholine at the neuromuscular junction (Hubbard and Yokota, 1964). I have shown that in adult zebrafish spinal cord, large diameter \( Tg(HB9:GFP)^+ \) (> 12 µm) motor neurons are mainly ChAT+: I analyzed 95 \( Tg(HB9:GFP)^+ \) cells and 176 ChAT+ cells (n = 9 sections, 3 animals) in unlesioned control animals. I observed 77 ChAT+/\( Tg(HB9:GFP)^+ \) cells. Thus, 81.1% of the \( Tg(HB9:GFP)^+ \) cells were ChAT+. Most of the ChAT+ cells are located ventro-lateral in the grey matter of the spinal cord (Fig. 2.1).

The diameter of ChAT+ cells ranges between ~7 and 35 µm. Further characterization of ChAT+ cells revealed a subpopulation of small cells in the dorsal horn. Among the ChAT+ cells, exclusively those in the dorsal horn possess pax2+ nuclei (Fig. 2.1). This pattern was consistent in unlesioned control fish and at 6 weeks post lesion (wpl). In unlesioned fish, I analyzed 47 ChAT+ cells and 79 pax2+ cells (n = 3 sections, 1 animal). I observed 3 ChAT+/pax2+ cells. 6.4% of the ChAT+ cells were pax2+. At 6 wpl, I analyzed 464 ChAT+ motor neurons and 1344 pax2+ cells. I identified 64 ChAT+ cells with a pax2+ cell nucleus (n = 47 sections, 8 animals). Thus, 13.8% of ChAT+ cells were pax2+. These observations are in agreement with previous studies reporting various subtypes of cholinergic population in all lamina layers of the grey matter of rats and cats (Phelps et al., 1984; Huang et al., 2000). Huang et al. (2000) suggest large and ventrally located ChAT+ cells to be motor neurons. Further
2. Cell types in the spinal cord of adult zebrafish

characterization of cholinergic cells in the zebrafish spinal cord might include mapping of ChAT\(^+\) cells to the different laminar layers. Double-labeling of ChAT and the transcription factor c-fos, which has been shown to be activated in ChAT\(^+\) cells during fictive locomotion in cats (Carr et al., 1995), might give further insight into the function of these cells. Retrograde tracing with dextran from peripheral muscle tissue might reveal which spinal ChAT\(^+\) subpopulation innervates muscles and are motor neurons.

![ChAT/pax2](image)

Fig. 2.1: A subpopulation of ChAT\(^+\) cells possess pax2\(^+\) nuclei. Double-labeling of ChAT (green) and pax2 (red) in spinal cross section of recovered fish at six weeks post-lesion caudal to the lesion site; dorsal is up. The labeling pattern at six weeks post-lesion is like the pattern in control unlesioned fish. Inset depicts an area with two ChAT\(^+\)/pax2\(^+\) cells shown in high magnification in the right column. Unfilled arrowheads point to ChAT\(^+\)/pax2\(^-\) cells, filled arrowheads point to ChAT\(^-\)/pax2\(^+\) cells. Arrows indicate double-labeled cells. Scale bars = 20 \(\mu\)m.
2. Cell types in the spinal cord of adult zebrafish

In the context of spinal lesion and motor neuron disease, the relevance of parvalbumin\(^+\) motor neurons has been shown: Parvalbumin\(^+\) motor neurons are resistant to degeneration in amyotrophic lateral sclerosis (ALS), which might be related to the calcium buffering capacity of parvalbumin (Elliott and Snider 1995; Van den Bosch, Schwaller et al. 2002). In the ventral horn, I observed $Tg(HB9:GFP)/parvalbumin^+$ cells (Fig. 2.2A,C). In the dorsal horn, cells were exclusively $Tg(HB9:GFP)/parvalbumin^+$ (Fig. 2.2B). I analyzed 77 large-diameter $Tg(HB9:GFP)^+ (> 12 \mu m)$ and 436 parvalbumin\(^+\) cells in unlesioned animals (n = 9 sections, 3 animals). I observed 17 $Tg(HB9:GFP)^+/parvalbumin^+$ cells. Thus, 24.7% of large-diameter $Tg(HB9:GFP)^+$ cells also expressed parvalbumin.

Fig. 2.2: Subpopulation of $Tg(HB9:GFP)^+$ cells express parvalbumin.

**A**: Double-labeling of parvalbumin (red) and GFP (green) in spinal cross section of unlesioned $Tg(HB9:GFP)^+$ transgenic fish with exclusively parvalbumin\(^+\) cells in the dorsal neuropil (B) and double-labeled cells in the ventral horn (C). Unfilled arrowheads point to $Tg(HB9:GFP)^+/parvalbumin^+$ cells, filled arrowheads point to $Tg(HB9:GFP)^+/parvalbumin^-$ cells. Arrows indicate double-labeled cells. The asterix indicates the central canal. Dorsal is up.

Scale bars = 20 \mu m.

In a recent publication, we showed that lost motor neurons are replaced and newly born motor neurons are integrated into the spinal network (Reimer et al., 2008). I contributed to this publication by demonstrating with terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL labeling) that large $Tg(HB9:GFP)^+$ motor neurons die after lesion. At 1
2. Cell types in the spinal cord of adult zebrafish

wpl (42 ± 15.1 cells/750µm; n = 3; p = 0.0035) and 2 wpl (40 ± 7.3 cells/750µm; n = 11; p < 0.0003), large-diameter $Tg(HB9:GFP)^+$ motor neurons were strongly reduced in number compared with unlesioned animals (133 ± 34.9 cells/750µm; n = 4) (Reimer et al., 2008). This reduction in number of $Tg(HB9:GFP)^+$ motor neurons suggests lesion-induced loss of motor neurons, which was confirmed by TUNEL labeling of 22.8 ± 11.39% of the $Tg(HB9:GFP)^+$ motor neurons at 3 d after lesion (n = 3) (Fig. 3A,B). In unlesioned animals, I could not detect any TUNEL+ signal (Reimer et al., 2008).

I have also provided data that ChAT+ cells follow a similar time course during recovery as large diameter $Tg(HB9:GFP)^+$ cells (Fig. 3.3D). After spinal lesion, I observed a tendency of decrease in the number of motor neurons at two weeks post-lesion compared with unlesioned animals. Between 2 wpl and 6 wpl, I could observe a tendency of increase in the number of large ChAT+ motor neurons (unlesioned: 484.8 ± 70.75, n = 5; 2 wpl: 249.3 ± 41.83, n = 3; 6 wpl: 498.6 ± 90.79, n = 4, n.s).

The labeling rate of all large ChAT+ cells with BrdU was 0.7 % at 2 wpl and 4.7% at 6 wpl (2 wpl: 1. 7 ± 1.67 ChAT+/BrdU+ cells, n = 3; 6 wpl: 23.6 ± 11.20 ChAT+/BrdU+ cells; n = 4, Fig. 2.3E). As mentioned before, I observed a tendency of increase between 2 wpl and 6 wpl in number of ChAT+ cells. Thus, the increase of ChAT+ cells between 2 wpl and 6 wpl can be explained by newly generated cells that are added on top of resident ChAT+ cells. I would only expect BrdU labeling in these newly generated cells. When this was taken into consideration, the labeling rate with BrdU of ChAT+ cells cells at 6 wpl was 9.5%. To investigate if these newly generated ChAT+ cells are integrated into the spinal neuronal network at 6 wpl, we combined labeling of BrdU, ChAT, and SV2 in the 1500 µm surrounding the lesion site. We found 29 ± 23.1 large BrdU+/ChAT+ cells (n = 3) covered with SV2+ contacts at a density that was comparable with that of motor neurons in unlesioned animals (Fig. 2.3C). Moreover, newly born ChAT+ cells bear long elaborate extensions, indicating that they are fully mature and differentiated.
2. Cell types in the spinal cord of adult zebrafish
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.3: ChAT+ motor neurons die after spinal lesion, are newly generated and integrated into the spinal circuit.

A, B: Lesion-induced death of motor neurons at 3 days post-lesion. Spinal cross sections are shown; dorsal is up. Tg(HB9:GFP)+ cells are shown in green, TUNEL signal in red and DAPI nuclear counter-stain in blue or white. The arrows point to a TUNEL+/Tg(HB9:GFP)+ cells, the nucleus is labeled with DAPI.

A: Overview; the asterisk indicates the central canal and the arrow indicates the area shown in higher magnification in B.

B: Triple-labeled cells are indicated by arrows. The arrowhead indicates a Tg(HB9:GFP)+ cell without TUNEL signal. TUNEL+ nuclei appear brighter in DAPI labeling than TUNEL- nuclei.

C: At 6 weeks after lesion, ChAT+/BrdU+ somata are decorated with SV2+ contacts (arrow). Inset (right) depicts a ChAT+ motor neuron decorated with SV2+ contacts in an unlesioned animal.

D: Quantification of numbers of ChAT+ cells in unlesioned fish and at two and six weeks after lesion.

E: Quantification of numbers of ChAT+ cells in at two and six weeks after lesion (white bar) and ChAT+/BrdU+ cells (black bar).

Scale bars A = 25 μm, B = 10 μm, C = 25 μm.

(Figure 2.3 C was generated in collaboration with Dr. Michell M. Reimer. He did the staining for SV2/ChAT/BrdU at 6wpl and I did the staining for ChAT/SV2 in the unlesioned control.)
At 2 wpl, newly born $Tg(HB9:GFP)^+$ motor neurons clearly differ from resident $Tg(HB9:GFP)^+$ by their small cell diameter and lack of ChAT expression (Reimer et al., 2008). I have shown that these small diameter $Tg(HB9:GFP)^+$ cells are mainly ChAT$^-$ at 2 wpl (Fig. 2.4). I analyzed 551 ChAT$^+$ cells and 1265 $Tg(HB9:GFP)^+$ cells of small diameter ($n = 18$ sections, 3 animals). Only 32 $Tg(HB9:GFP)^+$ cells were ChAT$^+$. Thus, 97.47% of the small-diameter $Tg(HB9:GFP)^+$ cells were ChAT$^-$, suggesting that they were undifferentiated motor neurons.

**Fig. 2.4:** Small-diameter $Tg(HB9:GFP)^+$ cells are ChAT$^-$. Double-labeling of ChAT and GFP in spinal cross sections of transgenic $Tg(HB9:GFP)^+$ fish at two weeks post-lesion; dorsal is up. Newly generated $Tg(HB9:GFP)^+$ motor neurons are ChAT$^-$. Empty arrowheads point to undifferentiated $Tg(HB9:GFP)^+$ motor neurons, the filled arrowhead indicates a ChAT$^+/Tg(HB9:GFP)^-$ differentiated motor neuron. Dots outline the central canal. Scale bar = 25 µm.

What is the origin of these newly generated motor neurons and which pathways are involved in their generation during regeneration? Reimer et al. (2009) could show that $Tg(Olig2:GFP)^+$ ependymo-radial glial cells are putative stem cells and progenitors for newly generated $Tg(HB9:GFP)^+$ motor neurons. We hypothesized that sonic hedgehog (shh) and downstream genes will be upregulated.
2.2.2 Expression of pMN transcription factors is retained in adult zebrafish and is increased after a spinal lesion

In a recent publication, we focused on the question of which pathway might be involved in motor neuron regeneration after a spinal lesion in adult zebrafish (Reimer et al., 2009). I contributed to this publication by showing that after lesion a pMN-like zone adjacent to the central canal increases in cell number and intensity of immunoreactivity of pax6 and nkx6.1. Also, the central canal itself increases in size. I also showed that shh is upregulated in a subpopulation of ependymo-radial glial cells distinct from motor neuron progenitors. I also provided data that patched1 is upregulated in motor neuron progenitors. Because motor neurons in the developing neural tube are derived from an olig2\(^{+}\), nkx6.1\(^{+}\), and pax6\(^{+}\) domain, I analyzed expression of these genes in the adult spinal cord in the unlesioned and lesioned situation, focusing on the Tg(olig2:egfp)\(^{+}\) ependymo-radial glial cells around the central canal, from which motor neurons regenerate (Reimer et al., 2008) (Fig. 2.5A). I found that, close to the lesion site, the central canal increased massively in size. The average circumference of the central canal in cross-section within 750 µm of the lesion site increased to almost 300% at 2 wpl (unlesioned: 33.4 ± 1.60, n = 7 animals; 2 wpl: 97.7 ± 10.50 µm, n = 7 animals; ANOVA, p = 0.0001) and remained enlarged at 6 wpl (89.5 ± 7.91 µm, n = 7 animals; ANOVA, p = 0.0002). In the eel, Dervan and Roberts (2003) describe that widening of the central canal after spinal cord lesion directly correlates with an increase in the number of ventricular cells (Dervan and Roberts, 2003). Ventricular cells are potential stem cells and are the major proliferating cell type in the lesioned spinal cord (Reimer et al., 2008). Thus, a widened central canal suggests an increase in symmetrical division of these cells that may lead to larger domains of progenitor cells.

Nkx6.1 immunoreactivity was found in a U-shaped pattern in ventricular cells around the ventral central canal in unlesioned adult animals, which is reminiscent of the embryonic expression pattern in the neural tube. Some parenchymal cells were also Nkx6.1\(^{+}\). At 2 wpl, Nkx6.1 immunoreactivity was still detectable in a U-shape around the central canal. However, more Nkx6.1\(^{+}\) ventricular cells were present and immunoreactivity was more intense. The
number of ventricular Nkx6.1-immunoreactive nuclear profiles in single optical sections increased from $7.5 \pm 1.45$ profiles/section ($n = 6$ animals) to $35.3 \pm 5.58$ at 2 wpl ($n = 6$ animals, $p = 0.0039$). In the parenchyma, we observed $4.2 \pm 0.83$ cells in unlesioned animals and $6.3 \pm 1.02$ profiles/section in lesioned animals; $p = 0.16$). In multiple labeling experiments, we found that Nkx6.1 immunoreactivity overlapped with the $Tg(\text{olig2:egfp})^+$ zone of the adult central canal and exceeded it slightly dorsally in the unlesioned and lesioned spinal cord (Fig. 2.5B). Thus Nkx6.1 is expressed at low levels in the ventral ventricular zone of the unlesioned adult spinal cord, and its expression is increased in the same relative position after a lesion.

Pax6 immunoreactivity was detectable in an inverted U-shape around the central canal and in some parenchymal cells in unlesioned animals. There was a ventral-high to dorsal-low gradient in Pax6 immunoreactivity. At 2 wpl this expression pattern was retained (Fig. 2.5B). However, the number of ventricular Pax6-immunoreactive profiles in single optical sections significantly increased from $9.3 \pm 2.19$ profiles/section ($n = 6$ animals) to $43.5 \pm 13.13$ at 2 wpl ($n = 6$ animals, $p = 0.0039$). In the parenchyma, we observed $3.7 \pm 0.80$ profiles/section in unlesioned animals and $8.3 \pm 1.15$ profiles/section in lesioned animals ($p = 0.0096$), indicating a moderate increase in the number of parenchymal Pax6+ cells. Co-labeling indicated that Pax6 immunoreactivity at the central canal overlapped with the $Tg(\text{olig2:egfp})^+$ zone and shared a ventral border with it in the unlesioned and lesioned spinal cord (Fig. 2.5B). Ventral Pax6 immunoreactivity also overlapped with dorsal Nkx6.1 immunoreactivity (Fig. 2.5B). Thus, Pax6 expression in the latero-dorsal aspect of the spinal cord is increased after a spinal lesion.

Overall, triple-labeling indicated that the motor neuron-generating $Tg(\text{olig2:egfp})^+$ zone was also Pax6+ and Nkx6.1+. Individual $Tg(\text{olig2:egfp})^+$ ependymo-radial glial cells were found to co-express both Nkx6.1 and Pax6 in single optical sections of the unlesioned and lesioned spinal cord, and their number was increased from $2.0 \pm 0.00$ profiles/section in unlesioned animals ($n = 2$) to $13.7 \pm 2.19$ profiles/section at 2 wpl ($n = 3$ animals). Parenchymal cells were rarely found to co-express $Tg(\text{olig2:egfp})$, Nkx6.1, and Pax6 (unlesioned: 0
profiles/section; lesioned: 0.3 ± 0.33 profiles/section). Thus a subset of ependymo-radial glial cells in the adult spinal cord retain low, but clearly detectable, expression of markers for the pMN zone, namely $Tg(\text{olig2}:\text{egfp})$, Nkx6.1, and Pax6 in specific domains. The number of cells co-expressing these genes and gene expression levels are increased, when motor neurons are generated after a lesion.
Fig. 2.5: Evidence for a pMN-equivalent zone in the ependymal layer of the adult spinal cord.

**A:** Schematic presentation of transcription factor domains set up in the developing vertebrate neural tube by a ventrodorsal gradient of Shh (modified after Vallstedt and Kullander, 2007). Different progenitor zones (p0–3) give rise to distinct classes of interneurons (V0–3). The pMN zone gives rise to motor neurons (MN).

**B:** Spinal cross sections at the level of the central canal (asterisks) are shown; dorsal is up. In the unlesioned (arrows) and lesioned spinal cord (brackets) Pax6/Tg(olig2:egfp)/Nkx6.1 co-expressing ependymo-radial glial cells are present. Expression of all markers is increased close to a spinal lesion site at 2 weeks post-lesion. Arrowheads point to oligodendrocytes. The star indicates the central canal. Scale bar = 20 μm.
2.2.3 shh is upregulated in a distinct class of proliferating ependymo-radial glial cells at the ventral midline of the spinal cord

It was shown that in the lesioned adult spinal cord, shh expression was increased in proliferating ventral ependymo-radial glial cells, directly adjacent to motor neuron progenitor cells. This increase was detectable by in situ hybridization as well as via analysis of transgene expression of Tg(shha:gfp)+ (Reimer et al., 2009).

I contributed to these findings by characterizing the Tg(shha:gfp)+ ependymo-radial glial cells: I could show that Tg(shha:gfp)+ ependymo-radial glial cells were distinct from Tg(olig2:egfp)+ motor neuron progenitor cells, as revealed in multiple labeling experiments: Pax6 immunoreactivity bordered ventrally on Tg(shha:gfp)+ ependymo-radial glial cells in the unlesioned and lesioned spinal cord (Fig. 2.6). Tg(shha:gfp)+/Pax6+ cells were not detected, in contrast to Tg(olig2:egfp)+ ependymo-radial glial cells which expressed Pax6 (Fig. 2.5). The fact that the ventral border of Pax6 expression in ventricular cells is coextensive with that of Tg(olig2:egfp)+ ependymo-radial glial cells indicates that pMN-like cells line the central canal immediately adjacent to the shha-expressing cells.

Fig. 2.6: Tg(shha:gfp)+ ependymo-radial glial cells co-express Nkx6.1, but not Pax6. Spinal cross sections at the level of the central canal (outlined by dots) are shown; Arrows point to an individual Tg(shha:gfp)+/Nkx6.1+/Pax6− cell. Scale bar = 15 μm.
2. Cell types in the spinal cord of adult zebrafish

2.2.4 Expression patterns of shh receptor patched suggests lesion-induced activity of the pathway in motor neuron progenitor cells

If shh signaling is active in the adult lesioned spinal cord, I hypothesized that the patched1 receptor will be expressed. Patched1 mRNA expression was increased at 2 wpl close to the lesion site from undetectable levels in unlesioned animals. The ventral zone, corresponding to the cells that express Tg(shha:gfp)+, was free of signal at 2 wpl. In the adjacent zone there was strong expression of patched1 mRNA that tapered off towards the dorsal spinal cord (Fig. 2.7A). Since strong expression of patched1 is an indicator of activity of the hh pathway, I determined whether Tg(olig2:egfp)+ motor neuron progenitors expressed patched1, using double labeling of patched1 mRNA in lesioned Tg(olig2:egfp) transgenic fish at 2 wpl. The region of strongest patched1 mRNA expression overlapped with Tg(olig2:egfp)+ ependymo-radial glial cells, consistent with the hypothesis that Shh-dependent transcription took place in Tg(olig2:egfp)+ motor neuron progenitor cells after a lesion (Fig. 2.7A). Events involved in motor neuron neurogenesis are summarized in Figure 2.7B.
2. Cell types in the spinal cord of adult zebrafish
Fig. 2.7: shh pathway gene *patched* is upregulated in ependymo-radial glial cells of the lesioned spinal cord.

**A:** Cross-sections of the adult spinal cord at the level of the central canal (asterisk) are shown; dorsal is up. In the lesioned, but not the unlesioned spinal cord, *Tg(olig2:egfp)*+ ependymo-radial glial cells express detectable levels of mRNA for *patched1* (arrow), an indicator of shh pathway activity.

**B:** Summary of known events associated with neuron regeneration in adult zebrafish. Schematic cross section through the spinal cord is shown (the central canal is represented by a grey oval). After a lesion, the central canal widens close to the lesion site, concomitant with an increase in numbers of ependymo-radial glial cells that also express higher levels of transcription factors. A ventro-dorsal shh signal, evidenced by a ventro-dorsal *patched1* (*ptc1*) mRNA expression gradient in ependymo-radial glial cells, contributes to production of Hb9+/islet-1/-2+/ChAT+ motor neurons (MNs) from pMN-like ependymo-radial glial cells (green) after a lesion (Reimer et al, 2009).

Scale bar = 15 µm.
2. Cell types in the spinal cord of adult zebrafish

2.2.5  **shh agonist does not increase number of newly-generated motor neurons**

Recently, we have shown that injection of cyclopamine (0.2mg/ml in a volume of 25 µl) – which is a shh antagonist – reduces the number of small-diameter Tg(HB9:GFP)$^+$ motor neurons. Also, treatment with cylopamine reduced proliferation (Reimer et al., 2009).

I hypothesized that injection of a shh agonist will increase the number of newly generated motor neurons and proliferation in ventricular cells. I used a novel unpublished shh agonist provided by the company Curis.

Fish were injected at 3, 6 and 9 days after lesion with at least 10mg/body weight of the agonist in PBS/DMSO. Controls were injected with PBS/DMSO. At 2 wpl, numbers of Tg(HB9:GFP)$^+$ motor neurons did not show any significant difference in fish that had received the agonist compared to vehicle-injected control fish (vehicle: 749.2 ± 219.15 cells/1500 µm, n = 7; agonist: 771.0 ± 63.52/1500 µm, n = 6; p > 0.05, Fig. 2.8). Thus, I was not able to show an effect of this particular agonist on motor neuron regeneration. For future studies, other shh pathway agonists might be tested, such as the smoothened agonist purmorphamine (Aanstad et al., 2009).

![Fig. 2.8: shh agonist does not increase number of tg(HB9:GFP)$^+$ motor neurons.](image)

**A:** Spinal cross sections of transgenic tg(HB9:GFP) fish are shown; dorsal is up; the dots outline the central canal. **B:** Treatment with shh agonist does not increase number of tg(HB9:GFP)$^+$ motor neurons.

Scale bar = 30 µm.
2. Cell types in the spinal cord of adult zebrafish

2.2.6 Tg(vsx1:GFP)+ cells are newly generated after lesion

To assess whether after a spinal lesion neurons other than motor neurons would be newly generated, we decided to use the transgenic line Tg(vsx1:GFP) (Kimura et al., 2008). Similar to HB9 which is a transcription factor expressed in undifferentiated motor neurons emerging from the pMN zone, vsx1 (also known as chox10-like) is a transcription factor that is expressed in undifferentiated V2 interneurons arising from the p2 zone.

To investigate the effect of a spinal lesion on the generation of vsx1+ neurons, we assessed the number of vsx1+ neurons in unlesioned control fish (Fig. 2.9A, D) and at 2 wpl (Fig. 2.9B,D) and at 6 wpl (Fig. 2.9C,D). In unlesioned Tg(vsx1:GFP) fish (Fig. 2.9A), very few small interneurons were observed in 1500 µm of spinal cord at midthoracic level. The number of Tg(vsx1:GFP)+ cells was significantly increased (24-fold) at 2 wpl and at 6 wpl (22-fold) compared to unlesioned control fish (ANOVA, p = 0.0213; post test Dunnett’s multiple comparison test, unlesioned: 26.5 ± 19.08 cells/1500 µm, n = 3; 2 wpl: 628.2 ± 80.9 cells/1500 µm, n = 12; 6 wpl: 573.0 ± 162.94 cells/1500 µm, n = 6; p < 0.05). Tg(vsx1:GFP)+ cells were located ventro-lateral of the central canal. Some of the Tg(vsx1:GFP)+ cells contacted the central canal. They were oval shaped and some of them had extended processes (Fig. 2.9B’,C’). Numbers of Tg(vsx1:GFP)+ cells between 2 wpl and 6 wpl did not differ significantly from each other.
Fig. 2.9: \( Tg(vsx1:GFP) \cdot \) cells are only found after lesion. Labeling of GFP in spinal cross sections of transgenic \( Tg(vsx1:GFP) \cdot \) fish; dorsal is up. 

A shows the section of a unlesioned animal devoid of any \( Tg(vsx1:GFP) \cdot \) cells. 

B shows a spinal cross section at two weeks post-lesion and C at six weeks post-lesion. Arrowheads point to cell bodies with central canal contact and arrows point to extending neurites. White insets outline areas that are depicted in high magnification in \( B', B'' \) and \( C' \). 

D: Quantification of cell numbers of \( Tg(vsx1:GFP) \cdot \) cells in unlesioned animals and at two and six weeks after lesion. Numbers of cells significantly increase after lesion. \(^*P < 0.05\)

The star (A) and the dots (B, B', B'' and C, C') indicate the position of the central canal. Scale bars = 15 µm.
To directly show that these $Tg(vsx1:GFP)^+ \text{ cells are newly born after lesion, I}$ injected BrdU at 0, 2 and 4 days after lesion.

At 2 wpl, 20.4 % of the $Tg(vsx1:GFP)^+ \text{ cells were labeled with BrdU (Fig. 2.10).}$

Not all $Tg(vsx1:GFP)^+ \text{ cells were double-labeled with BrdU. This was expected,}$

because BrdU is available for only two hours after injection and our BrdU injection scheme also labels only $\sim 25\%$ of newly generated $Tg(HB9:GFP)^+ \text{ neurons after a lesion (Reimer et al., 2008).}$

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**Fig. 2.10:** $Tg(vsx1:GFP)^+ \text{ cells are newly generated after lesion.}$

Labeling of GFP and BrdU in spinal cross section of transgenic $Tg(vsx1:GFP)^+ \text{ fish at two}$

weeks post-lesion. Dorsal is up. Inset outlines two $Tg(vsx1:GFP)^+ \text{ cells labeled with}$

BrdU shown in high magnification (right). Arrows point to double-labeled cells. Dots

indicate the central canal.

Scale bar = 15 $\mu$m.
2. Cell types in the spinal cord of adult zebrafish

Future experiments might include different injection schemes of BrdU at time points between 2 wpl and 6 wpl, to investigate if neurogenesis of \( Tg(vsx1:GFP)^+ \) cells is a continuous process after spinal lesion in adult zebrafish.

\( 2.2.7 \quad Tg(vsx1:GFP)^+ \) cells are distinct from HB9\(^+\) or islet-1/2\(^+\) motor neurons

After spinal lesion, we could detect newly born \( Tg(vsx1:GFP)^+ \) cells. We could observe morphological similarities between \( Tg(vsx1:GFP)^+ \) cells and recently described newly generated small \( Tg(HB9:GFP)^+ \) motor neurons (Reimer et al., 2008): both cell types share a similar shape and size of cell somata, and in some instances, contact the central canal; \( Tg(HB9:GFP)^+ \) motor neurons as well as \( Tg(vsx1:GFP)^+ \) cells show long axonal protrusions that elongate to the pial surface; they are located around the central canal in the ventral horn; both cell types highly increase in number at 2 wpl compared to unlesioned control fish. However, the \( Tg(vsx1:GFP)^+ \) cells appear to be located more dorsal in comparison to the \( Tg(HB9:GFP)^+ \) cells.

During development, Lee et al. have shown that \( Tg(HB9:GFP)^+ \) motor neurons and \( Tg(vsx1:GFP)^+ \) cells form distinct cell populations and are connected via a negative regulatory feedback-loop (Lee et al., 2008).
To establish that these two cell types are distinct from each other in regeneration, we performed immunohistochemical labeling of HB9 (Fig. 2.11A) and islet-1/2 (Fig. 2.11 B) in spinal cross sections of Tg(vsx1:GFP) fish at 2 wpl.

We analyzed 90 Tg(vsx1:GFP)+ cells and 208 islet-1/2+ cells (n = 12 sections, 2 animals). 95.6% of the Tg(vsx1:GFP)+ interneurons were islet-1/2-. We quantified 10 sections out of three animals with clearly detectable HB9+ immunohistochemical signal and Tg(vsx1:GFP)+ cells. I counted 120 Tg(vsx1:GFP)+ cells and 86 HB9+ cells. 91.67% of the Tg(vsx1:GFP)+ cells were HB9-.

Fig. 2.11: Tg(vsx1:GFP)+ cells are distinct from HB9+ or islet-1/2+ cells. Labeling of HB9 (red) in column A or Islet-1/2 (red) in column B in spinal cross section of transgenic Tg(vsx1:GFP)+ (green) fish at two weeks post-lesion. 91.7% of Tg(vsx1:GFP)+ cells are HB9+. 95.6% Tg(vsx1:GFP)+ cells are Islet-1/2-. Dots indicate the central canal. Unfilled arrowheads point to HB9+/Tg(vsx1:GFP) cells (A) or Islet-1/2+/Tg(vsx1:GFP) cells (B).

Scale bars = 25 µm.
To confirm that the transgenic line \emph{Tg(vsx1:GFP)}\(^+\) reliable reports expression of vsx1, Tatyana B. Dias performed \emph{in situ} hybridization for vsx1 mRNA in spinal cross sections of adult zebrafish (Fig. 2.12). She observed colocalization between \emph{Tg(vsx1:GFP)}\(^+\) and mRNA expression.

In summary, we conclude that \emph{Tg(vsx1:GFP)}\(^+\) are distinct from HB9\(^+\) and islet-1/2\(^+\) motor neurons and the transgene accurately reports V2 interneurons.

**Fig. 2.12**: GFP enhancement and \emph{in situ} hybridization for vsx1 mRNA in spinal cross sections of \emph{Tg(vsx1:GFP)} transgenic fish. \(A\): In unlesioned control animals, neither vsx1 mRNA nor \emph{Tg(vsx1:GFP)} transgene expressing cells are detectable. At two weeks post-lesion, rostral (\(B\)) and caudal (\(C\)) to the lesion site, vsx1 mRNA and \emph{Tg(vsx1:GFP)} transgene expression are increased. Arrows indicate colocalization of vsx1 mRNA and \emph{Tg(vsx1:GFP)} transgene expression. Asterisk indicates the central canal. Scale bar = 50 µm.

(This figure was generously provided by Tatyana B. Dias.)
2. Cell types in the spinal cord of adult zebrafish

2.2.8 Rostro-caudal difference in \(Tg(vsx1:GFP)^+\) cell numbers

Previous observations showed differences in axonal innervation rostral and caudal to the lesion (see chapter 1). These findings prompted us to investigate separately the numbers of \(Tg(vsx1:GFP)^+\) cells – in the rostral and caudal spinal cord, respectively. The number of newly generated \(Tg(HB9:GFP)^+\) motor neurons is higher in the rostral than in the caudal spinal cord at 2 wpl (Fig. 2.27B and see chapter 3). During development, V2 interneurons are generated at the expense of motor neurons (Yang et al., 2006; Lee et al., 2008). Genetic regulatory feedback mechanism drive a progenitor cell to develop into a motor neuron and depress meachansim that would propagate interneuron development, and the other way round. Knock-out of the motor neuron specifing fators LMO4 resulted in an increase in number of interneuron (Lee et al., 2008). Thus, we hypothesized that the rostral-caudal ratio of V2 interneurons might be the inverse of the rostral-caudal ratio of motor neurons during regeneration. In consequence, we expected to find more \(Tg(vsx1:GFP)^+\) cells caudal than rostral to the lesion site. Indeed, at 2 wpl, the number of \(Tg(vsx1:GFP)^+\) cells is significantly higher caudal than rostral to the lesion site (Mann-Whitney, one-tailed, rostral: 245.9 ± 35.42 cells/750 µm, n = 12; caudal: 382.3 ± 66.97 42 cells/750 µm, n = 12, p < 0.05, Fig. 2.13B,B').

![Image](image.png)
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.13: Numbers of Tg(vsx1:GFP)$^+$ cells are higher in the caudal than in the rostral half of the lesion site at two weeks post-lesion. Spinal cross section of transgenic Tg(vsx1:GFP)$^+$ fish before lesion (A), at two weeks post-lesion (B) and at six weeks post-lesion (B',C'). Quantification of Tg(vsx1:GFP)$^+$ cells. At two weeks post-lesion, numbers of Tg(vsx1:GFP)$^+$ cells are caudal significantly higher than rostral to the lesion site (B'). At six weeks post-lesion, number of Tg(vsx1:GFP)$^+$ cells do not differ any more rostral and caudal to the lesion site (C'). Arrowheads point to Tg(vsx1:GFP)$^+$ cells. Arrows and dots indicate the central canal. Scale bar = 15 µm.

This result suggests that during regeneration, motor neurons are generated at the expense of V2 interneurons. Interestingly, at 6wpl, numbers of Tg(vsx1:GFP)$^+$ cells no longer differed rostral and caudal to the lesion site (rostral: 293.9 ± 91.78 cells/750 µm, n = 6; caudal: 279.4 ± 81.97 cells/750 µm, n = 6; p > 0.05) (Fig. 2.13C,C'), possibly suggesting that axonal innervation of the caudal spinal cord reduces V2 interneuron generation there.

2.2.9 Tg(vsx1:GFP)$^+$ cells are different from pax2$^+$ interneurons

I hypothesized that in adult zebrafish spinal cord, pax2$^+$ cells and Tg(vsx1:GFP)$^+$ cells will form distinct cell populations in the regenerating spinal cord of adult zebrafish. In zebrafish embryonic spinal cord, Tg(vsx1:GFP) transgene expressing cells are in an intermediate progenitor stage that gives raise to pair-producing neuronal cells. The two resulting cellular siblings eventually mature to V2a and V2b interneurons – the first ones retain their Tg(vsx1:GFP) transgene expression, whereas the latter ones loses it eventually (Kimura et al., 2008).

During development, Tg(vsx1:GFP) transgene is expressed in CoPA neurons and CiAS neurons at 26 hpf (Yeo, 2009). CiA interneurons are considered to be homologous to V1 interneurons (Higashijima et al., 2004b; Sapir et al., 2004). They share common features, for example they release inhibitory transmitters such as glycine and GABA, they possess ascending ipsilateral axons and they control fast movement (Higashijima et al., 2004b; Li et al., 2004; Gosgnach et al., 2006). A combination of the transcription factor pax2 (comprising pax2a and pax2b) and pax8 determines the glycinergic and GABAergic fate of CiA interneurons (Batista and Lewis, 2008). Also in mice, pax2 is expressed in a subpopulation of interneurons including V1 interneurons (Burrill et al., 1997).
Thus, *pax2* expression is another shared feature of CiA interneurons and V1 interneurons.

In summary, during development, *Tg(vsx1:GFP)* transgene expressing cells are progenitors for V2 interneurons, whereas the transcription factor *pax2* specifies the cell fate of V1 interneurons. Thus, I did not expect to find colocalization between *Tg(vsx1:GFP)* transgene expressing cells and *pax2*+ labeling in the regenerating adult spinal cord.

We analyzed 162 *Tg(vsx1:GFP)*+ cells and 409 *pax2*+ cells (n = 9 section, 3 animals). We only observed 4 cells with colocalization of *Tg(vsx1:GFP)*+ and *pax2*. Thus, only a minority of *Tg(vsx1:GFP)*+ cells (2.3%) are *pax2*+ (Fig. 2.14). We can conclude that *Tg(vsx1:GFP)*+ cells and *pax2*+ cells are distinct cell populations in zebrafish spinal cord.

![Double-labeling of vsx1:GFP (green) and pax2 (red) in spinal cross section of recovered fish caudal to the lesion site. Dorsal is up. 97.7% of Tg(vsx1:GFP)+ cells were pax2-. Unfilled arrowheads point at Tg(vsx1:GFP)+/pax2- cells, filled arrowheads point to Tg(vsx1:GFP)/pax2+ cells. Dots outline the central canal. Scale bar = 10 µm.](image)

**Fig. 2.14: Tg(vsx1:GFP)+ cells are distinct from pax2+ cells.**

In the developing neural tube, V2 interneurons emerge from the *pax6+/nkx6.1+/olig2-* zone (Thaler et al., 2002 and see Fig. 2.15A). We hypothesized that we would find a similar pattern during regeneration. As
recently published, at 2 wpl, the expression of the transcription factors nkx6.1, pax6, and the Tg(olig2:GFP) transgene are strongly upregulated compared to unlesioned control fish (Reimer et al., 2009).

I analyzed 64 Tg(vsx1:GFP)$^+$ cells that did not contact the central canal and 12 Tg(vsx1:GFP)$^+$ cells with central canal contact at 2 wpl (n = 12 sections, 2 animals). From the cells devoid of ventricular contact, approximately a third (34.4%) of the Tg(vsx1:GFP)$^+$ cells were pax6$^+$/nkx6.1$^+$, half (50.0%) were pax6$^+$/nkx6.1$^-$ and a minority (3%) were pax6$^-$/nkx6.1$^+$. 12.5% of the Tg(vsx1:GFP)$^+$ cells colocalized with neither pax6 nor nkx6.1. The Tg(vsx1:GFP)$^+$ cells with central canal contact showed a similar ratio: roughly one third (33.3%) were pax6$^+$/nkx6.1$^+$, half (50.0%) were pax6$^+$/nkx6.1$^-$, none of them were pax6$^-$/nkx6.1$^+$ and 17% colocalized with neither pax6 nor nkx6.1 (Fig. 2.15B).

I analyzed 18 Tg(vsx1:GFP)$^+$ cells with central canal contact in Tg(vsx1:GFP)$^+$ x Tg(olig2:DsRed2) animals (n = 4 animals). Only one Tg(vsx1:GFP)$^+$ cell colocalized with Tg(olig2:DsRed2) (Fig. 2.15C).

Therefore, 95% of the Tg(vsx1:GFP) transgene expressing cells were negative for the Tg(olig2:DsRed2) transgene.

These data indicate that Tg(vsx1:GFP)$^+$ are regenerated from a p2-like zone during spinal cord regeneration in adult zebrafish. However, given that 50% of cells with central canal contact are nkx6.1$^-$, we cannot exclude that some neurons are generated from an even more dorsal nkx6.1$^-$/pax6$^+$ zone.

Events involved in motor neuron generation after a spinal lesion in adult zebrafish are summarized in Figure 2.15D.
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.15: Tg(vsx1:GFP)$^+$ cells originate from a p2-like zone.

**A**: Schematic presentation of transcription factor domains set up in the developing vertebrate neural tube by a ventro-dorsal gradient of Shh (modified after Vallstedt and Kullander, 2007). Different progenitor zones (p0-3) give rise to distinct classes of interneurons (V0-3). The p2 zone (pax6$^+$/nkx6.1$^+$/Olig2$^-$) gives rise to V2 interneurons.

**B**: Triple labeling of gfp (green), pax6 (red) and nkx6.1 (blue) in a spinal cross section of transgenic Tg(vsx1:GFP) fish at two weeks post-lesion. Arrowheads point to two adjacent Tg(vsx1:GFP)$^+$ cells that are pax6$^+$/nkx6.1$^+$, shown in high magnification.
2. Cell types in the spinal cord of adult zebrafish

C: Spinal cross section of a Tg(vsx1:GFP)X Tg(olig2:DsRed2) fish line at two weeks post-lesion. Filled arrowheads indicate two Tg(vsx1:GFP)+ cells that are located dorsal of the (olig2:DsRed2) transgene expressing zone. The empty arrowheads point to an oligodendrocyte. The dotted line outlines the central canal.

D: Summary of known events associated with neuron regeneration in adult zebrafish. Schematic cross section through the spinal cord is shown (the central canal is represented by a grey oval). After a lesion, the central canal widens close to the lesion site, concomitant with an increase in numbers of ependymo-radial glial cells that also express higher levels of transcription factors. A ventro-dorsal hh signal, evidenced by a ventro-dorsal patched1 (Ptc1) mRNA expression gradient in ependymo-radial glial cells, contributes to production of Hb9+/islet-1/-2+/ChAT+ motor neurons (MNs) from pMN-like ependymo-radial glial cells (green) after a lesion (Reimer et al, 2009) and likely also to V2 interneurons from a p2-like zone.

Scale bars A = 10 µm and B = 15 µm.

2.2.11 Pax2+ cells are newly generated but do not increase in number after lesion

For studying localization of putative interneurons in the spinal cord of adult zebrafish, I used the transgenic fish line Tg(pax2a:GFP) (Picker et al., 2002). Pax2a is a transcription factor associated with neuronal development and specification and is a transcription factor downstream of Notch signaling. During development in zebrafish, CoPA commissural neurons and CiA interneurons at 26 hours post fertilization (hpf) express Tg(pax2a:GFP) transgene (Yeo, 2009). Also in chick, pax2 is expressed in various spinal interneurons (Burrill et al., 1997). Interestingly, I find Tg(pax2a:GFP) transgene expressing neurons still to be present in the adult spinal cord (Fig. 2.17). Occasionally, I observe commissural projections running through spinal cross section (Fig. 2.17). Thus, I suggest that in adult zebrafish spinal cord, pax2 might still be a marker for possibly inhibitory interneurons that project to the contralateral spinal cord (Batista and Lewis, 2008). To confirm the reliability of this transgenic fish for study of the adult spinal cord, I performed immunohistochemical co-labeling with the pax2 antibody in spinal sections of unlesioned control animals and at 2 wpl. In unlesioned control animals, I analyzed 104.00 Tg(pax2a:GFP)+ cells and 479 pax2+ cells (n = 24 sections, 4 animals). I observed 81 Tg(pax2a:GFP)+ /pax2+ cells. Thus, 78.9% of the Tg(pax2a:GFP) transgene expressing cells colocalized with pax2 antibody labeling. Therefore, I suggest that the transgenic line Tg(pax2a:GFP) reliably reports pax2a expression. About 20% the Tg(pax2a:GFP)+ cells are not labeled.
with pax2 antibody. Thus, the transgene is possibly more sensitive than the antibody.

At 2 wpl, I analyzed 296 $Tg(\text{pax2a}:\text{GFP})^+$ cells and 942 pax2$^+$ cells ($n = 24$ sections, 4 animals). I observed 191 $Tg(\text{pax2a}:\text{GFP})^+$/pax2$^+$ cells. Thus, 64.5% of the $Tg(\text{pax2a}:\text{GFP})$ transgene expressing cells colocalized with pax2 antibody.

To investigate how pax2$^+$ cells are affected by spinal cord lesion, I analyzed numbers of pax2$^+$ cells in the dorsal and ventral spinal cord in unlesioned control animals, at 2 wpl and at 6 wpl (unlesioned: 2661.1 ± 392.67 cells/1500 µm, $n = 4$; 2 wpl: 2608.4 ± 56.25 cells/1500 µm, $n = 4$; 6 wpl: 2048.6 ± 371.81 cells/1500 µm, $n = 4$, Fig. 2.16 A-D).

I did not observe any significant differences in cell numbers. Separate analysis of the numbers of pax2$^+$ cells in the dorsal and ventral horn, respectively, did not reveal any differences either. At 6 wpl, 17.0% of the all pax2$^+$ cells were labeled with BrdU (348.6 ± 172.34, parvalbumin$^+$/BrdU$^+$ cells; 2048.6 ± 371.81 parvalbumin$^+$ cells, $n = 4$, Fig. 2.16B",C). Interestingly, when I calculate BrdU labeling rate separately for dorsal and ventral pax2$^+$ cells, I find a higher percentage of BrdU labeled cells in the ventral (30%) than in dorsal horn (10%), however the difference is not significant. As there is no increase in cell number between 2 wpl and 6 wpl, these results indicate a turn-over of pax2$^+$ cells. Further experiments might include labeling with BrdU at 2 wpl and TUNEL labeling combined with pax2 labeling at various time points between 2 wpl and 6 wpl.
2. Cell types in the spinal cord of adult zebrafish

- A - A'  
  no lesion  

- B - B' - B'''  
  six weeks post-lesion  

- C  
  pax2/BrdU

- D  
  pax2+ cells

Phase contrast
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.16: Pax2$^+$ cells are newly generated after lesion but do not increase in cell number.

**A:** Labeling of pax2 in spinal cross section of unlesioned control animals; dorsal is up. **A’** shows the phase contrast

**B, B’, B’’:** Double-labeling of pax2 (green) and BrdU (red) in spinal cross section at six weeks post lesion. **B** shows the channel for pax2, **B’** shows the phase contrast and **B’’** shows the double-labeling. Inset (C) outlines two pax2$^+$ cells labeled with BrdU. Arrowheads point to double-labeled cells. **D** Quantification of pax2$^+$ cells in unlesioned control fish and at two and six weeks post-lesion.

Scale bar = 15 µm.
2. Cell types in the spinal cord of adult zebrafish

2.2.12 *Tg(Pax2:GFP)* interneurons and parvalbumin* interneurons form distinct neuronal cell populations in the spinal cord of adult zebrafish

I wanted to investigate if pax2* interneurons and parvalbumin* interneurons are distinct cell populations in adult zebrafish spinal cord. In the embryonic spinal cord of zebrafish, most pax2* interneurons express the inhibitory neurotransmitter GABA or glycine (Batista and Lewis, 2008). In the adult spinal cord of mice, V1 derived interneurons express parvalbumin. Their neurotransmitter is also mainly GABA or glycine and not the excitatory neurotransmitter glutamate (Alvarez et al., 2005). These publications indicate that pax2 and parvalbumin colocalize in the same cell population. I analyzed 20 *Tg(pax2:GFP)* cells and 57 parvalbumin* cells within the same spinal sections of unlesioned control animals (n = 3 animals, Fig. 2.17). I did not observe any colocalization between *Tg(pax2:GFP)* transgene expression and parvalbumin immunoreactivity. Thus, they form two distinct cell populations. The neurotransmitter phenotype of *Tg(pax2:GFP)* cells and parvalbumin* neurons in the adult spinal cord of zebrafish remains to be determined, for example by double-labeling of parvalbumin/GABA or pax2/GABA. Also, the transgenic fish Glyt2:GFP (McLean et al., 2007b) might be used to test whether parvalbumin* or pax2* cells are glycinergic.

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Fig. 2.17: Double-labeling of GFP (green) and parvalbumin (red) in spinal cross section of transgenic *Tg(pax2a:GFP)* fish; dorsal is up. Inset A outlines cells in the dorsal horn and is shown as zoom-out in A’, A” and A”’ with the respective channels (green, red, merge). Inset B outlines cells in the dorsal horn and is shown as zoom-out in B’, B”.
and B'''' with the respective channels (green, red, merge). Empty arrowheads indicate Tg(pax2a:GFP)+/parvalbumin- cells, filled arrow heads indicate Tg(pax2a:GFP)-/parvalbumin+. No double-labeled cells were observed. Scale bars A = 25 μm, B = 15 μm.

2.2.13 Dorsal parvalbumin+ cells are not newly generated and do not die after lesion

To my knowledge, parvalbumin+ cells have been described neither in embryonic nor in adult zebrafish spinal cord to date.

In the adult spinal cord of mice, V1 derived inhibitory interneurons and motor neurons express parvalbumin (Alvarez et al., 2005). In zebrafish brain, life-long neurogenesis occurs in several brain regions: Parvalbumin+ cells are newly generated in the dorsal telencephalic areas (Grandel et al., 2006).

In adult zebrafish spinal cord, I have already described that a subpopulation of parvalbumin+ motor neurons is located ventro-lateral of the central canal. The diameter of the parvalbumin+ cells in the ventro-lateral portion ranges between ~ 12 and 17 μm. However, we also occasionally find smaller parvalbumin+ cells in the range of ~5 and 11 μm in the ventral horn. I analyzed large 30 parvalbumin+ cells and 49 small parvalbumin+ in the ventral horn of spinal cross section of transgenic Tg(HB9:GFP) fish (n = 7 sections, 3 animals). 24 large parvalbumin+ cells colocalize with Tg(HB9:GFP) transgene. Thus, the majority (~80%) of the large parvalbumin+ cells are likely to be motor neurons. In contrast, only one parvalbumin+ cells colocalized with Tg(HB9:GFP)+. Thus, the majority (~98%) of small ventral parvalbumin+ cells appear to be a cell population distinct from motor neurons. Interestingly, this result is in agreement with observations reported by Clowry et al. (2000): human fetal spinal cords harbor few parvalbumin positive, non-motor neurons (Clowry et al., 2000).

In the dorsal horn, solely small cells with a diameter of ~5 and 7 μm could be identified. These parvalbumin+ cells are always Tg(HB9:GFP). This suggests that the parvalbumin+ cells present a mixed population comprising exclusively parvalbumin+/Tg(HB9:GFP)- in the dorsal horn as well as a heterogeneous ventro-lateral cell population with both parvalbumin+/Tg(HB9:GFP)+ cells and parvalbumin+/Tg(HB9:GFP)- cells (Fig. 2.2 and Fig. 2.18A-C).
2. Cell types in the spinal cord of adult zebrafish

Numbers of parvalbumin$^+$ cells in unlesioned control fish, at 2 wpl and at 6 wpl in the dorsal horn did not show any significant differences (unlesioned: 638.9 ± 92.24 cells/1500 µm, n = 4; 2 wpl: 735.2 ± 76.68 cells/1500 µm; n = 6; 6 wpl: 716.7 ± 185.39 cells/1500 µm, n = 4, Fig. 2.18F). After injection with BrdU at day 0, day 2 and day 4 post-lesion, I observed 1.9 ± 1.85 BrdU labeled parvalbumin$^+$ cells/1500 µm at 2 wpl and 16.7 ± 5.55 cells/1500 µm parvalbumin$^+$/BrdU$^+$ cells at 6 wpl (Fig. 2.18D-F). At 2 wpl, 0.3% and at 6 wpl, 2% of the parvalbumin$^+$ cells were labeled with BrdU. In summary, these data indicate that parvalbumin$^+$ cells are rarely newly generated. The 2% labeling rate with BrdU suggests a turn-over of parvalbumin$^+$ cells, however only within a very small range. Thus, I conclude that parvalbumin$^+$ cells appear mainly unaffected by spinal lesion.
2. Cell types in the spinal cord of adult zebrafish

**A**

parvalbumin

no lesion

**B**

parvalbumin

**C**

parvalbumin/BrdU

six weeks

**D**

parvalbumin

**E**

parvalbumin/BrdU

**F**

- **F1**
  - 1000
  - 800
  - 600
  - 400
  - 200
  - 0

- **F2**
  - parvalbumin+ cells
  - parvalbumin+/BrdU+ cells

- **F3**
  - no lesion
  - 2 weeks
  - 6 weeks

- **F4**
  - n=4
  - n=6
  - n=4
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.18: Parvalbumin+ cells are rarely newly generated after lesion.
A: Labeling of parvalbumin (green) in spinal cross section of unlesioned fish; dorsal is up. Insets depict cells in the dorsal horn (B) and in the ventral horn (C) and are shown in high magnification (right).
D: Double-labeling of parvalbumin (green) and BrdU (red) in spinal cross sections of recovered fish at six weeks post-lesion. Inset depicts one double-labeled cell in the dorsal horn (E), shown in high magnification for parvalbumin (E”), BrdU (E’) and merge (E). Arrowheads point to parvalbumin+ cells and arrows point to parvalbumin+/BrdU+ cells. The star (A) and the dots (B) indicate the central canal.
F: Quantification of parvalbumin+ cells (white bar) and parvalbumin+/BrdU+ cells (black bar) in unlesioned control fish and at two and six weeks post-lesion. Scale bars = 20 μm.

2.2.14 GABA+ cells are not newly generated at two weeks post-lesion

Gamma aminobutyric acid positive (GABA+), similar to dopamine and 5-HT, are involved in modulation of the CPG by binding to the GABA\(_B\) receptor (Grillner, 2003; Schmitt et al., 2004; Grillner and Jessell, 2009).

To identify GABA+ neurons in the embryonic zebrafish spinal cord, Higashijima et al. (2004) performed \textit{in situ} hybridization for GAD65 and GAD67, the enzymes for synthesizing the inhibitory neurotransmitter GABA. They also performed immunohistochemistry for GABA and reported an almost complete overlap between GAD65/67 and GABA. Staining intensity strongly varied among adjacent cells in the same section – a phenomenon I also observed in the adult spinal cord. In the zebrafish embryo, Higashijima et al. (2004) identified GAD65/67 mRNA expressing cells in the mediodorsal region and in the ventral cord (Higashijima et al., 2004a). Those cells located in the ventral spinal cord contact the central canal and appear to be Kolmer-Agduhr (KA) cells. K. A. homologues seem to be present across the vertebrates, including mammalians. In xenopus, Dale et al. (1987) described for the first time the GABA+ phenotype of the KA cells (Dale et al., 1987). Recently, it was shown in zebrafish larvae that the K.A. cell are involved in spontaneous locomotion (Wyart et al., 2009). In adult spinal cord, I could also identify a GABA+ cell population with central canal contact. Their diameter ranges ~ 5 and 6 μm. They possess a round shape and an endfoot contacting the central canal. Occasionally, long neurites extend ventrally to the pial surface (Fig. 2.19A). Possibly, these cells represent the KA cells described during development. My observations are in line with those
made in lamprey. Lamprey also possess a GABA+ liquor contacting cell population within the spinal cord (Brodin et al., 1990). I observed widespread GABA+ staining within the spinal cord. It was conspicuous that GABA+ cells contacting the central canal showed the strongest signal intensity.

I wanted to investigate whether inhibitory GABA+ interneurons would be affected by spinal lesion. Their close proximity to the central canal suggests that they might be prone to lesion-induced events such as cell death or proliferation. I did not observe differences in cell number at 2 wpl compared to unlesioned control fish (unlesioned: 576.15 ± 47.20, n = 3; 2 wpl: 621.46 ± 7.18, n = 2). To test if GABA+ cells are newly generated after lesion, I injected BrdU at 0, 2 and 4 days post-lesion. I did not observe any BrdU labeled GABA+ cells at 2 wpl (Fig. 2.19B). Thus, at 2 wpl, central-canal contacting GABA+ cell appear to be unaffected by a spinal lesion.

Future studies might include comparison of GABA staining and GAD65/67 expression in the adult spinal cord and additional time points after lesion.
2. Cell types in the spinal cord of adult zebrafish

![Images of cell types in the spinal cord with GABA and BrdU markers, showing no lesion and two weeks post-lesion.]
Fig. 2.19: Central canal contacting GABA$^+$ cells are not affected by spinal lesion.

**A**: Labeling of GABA in spinal cross sections of unlesioned animals; dorsal is up.

**B**: Double-labeling of GABA and BrdU in spinal cross sections at two weeks post-lesion. Arrowheads point to central canal contacting GABA$^+$ cells. None of the GABA$^+$ cells is labeled with BrdU. Dots outline the central canal.
Scale bar = 10 µm.

### 2.2.15 GAD67:GFP$^+$ cells were identified in the ventral and dorsal horns in adult zebrafish spinal cord

The enzyme glutamate decarboxylase 1 (GAD67, now also named gab1b) is involved in the biosynthesis of the inhibitory neurotransmitter GABA. Two different genes for the enzyme GAD have been identified (Erlander et al., 1991).

I contributed to the generation of the transgenic fish line $Tg$(GAD67:GFP) (unpublished, Reimer MM, Kuscha V, Becker T) and started to study the expression pattern of the transgene. I observe $Tg$(GAD67:GFP)$^+$ cells in various areas in the brain. Figure 2.20A shows exemplary $Tg$(GAD67:GFP)$^+$ cells in the optic tectum. The optic tectum consists of several layers which are named – from the deepest layer to the most superficial layer – periventricular layer (SPV), stratum album centrale (SAC), stratum griseum centrale (SGC) and stratum fibrosum and griseum superficiale (SFGS) (Madigou et al., 2000).

$Tg$(GAD67:GFP)$^+$ cells appear to be accumulated in high density within the SPV and are sparse in the other layers. Some of the $Tg$(GAD67:GFP)$^+$ cells within the SPV bear elaborate neurites that project to the SAC, the SGC and the SFGS.

Future studies will include systematic description of $Tg$(GAD67:GFP) transgene expression within the brain.

Within the spinal cord, I also observed few $Tg$(GAD67:GFP)$^+$ cells in the dorsal and ventral horns (Fig. 2.20B). The cellular pattern that I observe in the adult spinal cord and tectum is in agreement with previous data by Dr. Michell M. Reimer showing the expression of GAD67 mRNA with *in situ* hybridization (Fig. 2.21A, B). In addition to cells, I observe $Tg$(GAD67:GFP)$^+$ neuropil in the dorsal horn. The $Tg$(GAD67:GFP)$^+$ neuropil does not cover the complete area of the dorsal horn (Fig. 20B). I do not observe any cells with central canal contact, as for GABA labeling. This is very surprising, as during development, GAD67 has been to shown to be required for GABA synthesis in KA cells (Yang et al., 2010).
To confirm colocalization between \textit{Tg(GAD67:GFP)} transgene, GAD67 mRNA and GAD67 protein, the next experiments will comprise \textit{in situ} hybridization for GAD67 and immuno-labeling with an antibody for GAD67 (Bae et al., 2009) in \textit{Tg(GAD67:GFP)} embryos and spinal cord sections of adult \textit{Tg(GAD67:GFP)} fish. Future experiments will also include a systematic study to monitor \textit{Tg(GAD67:GFP)} transgene expression at different time points throughout development.

**Fig. 2.20:** \textit{Tg(GAD67:GFP)}$^+$ cells in spinal cord and tectum are shown. GFP enhancement in an unpublished \textit{Tg(GAD67:GFP)} transgenic line in tectum (A) and spinal cord (B) in adult zebrafish. Arrows point to \textit{Tg(GAD67:GFP)}$^+$ cells. The asterix marks the central canal. Scale bar = 50 µm.
Fig. 2.21: GAD67^-mRNA expressing cells in tectum and spinal cord are shown. *In situ* hybridization for GAD67 mRNA in tectum (*A*) and spinal cord (*B*, dorsal is up) of adult zebrafish. Arrowheads point to GAD67^-cells.

(This figure was generously provided by Dr. Michell M. Reimer.)
2.2.16  *Tg(Olig2:GFP)*+ cells are newly born after spinal lesion

During development, oligodendrocytes and motor neurons emerge from the same ventral region within the neural tube (Park et al., 2002). Park et al. (2007) report that *Tg(Olig2:GFP)*+ ependymo-radial glial cells are slowly proliferating in post-embryonic zebrafish. They suggest that *Tg(Olig2:GFP)*+ ependymo-radial glial in post-embryonic zebrafish retain their potential for self-renewal and generation of oligodendrocytes by asymmetric cell divisions. However, in contrast to development, *Tg(Olig2:GFP)*+ ependymo-radial glial cells only produce oligodendrocytes but not neurons in the adult unlesioned spinal cord. During regeneration, Reimer et al. have already shown that lesion induces a developmental switch in the spinal cord: pMN-*Tg(Olig2:GFP)*+ ependymo-radial glial cells proliferate and generate motor neurons during regeneration (Reimer et al., 2008). Does lesion also lead to generation of more oligodendrocytes from *Tg(Olig2:GFP)*+ ependymo-radial glial cells than in the unlesioned fish?

Following a spinal lesion, 8 hours after a single injection of BrdU, I observe BrdU labeled *Tg(Olig2:GFP)*+ cells devoid of central canal contact in the parenchyma (Fig. 2.22). These preliminary results raise further questions: Are these *Tg(Olig2:GFP)*+ cells oligodendrocytes? Do numbers of newly generated oligodendrocytes increase during recovery compared to unlesioned control fish? Do the oligodendrocytes within the parenchyma emerge from the ependymo-radial glial zone? Or do these oligodendrocytes renew themselves? Are these newly born oligodendrocytes functional in terms of their myelination of axons? These questions might be answered in future studies. To clarify if the parenchymal BrdU labeled *Tg(Olig2:GFP)*+ cells give rise to oligodendrocytes or motor neurons, further experiments might include their characterization with oligodendrocyte cell lineage markers such as sox10 or Claudin K (Morita et al., 1999; Wegner, 1999; Wissmuller et al., 2006), or with motor neuron lineage markers such as HB9 and Islet-1/2. Quantification of BrdU labeled *Tg(Olig2:GFP)*+ cells after lesion compared to unlesioned fish might answer the question of whether regeneration promotes generation of *Tg(Olig2:GFP)*+ cells. Interestingly, Park et al. (2007) suggest that some oligodendrocytes in the grey
as well in the white matter are still proliferating in the spinal cord of post-embryonic zebrafish. Labeling of parenchymal $Tg(Olig2:GFP)^+$ cells with acute proliferation markers such as proliferating cell nuclear antigen (PCNA) or phospho-histone 3 and quantitative analysis after lesion compared to control animals might elucidate whether lesion enhances their ability for self-renewal.

Fig. 2.22: $Tg(Olig2:GFP)^+$ cells devoid of central canal contact are newly generated after spinal lesion. Double-labeling of GFP (green) and BrdU (red) in spinal cross section of $Tg(Olig2:GFP)$ transgenic fish at 9 days post-lesion; dorsal is up. The white arrow points to a BrdU labeled $Tg(Olig2:GFP)^+$ cell devoid of ventricular contact, shown in high magnification in the bottom row. The open arrow points to $Tg(Olig2:GFP)^+$ ependymo-radial glial cells labeled with BrdU. Dots outline the central canal.
Scale bars = 10 µm, 5 µm (in high magnification).
2. Cell types in the spinal cord of adult zebrafish

2.2.17 A large transcriptional regulatory region of growth associated protein GAP43 is required to drive GFP expression after spinal lesion

In the context of spinal plasticity after lesion, I performed a pilot study in collaboration with Dr. Udvadia (University of Milwaukee, USA) in Edinburgh. I compared the GFP expression in two transgenic Tg(GAP43:GFP) fish lines. These fish lines differ in the length of the transcriptional regulatory region of GAP43 to drive GFP:

Kusik et al. analyzed the full length 3.6-kb fugu gap43 regulatory sequence that shows conserved regions between mammals and fish (Kusik et al., 2010). The authors induced deletion mutations at the 5′-end of the 3.6-kb fugu gap43 regulatory sequence. Then, they tested how these mutations affect the expression pattern of the Tg(GAP43:GFP) transgene. The GfG43-708 promoter which comprised the proximal 708 bp part of the of the 3.6-kb fugu gap43 regulatory sequence drives transgene expression in the developing nervous system almost as strongly as the full-length promoter GfG43S/A. In contrast, during regeneration of the axons of retinal ganglion cells, the GfG43-708 promoter does not drive transgene expression. I hypothesized that after spinal lesion, Tg(GAP43:GFP) transgene expression will also require the full length promoter GfG43S/A.

Previous work detected GAP43 mRNA upregulation by in situ hybridization in the lesioned spinal cord. After lesion at the transition between brainstem and spinal cord, the number of GAP43 mRNA expressing cells in the spinal cord peaked in a region 750 µm caudal to the lesion site at 6 dpl (Becker et al., 2005). Therefore, I used the same time point and position within the spinal cord to compare the two different promoters. In unlesioned control animals, I could not detect any Tg(GAP43:GFP)+ cells, neither with the full length promoter GfG43S/A nor with the truncated promoter GfG43-708 promoter (n = 3 animals, Fig. 2.23A,B, left panels). With the full-length promoter GfG43S/A, I observed Tg(GAP43:GFP)+ fibers exclusively in the dorsal horn (Fig. 2.23A, left panel). After lesion, I observed Tg(GAP43:GFP)+ cells in addition to the Tg(GAP43:GFP)+ neuropil (Fig. 2.23A, right panel). At 6 dpl, the numbers of Tg(GAP43:GFP)+ cells...
were 573.5 ± 217.12 cells/750 µm (n = 3 animals) compared to 0 cells in unlesioned control fish (n = 3 animals).

For the truncated promoter GfG43-708, I did not observe GFP expression in the dorsal neuropil in unlesioned fish nor any Tg(GAP43:GFP)+ cells after lesion (n = 3 animals, Fig. 2.23B). These results indicate that parts of the sequence that were deleted in the GfG43-708 are necessary for transgene expression after a spinal lesion (Fig. 2.23C). Further experiments using transgenic fish with various other promoter sequences would allow narrowing down the minimal promoter elements.

Previously, Becker et al. reported an increase of GAP43 mRNA positive profiles from 0.6 ± 0.16 profiles/section in unlesioned animals to 16.8 ± 2.82 profiles/section at 6 dpl (Becker et al., 2005). In the transgenic fish line Tg(GAP43:GFP)+ with GfG43S/A promoter, I observed an increase from 0 cells/section to ~48 cells/section. Even though I detected more Tg(GAP43:GFP)+ cells after lesion than previously reported, cell numbers detectable with the transgenic fish or in situ hybridization are within the same magnitude, showing an increase in Tg(GAP43:GFP) transgene or GAP43 mRNA expressing cells after spinal lesion. Future studies should include direct confirmation of the transgene expression by in situ hybridization.
Fig. 2.23: Comparison of $Tg(GAP43:GFP)$ transgene expression driven by full length GfG43S/A or truncated GfG43-708 promoter after a spinal lesion. Spinal cross sections of a transgenic $Tg(GAP43:GFP)$ fish; dorsal is up. Fish were lesioned at the transition between brain stem and spinal cord. Analysis took place at six days post-lesion caudal to the lesion site.

A Transgene expression is driven by full-length promoter GfG43S/A. No cells can be detected in unlesioned animals (left). After lesion, GFP$^+$ cells can be detected in the ventral and dorsal horns. Arrowheads point to $Tg(GAP43:GFP)^+$ cells (in the right panel, image acquisition was taken with lower detection threshold than for the unlesioned fish – taking the image with same detection threshold as the unlesioned fish would have resulted in oversaturation of the cells.) The white square depicts $Tg(GAP43:GFP)^+$ neuropil in the dorsal horn. The asterix indicates the central canal.

B Transgene expression is driven by the GfG43-708 promoter. Neither GFP$^+$ cells nor GFP$^+$ neuropil are detectable in unlesioned fish as well as in lesioned animals. Scale bar = 20 µm.

C: The full length Fugu 3.6-kb gap43 promoter – which was used to drive expression in the transgenic line $Tg(GAP43:GFP)$ GfG43S/A – is represented by the black ruler at the top. Numbers on the ruler give the distance (bp) from the translation start site (ATG), and tss indicates the transcription start site. The violet bar marks the GfG43-708 promoter sequence (adjusted after Kusik et al., 2010).
2.2.18 Various cell types upregulate \textit{Tg(GAP43:GFP)} transgene

I have established that \textit{Tg(GAP43:GFP)} transgene is upregulated in cells within the spinal cord after a lesion when the \textit{Tg(GAP43:GFP)} is driven by the full length promoter GfG43S/A. We were interested to know whether a particular cell type upregulates \textit{Tg(GAP43:GFP)} transgene. I analyzed most of the cell types after a lesion at midthoracic level at 2 wpl. However, \textit{in situ} hybridization data of GAP43 mRNA expression indicate that GAP43 expression peaks at 6 dpl after a lesion at brainstem/spinal cord transition (Becker et al., 2005). Given the limitations of this pilot study, I decided to lesion at midthoracic level. Time point of analysis was either 6 dpl or 2 wpl. Future work would require systematic experiments including a comparison of different time points after lesion. Also, lesion at midthoracic level as well as lesion between brainstem and spinal cord should be performed for all different time points. Because GAP43 mRNA expression is higher after a lesion at the transition between brainstem and spinal cord than at a midthoracic level, and GAP43 mRNA expression decreased between 6 dpl and 18 dpl (Becker et al., 2005), a systematic comparison might be useful to elucidate which cell types upregulate GAP43 transgene. To investigate if ChAT\textsuperscript{+} cells or parvalbumin\textsuperscript{+} cells upregulate \textit{Tg(GAP43:GFP)} transgene, analysis took place at 6 dpl.

I analyzed 115 \textit{Tg(GAP43:GFP)}\textsuperscript{+} cells and 156 ChAT\textsuperscript{+} cells (n = 18 sections, 3 animals). I observed 18 double-labeled cells. Thus, 15.7\% of \textit{Tg(GAP43:GFP)} transgene expressing cells are ChAT\textsuperscript{+}, and 10.9\% of the ChAT\textsuperscript{+} cells express \textit{Tg(GAP43:GFP)} transgene (Fig. 2.24D,D').

Within the same sections, we analyzed 173 parvalbumin\textsuperscript{+} cells. I observed 8 parvalbumin\textsuperscript{+}/\textit{Tg(GAP43:GFP)}\textsuperscript{+} cells. Therefore, 7.0\% of the \textit{Tg(GAP43:GFP)}\textsuperscript{+} cells are parvalbumin\textsuperscript{+}, and 4.7\% of the parvalbumin\textsuperscript{+} cells upregulate \textit{Tg(GAP43:GFP)} transgene (Fig. 2.24A,A').

To investigate if pax2\textsuperscript{+} cells or 5-HT\textsuperscript{+} cells upregulate \textit{Tg(GAP43:GFP)} transgene, analysis took place at 2 wpl.

We lesioned at a midthoracic level and analyzed 750 \textmu{}m rostral and caudal to the lesion site and at a far caudal level. We analyzed 77 \textit{Tg(GAP43:GFP)}\textsuperscript{+} cells.
2. Cell types in the spinal cord of adult zebrafish

and 676 pax2\(^+\) cells (n = 17 sections, 2 animals) in the region 750 \(\mu\)m rostral and caudal to the lesion site. \(Tg(GAP43:GFP)^+\) transgene expression and pax2 immuno-reactivity only colocalized in two cells. Therefore, only 26.0% of the \(Tg(GAP43:GFP)^+\) cells express pax2 and only 3.0% of all pax2\(^+\) cells express \(Tg(GAP43:GFP)\) transgene (Fig. 2.24B,B'). Within the same two animals in different sections (n = 10), I quantified 64 \(Tg(GAP43:GFP)^+\) cells and 56 5-HT\(^+\) cells. None of the 5-HT\(^+\) cells showed \(Tg(GAP43:GFP)\) transgene expression (Fig. 2.24C,C'). Far caudal of the lesion site, I could not detect any \(Tg(GAP43:GFP)^+\) cells.

Sections of spinal cord lesioned between brainstem and spinal cord did not show any \(Tg(GAP43:GFP)^+\) /5-HT\(^+\) cells.

For further experiments, I will lesion at the transition of brainstem and spinal cord and analyze numbers of \(Tg(GAP43:GFP)^+\) cells 3.5 mm caudal to the brainstem. I will then be able to exclude \(Tg(GAP43:GFP)\) transgene expression due to injury of axons or dendrites of the respective cell. Moreover, analysis at a greater distance from the lesion site will make it possible to distinguish between resident neurons that upregulate \(Tg(GAP43:GFP)\) transgene as part of lesion-induced morphological plasticity and newly generated neurons. This is because newly generated neurons are only observed in the vicinity of the lesion site.
2. Cell types in the spinal cord of adult zebrafish

A
GAP43:GFP 5A parvalbumin

B
GAP43:GFP 5A pax2

C
GAP43:GFP 5A 5-HT

D
GAP43:GFP 5A ChAT

Phase contrast
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.24: Various cell types express Tg(GAP43:GFP) transgene after spinal lesion. Labeling of parvalbumin (A, red), pax2 (B, red), 5-HT (C, red) and ChAT (D, red) in spinal cross section of transgenic Tg(GAP43:GFP) fish; dorsal is up. The GfG43S/A promoter drives transgene expression. Fish were lesioned at midthoracic level. Analysis took place at six days post-lesion (A, D) or at two weeks post-lesion (B, C). Filled arrowheads point to cells positive for parvalbumin (A), pax2 (B), 5-HT (C) or ChAT (D) cells and negative for Tg(GAP43:GFP) transgene expression. Open arrowheads indicate Tg(GAP43:GFP)+ cells which are negative for the cell markers. Arrows point to double labeled cells. Inset in A, B and D depict double-labeled cells shown as in high magnification A, B and C, A’, B’, C’ and D’ show the amount of colocalization between GAP43 and the respective cell markers. Scale bars are A = 20 µm, inset in A = 5 µm, B = 20 µm, inset in B = 10 µm, C = 20 µm, D = 20 µm and high-magnification in D = 10 µm.
2.3 Discussion

2.3.1 Time courses of various mature and undifferentiated cell markers during recovery elucidate changes mainly in ventral but not in dorsal cell populations

I studied cell types along the dorsoventral axis in the adult zebrafish spinal cord after lesion in comparison to unlesioned control animals. Here, I discuss differences and similarities in their time course of cell numbers during recovery (see Fig. 2.25 to 2.27; Table 2.1 to 2.5).

To compare time courses of cell numbers of various markers during recovery, I have divided the cell markers I have studied into two main categories: markers of mature cells or markers of undifferentiated cells. Mature cell markers are expressed by differentiated neurons and have a distinct function as a neurotransmitter (5-HT, GABA), during synthesis of a neurotransmitter (ChAT) or have a defined biochemical function. For instance, parvalbumin is a marker of oxidative/excitatory-resistant motor neurons, probably due to its calcium-buffering capability (Elliott and Snider, 1995).

As examples for undifferentiated cell markers, I investigated the time course of the number of $Tg(\text{vsx1:GFP})$ transgene expressing cells and I will discuss them together with small diameter $Tg(\text{HB9:GFP})$ transgene expressing motor neurons (Reimer et al., 2008) – both HB9 and vsx1 are transcription factors which are generated depending on a shh gradient during development and are cell lineage markers mainly expressed in undifferentiated motor neurons or interneurons, respectively (Briscoe et al., 2000). However, in the case of HB9, assignment as “undifferentiated” based only on cell marker expression is ambiguous, as large diameter, ChAT expressing – and thus mature – $Tg(\text{HB9:GFP})$ transgene expressing motor neurons are still present in the adult zebrafish spinal cord.

To clarify this, we used as additional criteria the shape and size of the $Tg(\text{HB9:GFP})^+$ cells – small diameter $Tg(\text{HB9:GFP})^+$ cells are very rare in unlesioned spinal cord (Reimer et al., 2008). I did not observe any $Tg(\text{vsx1:GFP})^+$ cells in the unlesioned spinal cord. I use the absence of transgene expression in the adult spinal cord as a sufficient criteria to classify the $Tg(\text{vsx1:GFP})^+$ cells as undifferentiated.
I also studied pax2\(^+\) cells. Pax2 is a transcription factor required during neurogenesis in the dorsal neural tube (Helms and Johnson, 2003). Moreover, pax2 is expressed in the developing optic nerve and is necessary for fate decision between neurons and glia. However, pax2 is not only expressed in undifferentiated cells. For example, mature astrocytes still express pax2 (Soukkarieh et al., 2007). Interestingly, in adult zebrafish, I have observed pax2\(^+\) cells in the dorsal grey matter of the spinal cord in unlesioned control animals. Thus, differentiated spinal cells in zebrafish express pax2. The function of these pax2\(^+\) cells remains to be elucidated. Based on their dorsal position, I speculate that they might be sensory neurons. I suggest considering pax2 as marker for mature cells in the adult spinal cord. Figure 2.25 illustrates for all cell types that I have studied the distribution within the adult spinal cord in unlesioned fish and after lesion.
2. Cell types in the spinal cord of adult zebrafish

- Mature cell markers:
  - 5-HT
  - GABA
  - ChAT
  - parvalbumin
  - pax2

- Undifferentiated cell markers:
  - HB9-GFP
  - vsx1-GFP

*pre-existing*  *newly generated*

<table>
<thead>
<tr>
<th>pre-existing</th>
<th>newly generated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature cell markers</td>
<td>Undifferentiated cell markers</td>
</tr>
<tr>
<td>no lesion</td>
<td>no lesion</td>
</tr>
<tr>
<td>six weeks</td>
<td>two weeks</td>
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<tr>
<td>n.d.</td>
<td>n.d.</td>
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Fig. 2.25: Schematic overview about the distribution of various cell markers within the spinal cord of adult zebrafish. The left half of the spinal cord shows the pattern of cells in unlesioned fish, the right half of the spinal cord shows the pattern at two weeks or at six weeks after lesion.

Black dots symbolize cells present in the spinal cord in unlesioned animals; red dots symbolize newly added cells. The size of the dot represents approximately the relative diameter of the cells.

Among all mature cell markers I studied, 5-HT$^+$ cells are located most ventrally. They continuously increase in cell number until six weeks post-lesion (see Chapter 1). This is in agreement with observations in goldfish, showing that 5-HT$^+$ cells are newly born and increase in number after spinal lesion until 12 weeks post-lesion (Takeda et al., 2008).

Along the dorsoventral axis, ChAT$^+$ cells are the next ventral cell type adjacent to the 5-HT cells. They are more abundant than the 5-HT$^+$ cells and they are located in the ventro-lateral and ventro-medial of the ventro-dorsal midline. Few ChAT$^+$ cells with small diameter are located dorsal to the central canal and have pax2$^+$ cell nuclei. Tracing experiments from the peripheral muscle tissue would reveal whether this group of cells is a motor neuron subtype. Westerfield et al. classified motorneurons according to their size in the spinal cord of adult zebrafish. Small motor neurons were classified as secondary motor neurons. On the other hand, the relatively dorsal position of these ChAT$^+$/pax2$^+$ cells might indicate that they are primary motor neurons. Further studies, for example about the field of innervation of these cells, would help to clarify ambiguity (Westerfield et al., 1986).

Within our study, we do not observe significant changes in large ChAT$^+$ cells during recovery. However, increasing the n-numbers would likely reveal a significant reduction at two weeks, indicating that motor neurons are lost after lesion. I also expect a significant increase between two and six weeks post-lesion with higher n-numbers, in agreement with numbers of large Tg(HB9:GFP)$^+$ motor neurons (Reimer et al., 2008). Our data suggest that some motor neurons die and that newly generated motor neurons are integrated into the spinal network. These results are in line with earlier studies in guppy.
2. Cell types in the spinal cord of adult zebrafish

(Poecilia reticulata), in which large “ganglion cells” regenerate after lesion (Kirsch, 1951).

GABA+ cells with central canal contact are not changed in number at two weeks post-lesion. This is remarkable, as their location in proximity to the central canal suggests that they might be prone to lesion-induced events, such as cell death and proliferation. My findings are in contrast to observations made by Dervan et al (2003) in eel. Immediately after spinal cord transection, they observe a complete loss of GABA+ cells numbers adjacent to the lesion site. 11 days after injury, they report liquor-contacting GABA+ cells that are smaller in diameter compared to unlesioned control animals. Overall numbers of GABA+ cells remain reduced (Dervan and Roberts, 2003). Functional recovery is not discussed in this study, however other studies show functional recovery in eel after complete spinal cord transection (Doyle et al., 2001; Doyle and Roberts, 2004).

Within the dorsal horn, I investigated the time course of pax2+ cells and parvalbumin+ cells. Both cell population did not change in cell number until six weeks post-lesion, the latest time point I investigated in this study.

Pax2+ cells form a mixed cell population: Most of the cells are located the dorsal horn, however, I also occasionally observed pax2+ cells in the ventral horn. Separate analysis of dorsal and ventral pax2+ cell population did not show any differences in cell number for either of the subpopulation. Thus, the time course of pax2+ cells in Figure 2.26 depicts dorsal and ventral cells together.

Inner ear stem cells possess the ability to differentiate spontaneously into sensory neurons. The underlying mechanism is enhanced by retinoic acid and involves pax2 expression (Martinez-Monedero et al., 2008). Due to their location in the spinal cord, the dorsal pax2+ and parvalbumin+ cells might be spinal sensory neurons. Previously, it was shown that during spinal cord regeneration mRNA expression of retinoic acid target genes is upregulated (Reimer et al., 2009). It would be interesting to study whether additional administration of retinoic acid would enhance generation of pax2+ cells after spinal lesion.
2. Cell types in the spinal cord of adult zebrafish

In summary for mature cell markers, the ventral-most 5-HT$^+$ cell population, increases in number whereas the dorsal-most cell populations (pax2$^+$ and parvalbumin$^+$ cells) do not. Similar to pax2$^+$ and parvalbumin$^+$ cells, numbers of GABA$^+$ cells with central canal contact remain unaffected. ChAT$^+$ cells first show a tendency to decrease and then a tendency to increase (see Fig. 2.26 and Table 2.1).

The time course of $Tg(vsx1:GFP)^+$ cells is like the time course of $Tg(HB9:GFP)^+$ cells at two weeks post-lesion compared to unlesioned control animals. Few small diameter $Tg(HB9:GFP)^+$ cells and $Tg(vsx1:GFP)^+$ cells were observed in the unlesioned spinal cord and cell numbers have increased significantly at two weeks post-lesion. At six weeks post-lesion, the time course of $Tg(HB9:GFP)^+$ cells and $Tg(vsx1:GFP)^+$ cells differ (Reimer et al., 2008 and Kuscha et al., in preparation): the number of $Tg(HB9:GFP)^+$ cells is reduced again and is similar to unlesioned control fish (Reimer et al., 2008 and Kuscha et al., in preparation), whereas the number of $Tg(vsx1:GFP)^+$ cells remains at the level observed at two weeks post-lesion. Our data suggest that some of the small diameter $Tg(HB9:GFP)^+$ cells have differentiated into mature motor neurons (Reimer et al., 2008). The high number of $Tg(vsx1:GFP)^+$ cells at six weeks post-lesion suggest that $Tg(vsx1:GFP)^+$ cells are continuously generated after lesion (see Fig. 2.25 and Table 2.1).
2. Cell types in the spinal cord of adult zebrafish

![Graph showing cell types and marker expressions in spinal cord with lesion groups: no lesion, 2 weeks, 6 weeks for mature and undifferentiated cell markers.](image-url)
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.26: Overview of quantification of numbers of mature cells (5-HT, ChAT, GABA, parvalbumin, pax2, \textit{Tg(pax2a:GFP)}) and undifferentiated cells \textit{Tg(HB9:GFP)}, \textit{Tg(vsx1:GFP)}).

Time course of numbers of cells positive for the respective markers in unlesioned fish, two weeks and six weeks after lesion. 5-HT$^+$ cells increase continuously until six weeks post-lesion, ChAT$^+$ cells show a tendency of decrease at two weeks post-lesion and then a tendency of increase. All other differentiated cell markers do not show any changes in cell number during recovery. Numbers of cells positive for the undifferentiated cell marker \textit{Tg(HB9:GFP)} or \textit{Tg(vsx1:GFP)} have strongly increased at two weeks post-lesion compared to unlesioned animals. Numbers of \textit{Tg(HB9:GFP)} cells have decreased again at six weeks post-lesion, whereas numbers of numbers \textit{Tg(vsx1:GFP)} at six weeks post-lesion are comparable to numbers at two weeks post-lesion.

After spinal lesion, \textit{ssh} mRNA expression is increased in the ventral-most region of the central canal (Reimer et al., 2009). I could show that after spinal lesion, mRNA expression of \textit{patched1} – a target gene of shh signaling – is increased after lesion and forms a ventro-dorsal gradient around the central canal. It is tempting to speculate that the ventro-dorsal gradient of the shh receptor translates the sonic hedgehog signal accordingly. As a consequence, the cells located ventrally around the central canal would be more prone to react to sonic hedgehog than the cells located dorsally around the central canal. This might explain why cellular regeneration seems to appear mainly in the ventral half of the spinal cord.

2.3.2 Various cell types are newly generated but not in an uniform pattern

The documented time courses have elucidated whether and how numbers of various cell types have changed after spinal lesion. Increasing cell numbers of 5-HT$^+$ cells and \textit{Tg(vsx1:GFP)}$^+$ cells suggested that they are newly generated after lesion. However, constant parvalbumin$^+$, pax2$^+$ and GABA$^+$ cell numbers do not necessarily mean that these cell types are not newly generated after lesion. A balance between proliferation and cell death might also result in overall unchanged cell numbers. This is why I investigated BrdU labeling rates in addition to the time courses of cell numbers. To investigate which of the studied cell types are newly generated after lesion and to which extend – compared to others – I used the same injection scheme for a studied cell types – BrdU was injected at 0, 2 and 4 days post-lesion.
2. Cell types in the spinal cord of adult zebrafish

At two weeks post-lesion, ~20% of Tg(vsx1:GFP)$^+$ cells were labeled with BrdU – this is a similar labeling rate compared to ~25% BrdU labeled Tg(HB9:GFP)$^+$ motor neurons, suggesting that Tg(vsx1:GFP)$^+$ cells and Tg(HB9:GFP)$^+$ cells are newly generated at a similar time point and to a same extent. As the number of Tg(vsx1:GFP)$^+$ cells is still increased at six weeks after lesion, future experiments should include BrdU injection between two and six weeks post-lesion. Thereby, I might determine whether Tg(vsx1:GFP)$^+$ cells are continuously generated up to six weeks post-lesion.

Most of the mature cell types were not labeled with BrdU at two weeks post-lesion. The BrdU labeling rate for ChAT$^+$ cells was ~0.7%, for dorsal parvalbumin$^+$ cells ~0.3% and 0% for GABA$^+$ cells.

5-HT$^+$ cells are an exception – 27.5% of 5-HT$^+$ cells were labeled with BrdU at two weeks post-lesion. At six weeks post-lesion, the BrdU labeling rate for ChAT$^+$ cells was ~9.5%, for dorsal parvalbumin$^+$ cells ~2% and for dorsal pax2$^+$ cells ~10%. Of the 5-HT$^+$ cells, ~54% were labeled with BrdU.

In general, within each mature cell type, more cells are labeled with BrdU at six weeks compared to two weeks post-lesion. Higher n-numbers are required to show if these differences are significant. Possibly, the injected BrdU was incorporated into progenitor cells that have eventually differentiated into mature cells by 6 wpl.

The high labeling rate of 5-HT$^+$ cells and the increase in cell number is striking and distinguished them from other mature cell types. Which factors promote the regeneration of this cell type compared to others? Is the ventral-most position the factor that favors 5-HT$^+$ generation towards others? Hardly any dorsal parvalbumin$^+$ cells are newly generated, suggesting that they are unaffected by lesion. Interestingly, at a similar position in the spinal cord, pax2$^+$ cells show higher BrdU labeling rates. Further experiments are needed to confirm whether the BrdU labeling rate between pax2$^+$ and parvalbumin$^+$ cells significantly differs at six weeks post-lesion. Possibly, pax2$^+$ cells are more sensitive to lesion than parvalbumin$^+$ cells. Cell death and generation of pax2 cells might be tightly balanced to maintain cell numbers constant.
2. Cell types in the spinal cord of adult zebrafish

The low labeling rate of parvalbumin\(^+\) cells is particularly remarkable, as they are located adjacent to the dorsal midline that harbours high numbers of BrdU\(^+\) nuclei. Further studies are required to find out which cell type is newly generated within the dorsal midline.

In summary, I conclude that cellular regeneration after spinal lesion is not uniform but a highly regulated process, possibly depending on the ventro-dorsal position of a cell type within the spinal cord and of the marker expression of individual cell types (see Fig. 2.26).

Are any of these cell types replaced in mammals? Two weeks after excitotoxic injury in adult rodent brain, precursors from the subventricular zone give rise to newly born neurons. About 80\% of them survived up to six weeks post injury and differentiated into mature neurons – some of them expressed parvalbumin (Collin et al., 2005). In rats, transplantation of fetal hippocampal cells into injured adult hippocampus resulted in generation of parvalbumin expressing neurons (Mudrick and Baimbridge, 1991). Parvalbumin\(^+\) cells also play a direct role in regeneration. Overexpression of parvalbumin in mice increases survival rate of motor neurons after sciatic nerve crush in the hindlimb (Dekkers et al., 2002).

2.3.3 What are the progenitor cells for these newly generated cells?

We already could show that pax6\(^+\)/nkx6.1\(^+\)/Tg(olig2:GFP2)\(^+\) ependymo-radial glia cells are stem-cell like progenitors for motor neurons (Reimer et al., 2008; Reimer et al., 2009). Tg(vsx1:GFP)\(^+\) cells possibly emerge from a p2-like zone, however, the further fate of these cells remain unclear. 5-HT\(^+\) cells possibly arise from a p3-like zone (see Chapter 1). Progenitors for newly generated pax2 cells remain to be elucidated. It would be interesting to investigate whether different progenitors show a distinct spatio-temporal pattern. Using different time windows for BrdU injections might reveal whether BrdU labeling rates of individual cell types change or whether the ratio of BrdU labeling rates between various cell types remains constant.
Does the mammalian adult spinal cord possess similar progenitor cells?
In rat spinal cord, stem cells exist that can form neurospheres in vivo and in vitro. When transplanted into a suitable environment, they show self-renewal and multipotency. They differentiate into glia and neurons (Shihabuddin et al., 2000). Remarkably, lineage tracing experiments in mouse spinal cord revealed that ependymal glial cells are the main neurosphere forming cells. In vivo, they are able to differentiate into oligodendrocytes and scar-forming cells, but not into neurons. Their shape resembles ependymo-radial glia cells in zebrafish spinal cord. However, their processes do not extend to the pial surface but contact blood vessels (Meletis et al., 2008).

2.3.4  Rostro-caudal differences in cell numbers of V2 interneurons and motor neurons
At two weeks after lesion, I observed more Tg(vsx1:GFP)+ cells in the caudal spinal cord compared to the rostral half. In contrast, numbers of Tg(HB9:GFP)+ cells are higher rostral than caudal to the lesion site. These findings prompted us to hypothesize that Tg(HB9:GFP)+ motor neurons might be generated at the expense of Tg(vsx1:GFP)+ cells interneurons, as described in normal development. Further experiments were performed to test this hypothesis (see Chapter 3). Of the mature cell markers, I observed more pax2+ cells in the caudal than in the rostral half at two weeks post-lesion (see Fig. 2.27, Table 2.2).

2.3.5  Differences between recovered and non-recovered fish
When I compared numbers of cells in recovered and non-recovered fish rostral and caudal to the lesion site for each cell marker, I observed a significant difference in number solely for 5-HT+ cells (Table 2.4 and Table 2.5): caudal to the lesion site, numbers of 5-HT+ cells were significant increased in recovered fish compared to non-recovered fish at six weeks post-lesion (Table 2.5).
This observation is consistent with descending axons, present in much lower numbers in non-recovered fish caudal to the lesion site, stimulating lesion-induced generation of 5-HT+ cells and/or preventing death of these cells (see Chapter 1).
2. Cell types in the spinal cord of adult zebrafish

- Mature cell markers
  - S-HT
  - large ChAT
  - small ChAT
  - GABA

- Undifferentiated cell markers
  - HB9:GFP
  - vglut1:GFP
  - pax2
  - pax2:GFP

**n** values:
- S-HT: 19
- large ChAT: 3
- small ChAT: 3
- GABA: 2
- HB9:GFP: 22
- vglut1:GFP: 12
- pax2: 4
- pax2:GFP: 4

Statistical significance:
- *p < 0.05
- **p < 0.01
- ***p < 0.001
2. Cell types in the spinal cord of adult zebrafish

Fig. 2.27: Overview of quantification of cell numbers of mature cell markers (5-HT, ChAT, GABA, parvalbumin, pax2, Tg(pax2a:GFP)) and undifferentiated cell markers (Tg(HB9:GFP), Tg(vsx1:GFP)). The graphs depict numbers of each cell marker rostral and caudal to the lesion site at two weeks post-lesion. Only the undifferentiated cells markers Tg(HB9:GFP) (data generously provided by Dr. Michell M. Reimer) and Tg(vsx1:GFP) show significant rostral-caudal differences.

*P < 0.05, ***P < 0.001
Table 2.1: Dynamic changes of various cell populations in the spinal cord in unlesioned control fish and after lesion in 1500 µm (rostral and caudal together). Fish at six weeks post-lesion are recovered, if not stated otherwise. n.d. = no data

<table>
<thead>
<tr>
<th>Mature cell markers</th>
<th>No lesion</th>
<th>1 week</th>
<th>2 weeks</th>
<th>6 weeks</th>
<th>13 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>93.1 ± 14.17, n = 18</td>
<td>120.0 ± 18.39, n = 5</td>
<td>363.4 ± 30.41, n = 19*</td>
<td>471.2 ± 80.57, n = 10*</td>
<td>217.8 ± 16.70, n = 5</td>
</tr>
<tr>
<td>Large ChAT</td>
<td>484.8 ± 70.7, n = 5</td>
<td>n.d.</td>
<td>249.3 ± 41.83, n = 3</td>
<td>498.6 ± 90.79, n = 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Small ChAT</td>
<td>196.0 ± 55.59, n = 3</td>
<td>n.d.</td>
<td>185.3 ± 30.30, n = 3</td>
<td>259.7 ± 52.29, n = 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>GABA (ventricular)</td>
<td>576.2 ± 47.20, n = 3</td>
<td>n.d.</td>
<td>621.5 ± 7.18, n = 2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Parvalbumin (dorsal)</td>
<td>638.9 ± 92.24, n = 4</td>
<td>n.d.</td>
<td>735.2 ± 76.68, n = 6</td>
<td>716.7 ± 185.39, n = 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pax2 (dorsal and ventral)</td>
<td>2661.1 ± 392.67, n = 4</td>
<td>n.d.</td>
<td>2608.4 ± 56.25, n = 4</td>
<td>2048.6 ± 371.81, n = 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tg(Pax2a:GFP) (dorsal and ventral)</td>
<td>577.8 ± 97.29, n = 4</td>
<td>n.d.</td>
<td>822.2 ± 168.08, n = 4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Undifferentiated cell marker</th>
<th>No lesion</th>
<th>1 week</th>
<th>2 weeks</th>
<th>6 weeks</th>
<th>13 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tg(HB9:GFP) No behavior tested</td>
<td>20 ± 7.7, n = 4</td>
<td>n.d.</td>
<td>870 ± 106.8, n = 11*</td>
<td>251 ± 78.7, n = 6</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tg(Vsx1:GFP)</td>
<td>26.5 ± 19.08, n = 3</td>
<td>n.d.</td>
<td>628.4 ± 80.90*, n = 12</td>
<td>573.3 ± 162.94*, n = 6</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
### Table 2.2: Comparison of cell numbers rostral and caudal to the lesion of various cell types at two weeks post-lesion

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Mature cell markers</th>
<th>Undifferentiated cell markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rostral</td>
<td>caudal</td>
</tr>
<tr>
<td>5-HT</td>
<td>177.5 ± 12.41, n = 19</td>
<td>186.0 ± 21.91, n = 19</td>
</tr>
<tr>
<td>Large ChAT</td>
<td>103.0 ± 11.24, n = 3</td>
<td>146.0 ± 39.95, n = 3</td>
</tr>
<tr>
<td>Small ChAT</td>
<td>85.7 ± 19.34, n = 3</td>
<td>100.0 ± 12.00, n = 3</td>
</tr>
<tr>
<td>GABA ventricular</td>
<td>396.4 ± 34.88, n = 2</td>
<td>225.1 ± 42.07, n = 2</td>
</tr>
<tr>
<td>Parvalbumin dorsal</td>
<td>372.2 ± 33.06, n = 6</td>
<td>363.0 ± 54.86, n = 6</td>
</tr>
<tr>
<td>Pax2 dorsal and ventral</td>
<td>1083.4 ± 109.62, n = 4</td>
<td>1525.0 ± 130.08, n = 4</td>
</tr>
<tr>
<td>Tg(Pax2a:GFP) dorsal and ventral</td>
<td>377.8 ± 67.13, n = 4</td>
<td>444.4 ± 109.43, n = 4</td>
</tr>
<tr>
<td>Tg(HB9:GFP)</td>
<td>508.0 ± 39.22, n = 22</td>
<td>261.1 ± 17.14, n = 22</td>
</tr>
<tr>
<td>Tg(Vsx1:GFP)</td>
<td>245.9 ± 35.42, n = 12</td>
<td>382.3 ± 66.97, n = 12</td>
</tr>
</tbody>
</table>
Table 2.3: Comparison of cell numbers rostral and caudal of the lesion of various cell types at six weeks post-lesion in recovered fish. n.d. = no data

<table>
<thead>
<tr>
<th>Mature cell markers</th>
<th>rostral</th>
<th>caudal</th>
</tr>
</thead>
</table>
| 5-HT                | 365.0 ± 73.59, n = 6  | 253.7 ± 28.32, n = 6 | n.s.  
| Large ChAT          | 237.5 ± 47.97, n = 4  | 261.1 ± 64.87, n = 4 | n.s.  
| Small ChAT          | 79.2 ± 20.59, n = 4   | 180.6 ± 40.92, n = 4 | n.s.  
| GABA ventricular    | n.d.                  | n.d.                 |      
| Parvalbumin dorsal  | 480.6 ± 88.00, n = 4  | 502.8 ± 102.4, n = 4 | n.s.  
| Pax2 dorsal and ventral | 873.6 ± 179.26, n = 4 | 1175.0 ± 199.30, n = 4 | n.s.  
| Tg(Pax2a:GFP) dorsal and ventral | n.d.                | n.d.                |      
| Undifferentiated cell markers | Tg(HB9:GFP) | n.d. | n.d.  
| Tg(Vsx1:GFP)        | 293.9 ± 91.78, n = 6  | 279.4 ± 81.97, n = 6 | n.s.  

### 2. Cell types in the spinal cord of adult zebrafish

Table 2.4: Comparison of cell numbers of various cell types rostral to the lesion site in recovered and not recovered fish at six weeks post-lesion. n.d. = no data

<table>
<thead>
<tr>
<th>Mature cell markers</th>
<th>recovered</th>
<th>non-recovered</th>
<th>n.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-HT</strong></td>
<td>365.0 ± 73.59</td>
<td>292.6 ± 25.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 6</td>
<td>n = 6</td>
<td></td>
</tr>
<tr>
<td><strong>Large ChAT</strong></td>
<td>237.5 ± 47.97</td>
<td>208.3 ± 31.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td><strong>Small ChAT</strong></td>
<td>79.2 ± 20.59</td>
<td>77.8 ± 25.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td><strong>GABA ventricular</strong></td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td><strong>Parvalbumin dorsal</strong></td>
<td>480.6 ± 88.00</td>
<td>452.8 ± 18.91</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td><strong>Pax2 dorsal and ventral</strong></td>
<td>873.6 ± 179.3</td>
<td>908.3 ± 77.56</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>n = 4</td>
<td>n = 4</td>
<td></td>
</tr>
<tr>
<td><strong>Tg(Pax2a:GFP) dorsal and ventral</strong></td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Undifferentiated cell markers</th>
<th>recovered</th>
<th>non-recovered</th>
<th>n.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tg(HB9:GFP)</strong></td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td><strong>Tg(Vsx1:GFP)</strong></td>
<td>293.9 ± 91.78</td>
<td>29.0 ± 19.38</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>n = 6</td>
<td>n = 2</td>
<td></td>
</tr>
</tbody>
</table>
2. Cell types in the spinal cord of adult zebrafish

Table 2.5: Comparison of cell numbers of various cell types caudal to the lesion site in recovered and non-recovered fish at six weeks post-lesion. n.d. = no data

<table>
<thead>
<tr>
<th>Cell Markers</th>
<th>Mature cell markers</th>
<th>Undifferentiated cell markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>recovered</td>
<td>Non-recovered</td>
</tr>
<tr>
<td>5-HT</td>
<td>253.7 ± 28.32 n=6</td>
<td>111.1 ± 18.59 n = 6</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Large ChAT</td>
<td>261.1 ± 64.87 n = 4</td>
<td>169.4 ± 32.51 n = 4</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Small ChAT</td>
<td>180.6 ± 40.92 n = 4</td>
<td>105.6 ± 32.24 n = 4</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>GABA ventricular</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Parvalbumin dorsal</td>
<td>502.8 ± 102.4, n = 4</td>
<td>494.4 ± 67.97, n = 4</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Pax2 dorsal and ventral</td>
<td>1175.0 ± 199.30, n = 4</td>
<td>888.9 ± 73.04, n = 4</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
<tr>
<td>Tg(Pax2a:GFP) dorsal and ventral</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tg(HB9:GFP)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tg(Vsx1:GFP)</td>
<td>279.4 ± 81.97 n = 6</td>
<td>111.4 ± 72.68 n = 2</td>
</tr>
<tr>
<td></td>
<td>n.s.</td>
<td></td>
</tr>
</tbody>
</table>
### 2. Cell types in the spinal cord of adult zebrafish

Table 2.6: Overview and characterization of used cell markers

<table>
<thead>
<tr>
<th>Cell Marker (Antibody or transgenic line)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>Neurotransmitter, modulates CPG</td>
</tr>
<tr>
<td>ChAT</td>
<td>Enzyme for synthesis of acetylcholine (neurotransmitter), found in the motor neurons in spinal cord</td>
</tr>
<tr>
<td>GABA</td>
<td>Neurotransmitter of inhibitory interneurons</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>Ca(^{2+}) binding protein expressed by interneurons and some motor neurons</td>
</tr>
<tr>
<td>Pax2</td>
<td>Transcription factor that specifies cell fates, for example GABA(^{-}) and glycine(^{-}) inhibitory interneurons in spinal cord</td>
</tr>
<tr>
<td>Tg(HB9:GFP)</td>
<td>Transcription factor involved in specification of motor neurons in the spinal cord during development, also found in regeneration</td>
</tr>
<tr>
<td>Tg(Vsx1:GFP)</td>
<td>Transcription factor involved in specification of excitatory V2 interneurons in the spinal cord, during development, also found in regeneration</td>
</tr>
</tbody>
</table>
2. Cell types in the spinal cord of adult zebrafish

2.3.6 Regeneration is not a mere recapitulation of development

Possibly, adult ependymo-radial glial cells might be the equivalent of embryonic neuronal tube progenitor cells, based on the similar expression pattern of the key transcription factors *Tg(Olig2:GFP)*, *nkx6.1* and *pax6*. Their relative distribution with partial colocalization around the central canal is comparable to the expression pattern around the neural tube. In addition, adult ependymo-radial glia cells and embryonic neuronal tube progenitor cells share similar cell morphology. Both contact the central canal and bear long processes that extend to the pial surface. After spinal lesion, pMN-like ependymo-radial glia cells increase in number. In summary, I find that the ventral organization of the adult spinal cord during regeneration resembles the ventral developing spinal cord with regard to transcription factor expression and cell morphology. In contrast, we can provide accumulating data that the dorsal adult spinal cord does not appear to restore its developmental pattern.

During regeneration, previous work has already suggested a lack of dorsal cell markers during regeneration: the transcription factors BMP2 and BMP4 are below detection threshold with in situ hybridization in adult spinal cord sections of unlesioned control animals as well as after lesion (Inga Soerensen, Diploma thesis, 2005, Medizinische Hochschule Hannover, Germany). The same observations apply for neurogenin1 and mash1 (Inga Soerensen, Diploma thesis, 2005, Medizinische Hochschule Hannover, Germany).

My results are in agreement with these previous findings: I cannot detect the transcription factors pax3 (tested with in situ hybridization) and pax 7 (tested with in situ hybridization and immunohistochemical labeling) within adult spinal cord, neither in control nor in lesioned fish, whereas positive controls in zebrafish embryos using the same probes and antibodies were in agreement with published expression patterns. Furthermore, I performed in situ hybridization for two wnt family members, Tcf7 and wnt3a. Occasionally, I was able to detect a few cells in the parenchyma in unlesioned spinal cord as well as after lesion. However, no difference in expression was detectable after lesion compared to unlesioned control fish.
2. Cell types in the spinal cord of adult zebrafish

In summary, I suggest the upregulation of shh after spinal lesion is strong enough to expose dorsal regions of the spinal cord to shh. These concentrations of shh are possibly high enough to repress expression of dorsal markers, as reported during development for pax3 and pax7 (Tanabe and Jessell, 1996; Ericson et al., 1997). Thus, we propose that the spinal cord might be ventralized after spinal lesion. Therefore, regeneration is not a pure recapitulation of development.

It would be interesting to investigate if overexpression of BMP and wnt signaling initiates upregulation of dorsal cell markers and whether, in consequence, generation of dorsal cells would be enhanced, possibly at the expense of ventral cells.

Do mammalian progenitor cells in the adult spinal cord possess a dorso-ventral polarity?

Yamamoto et al. (2001) observe transient upregulation of the transcription factors pax6 but not olig2 or nkx2.2 in adult neuronal progenitor cells of rat spinal cord. In contrast to my findings, no clear ventro-dorsal gradient is detectable. One week after lesion, pax6 expression has already decreased. Also, the neuronal progenitor cells fail to generate motor neurons in vivo. Also in vitro, when these progenitor cells were exposed to conditions which favor neurogenesis in embryonic progenitor cells, they did not give rise to motor neurons (Yamamoto et al., 2001). Following contusion injury in adult mouse spinal cord, widespread shh mRNA upregulation in the grey and white matter was reported (Chen et al., 2005). In the human spinal cord, Dromard et al. (2008) describe nkx6.1 expressing cells around the central canal. In vitro, these authors were able generate neuronal precursor cells from dissociated spinal cord culture. These neuronal progenitors differentiated into GABA+ cells and glia (Dromard et al., 2008).

2.3.7 Future functional characterization of cell types

We focused on the anatomical description of the distribution of these various cell markers. Further characterization of the functional properties of the cell
2. Cell types in the spinal cord of adult zebrafish

types will require the identification of the neurotransmitter phenotype of the respective cell types.
Combination of immunohistochemistry and calcium imaging indicators, as recently described in the cortex of mice, would allow us to elucidate electrical properties of the cells.
Genetic or laser ablation of a particular cell type and analysis of the effect on the motor output would help to clarify the function of a cell type within the network.
3. Supraspinal descending dopaminergic axons control motor neuron regeneration in the lesioned spinal cord of adult zebrafish

3.1 Introduction

In the spinal cord, ependymal cells with radial processes act as progenitor cells after lesion, but they only generate glial cells (Meletis et al., 2008). This is despite their potential for neurogenesis, manifested when transplanted into neurogenic regions of the adult brain (Shihabuddin, 2002). Thus, elucidating environmental signals that stimulate adult spinal progenitor cells to generate motor neurons is of great importance in the context of conditions in which neurons are permanently lost, such as motor neuron disease or spinal cord injury (Rothstein, 2009). The spinal cord of adult zebrafish contains ependymo-radial progenitor cells, which are morphologically similar to mammalian stem cells (Meletis et al., 2008). As in mammals, no neurogenesis has been observed in the uninjured spinal cord of adult zebrafish. This is in contrast to constitutively active adult neurogenic zones, which only exist in the brain of zebrafish (Chapouton et al., 2006; Kaslin et al., 2009; Chapouton et al., 2010) and mammals (Gould, 2007). Remarkably, after spinal cord transection, quiescent Tg(olig2:GFP)+ ependymo-radial progenitor cells in the ventro-lateral cord generate Tg(HB9:GFP)+ motor neurons in zebrafish (Reimer et al., 2008). The developmental morphogen shh is one of the signals that stimulates adult motor neuron regeneration locally. Shh is upregulated in the ventral regenerating spinal cord, Tg(olig2:GFP)+ progenitors upregulate the hedgehog (hh) target gene patched1 (ptc1) and blocking hh signaling with cyclopamine reduces lesion-induced motor neuron generation (Reimer et al., 2009).

The possible influence of descending axons on motor neuron regeneration has not been addressed. In the developing fish spinal cord dopaminergic axons are exclusively derived from diencephalic neurons (McLean and Fetcho, 2004; Kastenhuber et al., 2010). We decided to analyze the role of brain-derived dopamine for adult motor neuron regeneration, because dopamine signaling
has been shown to influence adult neurogenesis in the mammalian forebrain (Borta and Hoglinger, 2007). However, the effect of dopamine is not clear. Some studies support a promoting (Baker et al., 2004; Hoglinger et al., 2004), while others suggest an inhibiting action (Kippin et al., 2005; Peng et al., 2008; Park and Enikolopov, 2010) on adult neurogenesis, possibly depending on the brain region studied and the experimental paradigm used.

Here we demonstrate an important asymmetry between the rostral and caudal lesioned spinal cord of adult zebrafish. Spinal progenitor cells rostral to a spinal lesion site express higher levels of *ptc1* and *drd4a* and generate twice as many motor neurons. This correlates with the presence of descending TH1 axons only rostral to the lesion site. Gain-and loss-of-function experiments confirm a decisive influence of dopamine on the above parameters. Thus, we show for the first time the important influence of descending axons on the plasticity of adult spinal progenitor cells and identify dopamine as novel signal that stimulates motor neuron regeneration from these cells via specific receptors.

### 3.2 Results

The aim of this study was to determine whether descending dopaminergic axons from the brain could influence motor neuron regeneration from ependymo-radial glial progenitor cells in the lesioned spinal cord of adult zebrafish.

#### 3.2.1 TH1+ axon numbers and *drd4a* receptor expression are increased in the rostral lesioned spinal cord

In the lesioned spinal cord, motor neuron regeneration occurs rostral and caudal close to the transection site and peaks at 2 weeks post-lesion (Reimer et al., 2008). Therefore we analyzed TH1+ axon distribution and dopamine receptor expression at 2 wpl in these locations. In the spinal cord immediately rostral to the spinal lesion site (750 µm), the number of TH1+ axon profiles was significantly increased by 42% compared to the unlesioned spinal cord. In contrast, caudal to the lesion site, few TH1+ axon profiles were detectable (4%
of unlesioned; Fig. 3.1 A,B). These data are consistent with sprouting of TH1+ axons rostral to the lesion site and Wallerian degeneration of severed distal axonal segments caudal to the lesion site. Even though TH1+ axons eventually regenerate into the caudal spinal cord after a lesion, this regeneration occurs later than 2 wpl (see Chapter 1). In Tg(olig2:GFP) transgenic fish, the adult Tg(olig2:GFP)+ ependymo-radial glial progenitor cells for motor neurons are labeled (Reimer et al., 2008). Double-labeling with the TH1 antibody revealed TH1+ axons that were in contact with the radial processes of the progenitor cells (Fig. 3.1C). Dopamine from these axons and more distant axons might reach progenitor cells rostral to the lesion site by volume transmission (Fuxe et al., 2010). However, caudal to the lesion site progenitor cells are not exposed to dopamine for the first 2 wpl. Adult neurogenesis is mediated mainly by D2-like receptors (Borta and Hoglinger, 2007). Therefore, we determined expression of known D2-like receptors in the lesioned adult spinal cord of zebrafish. We performed in situ hybridizations for drd2a, drd2b (formerly drd2c), drd2l (formerly drd2b), drd3, drd4a and drd4b (formerly drd4c) (Boehmler et al., 2004; Boehmler et al., 2007). We found a strong increase of expression only for drd4a in the entire ventricular zone of ependymo-radial glial cells in the 750 µm rostral to the lesion site. Expression was increased from undetectable levels in unlesioned fish. Caudal to the lesion site no expression was detectable (Fig. 3.1D). Expression of the other receptors was low or undetectable in the ependymal layer of the lesioned spinal cord (data not shown). Drd4a expression in ependymo-radial glial cells overlaps with the region in which lesion-induced spinal neurogenesis takes place rostral to the lesion site. Thus lesion-induced upregulation of drd4a could directly mediate a dopamine signal in ependymo-radial progenitor cells, including motor neuron progenitor cells, in the rostral spinal cord.
3. Descending dopaminergic axons control motor neuron regeneration

Fig. 3.1: TH1+ axons and drd4a in situ hybridization signal are present rostral, but not caudal to the spinal lesion site. **A, B:** Spinal cross sections show an increase in TH1 immuno-labeled profiles rostral and almost complete absence of profiles caudal to the lesion site at 2 weeks post-lesion. *P < 0.05; ***P < 0.001. **C:** In cross sections of the rostral lesioned spinal cord, Tg(olig2:GFP)+ somata (arrow) contact the central canal (outlined by dots). Radial processes of these cells are close to TH1+ profiles (arrowheads) at 9 days post-lesion. **D:** Cross sections through the spinal cord are shown; dorsal is up; central canal is indicated by asterisks. The drd4a in situ hybridization signal is strongly increased in the entire ventricular zone rostral, but not caudal to a spinal lesion, at 2 weeks post-lesion.

Scale bars: **A** = 40 µm; **C** = 5 µm; **D** = 20 µm.

(Figure 3.1D was generously provided by Tatyana B. Dias.)

3.2.2 Almost twice as many motor neurons are newly-generated rostral than caudal to a spinal lesion site

We reasoned that if dopamine has a positive influence on lesion-induced generation of motor neurons there should be more newly-generated motor neurons rostral than caudal to a spinal lesion site. This is because the dopamine signal and drd4a receptor expression on spinal progenitors were only detectable rostral to the lesion site. We have shown previously that small Tg(HB9:GFP)+ cells, observable in appreciable numbers only after a spinal lesion, label with BrdU and are bona fide newly-generated motor neurons (Reimer et al., 2008). We found that at 2 wpl rostral to a spinal lesion site, almost twice as many small Tg(HB9:GFP)+ motor neurons were newly generated than caudal to it (Fig. 3.2A,B).
3. Descending dopaminergic axons control motor neuron regeneration

Fig. 3.2: Numbers of newly-generated motor neurons, but not of 5-HT+ cells, differ between the rostral and caudal part of the lesioned spinal cord. Spinal cross sections at 2 weeks post-lesion are shown; dorsal is up; arrowheads indicate cell bodies; asterisks or dotted lines indicate the central canal. A, B: Small intensely labeled Tg(HB9:GFP)+ motor neurons are rarely found in unlesioned animals. More Tg(HB9:GFP)+ motor neurons (1.9 fold; arrowheads) are generated rostral than caudal to a spinal lesion site (***P < 0.0001, Mann-Whitney U-test). C, D: Numbers of 5-HT+ neurons (arrowheads) are strongly increased compared to those in unlesioned animals, but there is no difference (P > 0.05) in the numbers of 5-HT+ neurons between the rostral and caudal part of the spinal cord.

Scale bar = 15 µm.

(Figure 3.2A was generously provided by Dr. Michell M. Reimer.)

In comparison, 5-HT neurons, another cell type generated in large numbers after a spinal lesion in teleost fish (Takeda et al., 2008), were generated equally rostral and caudal to the lesion site (Fig. 3.2C,D). This suggests specificity of the differences in numbers of newly-generated motor neurons. Thus higher numbers of newly-generated motor neurons rostral to a spinal lesion site are consistent with the hypothesis that dopamine promotes motor neuron regeneration.

3.2.3 Ablation of TH1+ axons decreases the number of newly-generated motor neurons rostral, but not caudal to a spinal lesion

To directly test whether TH1+ axons influence motor neuron regeneration we ablated TH1+ axons with the specific toxin 6-hydroxydopamine (6-OHDA) that has been shown to selectively target dopaminergic neurons (Ding et al., 2004). Treating unlesioned animals with a single intraperitoneal injection significantly reduced the number of TH1+ axons by 49% at 3 days following the injection (Fig. 3.3A). 5-HT axons were unaffected, supporting relatively selective action of the toxin.

Experimental animals received a single injection of the toxin one day before spinal cord transection. Analysis of motor neuron numbers at 2 wpl included only individuals in which the number of TH1+ axons was lower than 50% of the average of lesioned control animals. Counting HB9 immuno-positive or Tg(HB9:GFP)+ motor neurons indicated a significant 43% reduction in the number of newly-generated motor neurons rostral to the lesion site to a level that was comparable to that caudal to the lesion site. Caudal to the lesion site, 6-
OHDA treatment had a small but not significant effect on the number of newly-generated motor neurons (Fig. 3.3B). This indicates that potential unspecific toxic effects of 6-OHDA on motor neuron regeneration in the absence of TH1⁺ axons are small. These observations suggest that TH1⁺, mostly dopaminergic axons promote generation of motor neurons in the lesioned spinal cord.
3. Descending dopaminergic axons control motor neuron regeneration

A

TH1

control 6-OHDA

number of TH1+ profiles

n=12 n=12

control 6-OHDA

5-HT

n=3 n=3

B

control 6-OHDA

rostral

% of control

n=14 n=11

caudal

% of control

n=14 n=11

n.s.

* **
3. Descending dopaminergic axons control motor neuron regeneration

Fig. 3.3: 6-OHDA destroys TH1+ axons, but not 5-HT+ axons, and selectively reduces the number of newly-generated motor neurons rostral to the lesion site. **A**: Complete cross sections through the spinal cord are shown; dorsal is up. For control and 6-OHDA treatment, respectively, double immunofluorescent labeling of the same tissue section is shown. Whereas an intraperitoneal injection of 6-OHDA significantly (**P < 0.01) reduces the number of TH1+ axons, no effect was detectable on 5-HT+ axons (P > 0.05).

**B**: Spinal cross sections in the area of the central canal (dotted lines) of Tg(HB9:GFP) transgenic fish are shown at 2 weeks post-lesion. Depletion of TH+ axons using 6-OHDA reduces the number of newly-generated motor neurons only rostral to the lesion site. Values are given as percentage of cell numbers rostral to the lesion site in control animals (*P < 0.05).

Scale bars: **A** = 50 µm, **B** = 15 µm.

(Fig. 3.3A was generously provided by Dr. Angela L. Scott. Fig 3.3B was generated by Scott. Dr. Angela L. Scott and me in collaboration.)
3. Descending dopaminergic axons control motor neuron regeneration

3.2.4 6-OHDA does not increase numbers of V1 interneurons rostral to the lesion site

During development, V2 interneurons are generated at the expense of motor neurons (Yang et al., 2006; Lee et al., 2008). We found that at 2 wpl rostral to a spinal lesion site, almost twice as many small Tg(HB9:GFP)$^+$ motor neurons were newly generated than caudal to it (Fig. 3.2 A,B). In contrast, 1.6 fold more Tg(vsx1:GFP)$^+$ cells were newly generated caudal to the lesion site than rostral to it (see Chapter 2). Also, we have shown that NPA increases and 6-OHDA reduces numbers of Tg(HB9:GFP)$^+$ motor neurons rostral to the lesion site. Thus, we hypothesized that Tg(HB9:GFP)$^+$ neurons and Tg(vsx1:GFP)$^+$ cells might be generated at the expense of each other after lesion. Thus a manipulation that increases the number of Tg(HB9:GFP)$^+$ neurons would result in a decrease of the number of Tg(vsx1:GFP)$^+$ cells, and vice versa. Therefore, we hypothesized that treatment with 6-OHDA would increase the number of Tg(vsx1:GFP)$^+$ cells rostral to the lesion site. However, numbers of Tg(vsx1:GFP)$^+$ cells did not change rostral to the lesion (rostral, NaCl: 486.7 ± 93.64, n = 9; rostral, 6-OHDA: 436.0 ± 65.69, n = 8; n.s.). Caudal of the lesion site, numbers of Tg(vsx1:GFP)$^+$ were even significantly reduced (caudal, NaCl: 534.0 ± 50.13, n = 9; rostral, 6-OHDA; 348.8 ± 51.97, n = 8; p = 0.0432).

Therefore, it is tempting to speculate that more Tg(vsx1:GFP)$^+$ cells are generated rostral but not caudal to the lesion site. In addition, an unspecific toxic action might reduce overall cell numbers rostral and caudal to the lesion site. Then, the net effect would be no significant difference in cell numbers rostral to the lesion site and a reduction caudal to the lesion site. Future experiments might include injection of NPA in Tg(vsx1:GFP)$^+$ transgenic fish to investigate whether cell numbers are reduced rostral but not caudal to the lesion site.
3. Descending dopaminergic axons control motor neuron regeneration

3.3 Discussion

In this study, we have shown that dopamine derived from cells in the diencephalon is involved in regeneration of spinal motor neurons from ependymo-radial progenitor cells in adult zebrafish, as ablation of the dopaminergic source resulted in reduction of newly generated motor neurons. Thus, we also wanted to test whether stimulating dopamine signaling promotes motor neuron regeneration. Dr. Michell M. Reimer used repeated intraperitoneal injections of R(−)-Propylnorapomorphine hydrochloride (NPA) during regeneration and determined numbers of newly-generated motor neurons at 2 wpl. NPA is a potent D2-like receptor agonist (Lahti et al., 1993). He observed that NPA treatment led to a significant 50% increase in the number of \(Tg(HB9:GFP)\)+ motor neurons rostral to the lesion site. However, caudal to the lesion site, no change in the number of newly-generated motor neurons was observed. This is consistent with low expression levels of \(drd4a\) caudal to a lesion site (unpublished data). Overall, these results suggest that a dopamine signal from supraspinal descending TH1+ axons contributes to motor neuron regeneration in the lesioned adult spinal cord of zebrafish.

Which signaling pathway is activated by dopamine and promotes motor neuron regeneration?

We have previously shown that hh signaling is involved in regeneration of motor neurons (Reimer et al., 2009). Thus we asked whether dopamine signaling would increase expression of \(ptc1\), an important target gene of the hh pathway. Using PCR, Dr. Angela L. Scott observes that in the lesioned spinal cord, \(ptc1\) expression was increased at 5 dpl both rostral and caudal to the lesion site, confirming earlier observations by in situ hybridization at 14 dpl. Notably, \(ptc1\) levels were higher rostral than caudal to the lesion site. To directly test whether dopamine signaling augments \(ptc1\) expression we injected animals with NPA. A single injection of NPA at 4 dpl further increased \(ptc1\) expression both rostral and caudal to the lesion site by 5 dpl. As patched is down-stream gene of shh signaling, this indicates an interaction of dopamine signaling with
3. Descending dopaminergic axons control motor neuron regeneration

the hh pathway. However, caudal levels still remained lower than rostral levels (unpublished data).

Which receptor mediates dopamine signaling and can dopamine influence its own receptor expression?

Dr. Angela L. Scott observes with semi-quantitative PCR confirmed a significant increase of *drd4a* mRNA expression rostral (p = 0.0289), but not caudal to the lesion site (p = 0.3823) at 5 dpl compared to expression levels in the unlesioned spinal cord, thereby confirming *Drd4a in situ hybridization* signal (unpublished data).

Given the much higher expression levels of the *drd4a* receptor rostral than caudal to the lesion site we next asked whether dopamine signaling could influence expression of its own receptor. After a single injection of NPA, Dr. Angela L. Scott found a small but significant increase of *drd4a* receptor expression rostral to the lesion site. However, caudal to the lesion site the drug had not influence. Thus, stimulating the dopamine pathway increases expression of *ptc1* and *drd4a*, but this influence is lower (*ptc1*) or absent (*drd4a*) caudal to the lesion site (unpublished data).

In sum, we provide evidence that dopamine promotes motor neuron regeneration by interacting with the hh pathway. We also show that the *drd4a* receptor is significantly upregulated in progenitor cells only in the presence of descending axons after a lesion. Overall, this shows a previously unknown influence of descending axons on adult spinal progenitor cell plasticity.
Fig. 3.4: Summary scheme of a lateral view of a lesioned spinal cord showing how TH1⁺ axons and drd4a receptor are present only rostral to a spinal lesion site at 2 weeks post-lesion, whereas Shh signaling and proliferation is also increased caudal to a spinal lesion site (Reimer et al., 2009).

Dopamine probably acts directly on spinal progenitor cells. This is suggested by detectability of increased expression of the drd4a receptor only in the ventricular zone of the lesioned rostral spinal cord. The ventricular zone consists of ependymo-radial glial cells, which are the only cells to proliferate after a spinal lesion. The ventricular zone contains slowly proliferating cells and gives rise to motor neurons from an Tg(olig2:GFP)⁺ zone, as shown by short term lineage tracing (Reimer et al., 2008). Consistent with the direct action of dopamine on progenitor cells, we show here that TH1⁺ axons are located in close proximity to the radial processes of Tg(olig2:GFP)⁺ ependymo-radial glial cells. However, volume transmission of dopamine may reach progenitor cells from even more distant sources (Fuxe et al., 2010). Direct actions of dopamine on progenitor cells has also been suggested for adult neurogenesis in the cerebral cortex (Hoglinger et al., 2004). Alternatively, altered activity of the spinal neuronal network reacting to the dopamine signal could indirectly stimulate motor neuron regeneration from progenitor cells (Spitzer, 2006).

What is the mechanism of dopamine action? Intriguingly, we observed that the hh target gene ptc1, which is upregulated after a lesion, is further upregulated
3. Descending dopaminergic axons control motor neuron regeneration

by the dopamine agonist NPA. Ptc1 is mainly expressed in Tg(olig2:GFP)+ependymo-radial cells, the adult motor neuron progenitors, after a lesion (Reimer et al., 2009). This suggests that dopamine signaling may converge on the hh signaling pathway in these progenitor cells. We have already shown that hh signaling is important for motor neuron regeneration by increasing proliferation of the progenitor cells and possibly acting on differentiation of precursor cells (Reimer et al., 2009). Thus dopamine probably acts in a similar way. The action of dopamine may also involve growth factors shown to mediate dopamine action on adult neurogenesis (Mori et al., 2008; Hasbi et al., 2009). These could be released by auto- or paracrine mechanisms in the ventricular zone or by the spinal network reacting to the dopamine signal with altered activity (Spitzer, 2006).

There is evidence for some specificity of the dopamine signal for motor neuron generation. We conclude this from the fact that during unperturbed regeneration more motor neurons are generated rostral to the lesion site than caudal to it, whereas 5-HT neurons are generated in comparable numbers. However, the drd4a receptor is apparently upregulated around the entire central canal and not only in the ventro-lateral zone of motor neuron progenitors. Thus regeneration of cell types originating from other progenitor zones around the central canal could also be influenced by dopamine.

Does dopamine regulate the expression of its receptor on ependymo-radial glial cells? A robust lesion-induced upregulation of drd4a was only observed in ventricular cells of the spinal cord rostral to the lesion site, suggesting that the presence of descending axons is necessary for the upregulation of drd4a. A small but significant increase of drd4a detectability by PCR rostral to the lesion site after NPA injection suggests a contribution of dopamine to the regulation of its receptor. However, caudal to the lesion site NPA did not lead to increased drd4a expression or motor neuron regeneration, suggesting that other signals derived from descending axons play a major role in drd4a regulation on progenitor cells after spinal lesion.

Could dopamine also act on mammalian spinal progenitor cells? It would be interesting to determine, whether ependymal cells in the adult spinal cord of
mammals also re-express dopamine receptors after a spinal lesion. These cells are morphologically similar to progenitors in the zebrafish spinal cord and also show lesion-induced proliferation (Meletis et al., 2008). However, the inhibitory environment of the spinal cord may prevent dopamine-mediated neuronal regeneration (Barnabe-Heider et al., 2010).

To our knowledge, this is the first report showing that descending axons strongly influence the plasticity of adult spinal progenitor cells in the lesioned spinal cord of an adult vertebrate. We identify dopamine as one of the important signals and provide evidence that dopamine’s action is mediated by the hh pathway. This opens up new avenues towards programming adult progenitor cells towards generation of neuronal cell types lost after injury or in disease.
4. Materials and Methods

4.1 Materials

4.1.1 Primary Antibodies

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4. Materials and Methods

4.1.2 Secondary Antibodies

All secondary antibodies were purchased from Jackson Immuno Research.

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### 4.1.3 Chemicals and Products

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<td>Glycine</td>
<td>Sigma-Aldrich (UK)</td>
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<td>Glycerol</td>
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<tr>
<td>Histoacryl</td>
<td>Braun (Tuttlingen, D)</td>
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<td>NaOH</td>
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<td>NBT/BCIP tablets</td>
<td>Sigma (St. Louis, Missouri, US)</td>
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<td>Sigma-Aldrich (UK)</td>
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<td>Methanol</td>
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<td>Microscope slide, superfrost plus</td>
<td>VWR (UK)</td>
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<tr>
<td>Potassium disulphate (KH₂PO₄)</td>
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<td>Roche Diagnostics (Mannheim, D)</td>
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<td>Purified Agar</td>
<td>OXOID (Hampshire, UK)</td>
</tr>
<tr>
<td>Ready-Load™1Kb Plus DNA Ladder</td>
<td>Invitrogen (UK)</td>
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<td>RNaseZAP</td>
<td>Sigma-Aldrich (UK)</td>
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<td>Sea Salt &quot;Carol Pro Salt&quot;</td>
<td>Aqua Schwarz (Goettingen)</td>
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<td>Sodium Borohydride (NaBH₄)</td>
<td>Sigma-Aldrich (UK)</td>
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<td>Sigma-Aldrich (UK)</td>
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<td>Tween 20</td>
<td>Sigma-Aldrich (UK)</td>
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<td>Triethanolamine, 98%</td>
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<td>Triton-X 100</td>
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<td>24-Well Tissue culture treated multiwell plates for adherent cell lines</td>
<td>Greiner Bio-One (UK)</td>
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<tr>
<td>Yeast t-RNA</td>
<td>Roche (Mannheim, D)</td>
</tr>
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</table>
4. Materials and Methods

4.1.4 Enzymes

<table>
<thead>
<tr>
<th>Enzyme/Reagent</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction endonucleases, various (5-20 U/µl)</td>
<td>New England Biolabs UK Ltd. (Hitchin, Hertfordshire, UK)</td>
</tr>
<tr>
<td>PfuUltraTM HF DNA Polymerase</td>
<td>Stratagene (Amsterdam, NL)</td>
</tr>
<tr>
<td><em>Taq</em> DNA Polymerase with Standard Taq Buffer</td>
<td>New England Biolabs UK Ltd.</td>
</tr>
<tr>
<td>SuperScript IIITM RT</td>
<td>Invitrogen Ltd. (Paisley, UK)</td>
</tr>
<tr>
<td>Ribonuclease Inhibitor RNasin®Plus RNase Inhibitor</td>
<td>Invitrogen Ltd. (Paisley, UK)</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Roche Diagnostics Ltd. (Burgess Hill, UK)</td>
</tr>
</tbody>
</table>

4.1.5 Bacterial strains and Bacterial media

E. coli XL-Blue or DH5α (Stratagene, UK) bacteria cells were used. All bacterial media were autoclaved before use. If necessary, ampicilline (50µg/ml) or kanamycine (30µg/ml) was added.

<table>
<thead>
<tr>
<th>Bacterial growth media encapsulated media LB medium</th>
<th>Supplier/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Agar Miller Fisher BioReagents</td>
<td>Fisher Scientific (UK)</td>
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4. Materials and Methods

4.1.6 Buffers and Medias

<table>
<thead>
<tr>
<th>Buffer/Solution</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking Solution (vibratome section in situ hybridization)</td>
<td>1% blocking reagent (Boehringer/Roche) in PBS-Tween 0.1% Prewarm – Do not boil</td>
</tr>
<tr>
<td>Hybridization Buffer</td>
<td>1. 5ml formamide 2.5ml 20x SSC 10µl Tween 100µl yeast tRNA (100mg/ml) 2.38ml DEPC-H2O 10µl heparin (50mg/ml)</td>
</tr>
<tr>
<td>Paraformaldehyde solution</td>
<td>Paraformaldehyde 1 M NaOH 10x PBS</td>
</tr>
<tr>
<td>Sodium Citrate Buffer, 10 mM</td>
<td>1.47 g Sodium citrate 500 ml 1x PBS Adjust pH to 6.</td>
</tr>
<tr>
<td>10X phosphate buffered saline (PBS)</td>
<td>4 g KCl 4 g KH₂PO₄ 28.39 g Na₂HPO₄ 160 g NaCl Add to 2000ml H₂O Adjust to pH 7.4</td>
</tr>
<tr>
<td>Tris-acetate-EDTA (TAE) buffer10X</td>
<td>2M Tris-acetate 100mM EDTA pH 8.5</td>
</tr>
<tr>
<td>Saline sodium citrate (SSC) buffer20x</td>
<td>3M NaCl 0.3M Na-citrate Adjust to pH 7.0</td>
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</table>

4.1.7 Kits

<table>
<thead>
<tr>
<th>Name of the Kit</th>
<th>Company</th>
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</thead>
<tbody>
<tr>
<td>In situ Cell death detection Kit, AP</td>
<td>Roche Diagnostics (Mannheim, D)</td>
</tr>
<tr>
<td>In situ Cell death detection Kit, TMR</td>
<td>Roche Diagnostics (Mannheim, D)</td>
</tr>
<tr>
<td>GFXTM Micro Plasmid Prep Kit27-9601-02</td>
<td>GE Health Care (Little Chalfont, UK)</td>
</tr>
<tr>
<td>MEGAscriptTM (T3/T7/SP6)</td>
<td>Ambion (Cambridge, UK)</td>
</tr>
<tr>
<td>HiSpeed® Plasmid Midi Kit</td>
<td>Quiagen (UK)</td>
</tr>
</tbody>
</table>
4. Materials and Methods

4.1.8 Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance</td>
<td>Acculab Sartorius group (UK)</td>
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<tr>
<td>Centrifuges 3K30C</td>
<td>Sigma Laborzentrifugen GmbH (Osterode am Harz, D)</td>
</tr>
<tr>
<td>Table Centrigue, 1-13</td>
<td>Sigma (D)</td>
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<tr>
<td>Confocal microscope, LSM 510</td>
<td>Zeiss (D)</td>
</tr>
<tr>
<td>Confocal microscope, LSM 750</td>
<td>Zeiss (D)</td>
</tr>
<tr>
<td>Fluorescence Microscope</td>
<td>Zeiss (D)</td>
</tr>
<tr>
<td>Hybridizer UVP HB-1000</td>
<td>Jencons PLS (East Grinstead, UK)</td>
</tr>
<tr>
<td>NanoDrop ND1000,</td>
<td>Thermo Scientific (USA)</td>
</tr>
<tr>
<td>pH Meter HP220</td>
<td>Mettler-Toledo (UK)</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Eppendorf (UK)</td>
</tr>
<tr>
<td>Shaking incubator MaxQ Mini 4450</td>
<td>Barstead Lab Line (UK)</td>
</tr>
<tr>
<td>Stereomicroscope, KL 1500 LCD</td>
<td>Zeiss (D)</td>
</tr>
<tr>
<td>Stirling Mixer</td>
<td>Sandrest Ltd. (UK)</td>
</tr>
<tr>
<td>Thermostat 5320</td>
<td>Eppendorf (UK)</td>
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<tr>
<td>Vibrotome, HM-650V</td>
<td>Microme (D)</td>
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<tr>
<td>Vortext-Genie 2</td>
<td>Scientific Industries (New York, USA)</td>
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<tr>
<td>Waterbath</td>
<td>Fisherbrand (UK)</td>
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4.1.9 Plasmids for probe making

<table>
<thead>
<tr>
<th>Gene</th>
<th>Promoter</th>
<th>Restriction site for linearisation</th>
<th>Provided by</th>
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<tbody>
<tr>
<td>GAD67</td>
<td>T3</td>
<td>EcoRI</td>
<td>Dr. Michell M. Reimer</td>
</tr>
<tr>
<td>Patched 1</td>
<td>T3</td>
<td>BamHI</td>
<td>Sudeh Riahi</td>
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<tr>
<td>Pax 3</td>
<td>T7</td>
<td>BamHI</td>
<td>Dr. Richard Dorsky, US</td>
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<tr>
<td>Pax 7</td>
<td>T7</td>
<td>EcoRI</td>
<td>Dr. Richard Dorsky, US</td>
</tr>
<tr>
<td>Vsx1</td>
<td>T3</td>
<td>Xhol</td>
<td>Dr. Shin-ichi Higashiima</td>
</tr>
</tbody>
</table>

4.1.10 Paraformaldehyde solution

For a final volume of 100 ml of a 4% paraformaldehyde solution, 4 g of paraformaldehyde powder and 2-3 drops of NaOH were added to approx. 20 ml of distilled water. The mixture was heated under a fume hood on a hot plate with a stir bar. When the solution became clear, it was immediately removed.
from the plate. The temperature of the solution should not exceed 60°C. In case the solution did not clear, more NaOH was added. Then, 10 ml of 10x PBS was added and filled up to 100 ml with distilled water. After filtration, the pH was checked with pH stripes (pH = 7.0-7.4). The solution was stored at 4°C and used for 1 to 2 weeks.

4.1.11 Transgenic fish lines

I used wik (wildtype) and Tg(HB9:GFP) (Flanagan-Steet et al., 2005), Tg(shha:GFP) (Shkumatava et al., 2004), Tg(olig2:GFP) (Shin et al., 2003), Tg(pax2a:GFP) (Picker et al., 2002), Tg(GAP43:GFP), GfG43S/A and Tg(GAP43:GFP), GfG43-708 (Kusik et al., 2010), Tg(vsx1:GFP) (Kimura et al., 2008), Tg(olig2:DsRed2) (Kucenas et al., 2008) and Tg(GAD67:GFP) (unpublished) transgenic fish line. I crossed Tg(vsx1:GFP) X Tg(olig2:DsRed2).

4.2 Methods

4.2.1 Fish maintenance

Zebrafish (Danio rerio) were kept at 26.5°C, 14-hour light and 10-hour dark cycle. They were fed three times a day, with dry flakes, ZM pellets (ZM Ltd., UK) and Artemia salina larvae (Aqua Schwarz, D). The fish were bred and raised according to standard protocols and all experiments have been approved by the British Home Office (Westerfield, 1989).

4.2.2 Lesion and injections

Before surgery to lesion the spinal cord, fish were kept for 48 hours (h) in 1300µS salt water to adapt them to a high salt concentration. Fish were kept in 1300µS salt water after surgery to minimize the infection risk. Adaptation is necessary to avoid additional stress after surgery. As previously described, fish were anesthetized with MS222 (Sigma, St. Louis, MO) diluted 1:5000 (Becker et al., 1997). During surgery, fish were kept on ice for additional analgesia and to constrict blood vessels in order to minimize bleeding. A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord
was completely transected under visual control 4 mm caudal to the brainstem-spinal cord junction or at the transition between brainstem and spinal cord. The wound was closed with Histoacryl (Braun, Melsungen, Germany). Until d5 after lesion, fish were kept in the dark without food to avoid unnecessary stimuli and to optimize the healing process.

BrdU (B9285, Sigma) was injected intraperitoneally at a concentration of 5 mg/ml in a volume of 25 µl at 0, 2 and 4 dpl. Analysis took place at 14 dpl or at 42 dpl. Cyclopamine and tomatidine were dissolved in 45% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) and injected at a concentration of 0.2 mg/ml in a volume of 25 µl (equaling 10 mg/kg) at 3, 6 and 9 dpl (Reimer et al., 2009).

NPA (D027, Sigma) was injected intraperitoneally at a concentration of 166 µg/ml in a volume of 25 µl (8.3 mg/kg body weight) at 3, 6 and 9 dpl. Analysis took place at 14 dpl.

6-OHDA (H4381, Sigma) was injected intraperitoneally at a concentration of 2.5 mg/ml in a volume of 25 µl (equaling 125 mg/kg body weight) 24 h before the spinal cord was transected. Analysis took place at 14 dpl.

4.2.3 Tissue preparation of adult zebrafish

For in situ hybridization and for all immunohistochemical labelings, apart from dopamine labeling, tissue was fixed in the following way: fish were anesthetized with MS222 (Sigma) diluted 1:1000 in PBS, perfused by inserting a needle connected to a syringe into the bulbus arteriosus through the heart. Once the blood was flushed out with 1xPBS, the syringe was replaced with a syringe containing 4% paraformaldehyde and 3ml/3min was flushed through the fish. Fixation for labeling of dopamine required a mixture of 5% glutaraldehyde, 1% metabisulfite and 1xPBS/1% metabisulfite for perfusion. Fixation for simultaneous labeling of tyrosine hydroxylase and dopamine required a mixture of 2% paraformaldehyde/2.5% glutaraldehyde, 1% metabisulfite and 1xPBS/1% metabisulfite for perfusion.
4. Materials and Methods

4.2.4 Tissue preparation of embryonic zebrafish

If not stated differently, washing steps were 5 minutes (min) and performed at room temperature (RT). 24 hpf embryos were dechorinated with watchmaker forceps in autoclaved fish water. For subsequent in situ hybridization or terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL), the solution was replaced with 4% paraformaldehyde in 1xPBS and embryos were fixed at 4°C overnight. Embryos were washed 4x with PBS/0.1% Tween (PBST 0.1). Then, embryos were incubated with 100% methanol. Fixed embryos were transferred into fresh 100% methanol and stored at –20°C for at least 30 mins.

4.2.5 Immunohistochemical labeling in vibrating blade microtome sections

Immunohistochemistry on 50 µm vibrating blade microtome sections has been described previously (Reimer et al., 2009). Apart from labeling of BrdU or dopamine, the following protocol was used: if not stated otherwise, washing steps were 5 min at RT. Spinal cord or brain was embedded in agar 4% in PBS, cut into 50 µm thick sections on the vibratome and sections were collected in 24-well plates in PBS. For labeling of synaptophysin, parvalbumin and ChAT, antigen retrieval was performed by incubating at 80°C in citrate buffer (10mM sodium citrate in PBS, pH 6.0) for 30 min. After washing in PBS/Triton-X 100 0.1% (PBSTx-0.1), sections were incubated in 50mM glycine in PBSTx-100 for 10 min followed by another 15 min wash step in PBSTx-0.1. For blocking, sections were incubated in either 1.5% donkey gamma globulin (labeling of ChAT or synaptophysin) or normal goat serum for 30 min. Incubation with up to three different primary antibodies in PBSTx-0.1 was performed overnight at 4°C. Subsequently secondary antibodies (diluted 1:200) were used. If required, sections were incubated in bis-benzimide (Sigma) in PBS to stain the nuclei. Finally, the sections were mounted in 90% glycerol/PBS. Specific binding of the secondary antibody was controlled by leaving out the primary antibody in alternating sections.

For labeling of BrdU, I used the following protocol:
4. Materials and Methods

after producing the sections, I washed these in PBS/ 0.5% Triton-X-100 (PBStx-0.5) for 1 h at RT, then washed 3x in PBS and 1x with HCl 37% diluted 1:8 in H2O. Then, sections were incubated in HCl conc. diluted 1:8 in H2O at 37°C in the waterbath for 20 min. After washing 6x in PBS, sections were incubated in 50 mM glycine in PBS Triton X-100 0.3% (PBStx-0.3) for 10 min followed by another 15 min wash in PBStx-0.3. For blocking, sections were incubated in 1.5% donkey gamma globulin in PBStx-0.3 for 1h. Subsequent steps were performed as described above using PBStx-0.3 instead of PBStx-0.1.

For simultaneous detection of dopamine and TH1 primary and secondary antibodies were simultaneously applied and a modified protocol was used for primary antibody incubation: floating sections of spinal cords (50 µm in thickness) of perfusion-fixed animals (2% paraformaldehyde/2.5% glutaraldehyde, 1% metabisulfite) were washed 2x in PBS/1% metabisulfite (adjust pH to 7.4) with 0.1% Triton X-100 (PBS Mb Tx) for 15 min each. Then, sections were incubated in 0.1% NaBH₄ in ddH₂O for 30 min followed by two washing steps of 15min each with PBS Mb Tx. Sections were blocked in 1.5% normal goat serum in PBS Mb Tx for 30 min. Then, primary antibodies were diluted in PBS Mb Tx and incubated with the sections overnight at 4°C. Secondary antibody incubation followed the standard protocol.

4.2.6 Immunohistochemical stainings in whole mount embryos

To detect protein expression patterns in 24 hpf embryos, whole-mount immunohistochemistry was performed. Animals were dechorionated and yolks were pierced with insect pins. Embryos were incubated in 4%PFA containing 1% (v/v) DMSO for 45 min and then washed with PBS.

Embryos were incubated with blocking buffer to prevent the unspecific binding of the primary antibody for 30 min at RT. Primary antibodies were diluted in blocking buffer and applied to embryos at 4°C overnight. Unbound primary antibody was removed by three washing steps with PBS. To visualize the primary antibodies, secondary antibody were diluted 1:200 in blocking buffer and applied to embryos overnight at 4°C. Unbound secondary antibody was removed by washing step with PBS. Finally embryos were washed by PBS and
cleared in an ascending glycerol series (30, 50, 70% glycerol in 1xPBS). Embryos were mounted in 70% glycerol/PBS.

4. Materials and Methods

4.2.7 In situ hybridization on 50µm in vibrating blade microtome sections

The method for non-radioactive in situ hybridization on 50 µm sections has already been described (Lieberoth et al., 2003).

Work areas and used material and solutions were kept RNAse-free. Work areas were cleaned with RNAaseZAP (Sigma-Aldrich) and solutions were made up with RNA-free water. If not stated otherwise, washing steps were 5 min.

Sections were transferred into 24-well-plates, washed twice in PBST-0.1 and then digested with proteinase K (stock concentration: Roche PCR Grade, 0.3 µg/ml in 0.1 M Tris pH 8 and 0.05 M EDTA; working solution 0.7µl/ml) for 9 min at RT.

After washing twice with glycine/ PBST-0.1 (2mg/ml), tissue was fixed in PFA for 20 min. After washing 4x in PBST-0.1, supernatant was removed and sections were washed once in 300µl whole mount hybridization buffer. After replacing with fresh whole mount hybridization buffer (500µl per well), the plate was wrapped with parafilm. The following prehybridizing step lasted for at least 3 h at 55°C. Digoxygenin labeled probes (1:1000 or 1:500 in whole mount hybridization buffer) were heated at 80°C for 10 mins in the water bath. Probes were briefly centrifuged and chilled on ice for 2 mins. Prehybridization buffer in the wells was replaced by probe/hybridization buffer solution. Plates were wrapped with parafilm and incubated at 55°C overnight.

The following steps were at 55°C. The solutions were preheated for 10 min in the incubator. Sections were washed twice in 50% formamide/2xSSC + 0.1% Tween for 30 mins each, once in 1x SSC + 0.1% Tween for 15 mins and twice in 0.2 x SSC + 0.1% Tween for 30 mins each.

Then, the sections were blocked for 1h in 10% (10g blocking reagent in 100ml PBST) Boehringer Roche blocking reagent at room temperature (500µl per well). Anti-DIG alkaline phosphatase coupled fab fragments (Boehringer/Roche) were diluted 1:2000 in blocking reagent (1:2000), added to
the sections and left overnight at 4°C. The next day, the sections were washed 6x for 20 min each in PBST on a shaker and then once in PBS. Staining solution was prepared by adding one NBT/BCIP tablet (Sigma) in 10 ml ddH$_2$O. Sections were washed 6x 20 mins each in PBST on the shaker. Then they were washed once in staining solution. Afterwards, the staining solution was exchanged and sections were incubated for 30 mins to overnight, depending on the kinetics of signal development. Finally the staining solution was removed, the sections were washed several times in PBS. When the experiment was finished without subsequent immunohistochemical staining, 70% Glycerol/PBS was added and sections were mounted onto slides in 70% Glycerol/PBS. When subsequent immunohistochemical staining was performed, sections were washed several times in PBS and immunohistochemical labeling was performed as described in 4.2.5, starting with the incubation step in 50 mM glycine.

4.2.8 Whole mount in situ hybridization

If not stated otherwise, washing steps were 5 min and performed at RT. Fixed embryos (4.2.4) were distributed into autoclaved eppendorf tubes (8-9 embryos per tube) and washed in 75%, 50%, 25% methanol in PBST-0.1. Then, they were washed twice in PBST-0.1. The subsequent protocol was the same as the one used for vibratome sections in situ hybridization up to the step in which the staining solution is removed. After washing several times in PBS, embryos were either frozen for subsequent sectioning on the cryostat or cleared in an ascending gradient of glycerol with PBS (30%, 50% and 70% glycerol in PBS). Once they had sunk to the bottom, the next higher concentration of glycerol was used. Slides were prepared with 4 drops of vaseline as spacers and embryos were mounted in 70% glycerol/PBS.

4.2.9 TUNEL staining in embryos

We used In Situ Cell Death Detection Kit, TMR red, TUNEL (Roche Applied Science). If not indicated otherwise, washing steps were 5 min and at RT. Fixed embryos were washed four times in PBStx-0.1 and then rehydrated in methanol series (75%-50%-30% in PBStx-0.1). After washing 3 times in PBStx-
0.1, embryos were digested with 0.7µl/ml proteinase K (stock concentration: Roche PCR Grade, 0.3 µg/ml in 0.1 M Tris pH 8 and 0.05 M EDTA) in PBStx-0.1 for 10 min. Then, the embryos were rinsed with 2mg/ml glycine in PBStx-0.1 and fixed in 4% PFA for 20 min.

After washing 6x in PBStx-0.1 (each 5 min), embryos were transferred into the prepared reaction mix (45 µl transferase buffer and 5 µl terminal transferase), incubated 1 h on ice and 1 h at 37°C in the waterbath.

Then, embryos were washed in PBS, incubated in PBS/bis-benzimididine and again washed in PBS. They were cleared in ascending glycerol series and mounted as described for whole mount in situ hybridization.

### 4.2.10 TUNEL staining in 50 µm vibrating blade microtome sections

TUNEL has been established for spinal cord section in zebrafish by modifying and combining different protocols described in literature (Labat-Moleur et al., 1998; Bessert and Skoff, 1999; Cole and Ross, 2001). Tissue preparation and sectioning was performed as described for immunohistochemical labeling.

Sections were incubated for 5 min in PBStx-0.1 and 10 min in PBStx-0.3 at RT. After a proteinase K digest (Roche PCR Grade, stock solution: 0.3µg/ml in 0.1M Tris pH 8 and 0.05M EDTA, working solution: 0.7µl/ml in PBSTX) in a water bath at 37°C for 15 min, subsequent washes with PBStx-0.1 twice 5 min were followed by an incubation step in 0.1% sodium citrate in PBStx-0.1 for 10 min on ice. After washing the sections in PBStx-0.1 twice 5 min, the terminal transferase reaction (Roche Applied Science, Cell death detection TMR Red) was carried as described above in embryos, with the only difference that the transferase reaction was extended to 1.5 h. After subsequent washing steps in PBS, sections were either directly mounted using a mounting media containing 2.5 % w/v DABCO, 50mM Tris pH8 and 90% glycerol or processed for subsequent immunohistochemical staining. DABCO was used to protect the staining from bleaching during confocal imaging. Susequent immunohistochemical stainings were performed to enhance GFP in transgenic Hb9:GFP fish to be able to colocalize TUNEL staining in Hb9:GFP motor neurons.

As controls, we performed TUNEL in zebrafish brain to detect apoptotic cells in
the cerebellum (Ampatzis and Dermon, 2007). In addition, we analyzed the tectum that was lesioned with a glass needle. As negative control, the enzyme was left out from alternating sections.

4.2.11 Image acquisition and figure plates

For documentation and analysis, I used either a Zeiss LSM 510 or a Zeiss 750 LSM Confocal Microscope using 20x and 63x oil immersion lenses. Images were processed with Zeiss LSM Image Browser and Image J. I composed the shown figure plates using Photoshop using raw data I have generated or using raw data provided by other people, which is stated at the respective figure plate.

4.2.12 Fiber quantification

For axon quantifications, we used a Zeiss LSM 510 Confocal Microscope using a 20x Apochromate 0.75 lens for acquiring images. TH1+ profiles were quantified applying the Feature J Hessian plug-in for Image J (smallest eigenvalue Hessian tensor; smoothing scale to 1.0; http://rsbweb.nih.gov/ij/plugins/index.html) on maximum projection z-stacks (15 optical sections, thickness 1.5 µm) of whole spinal 50 µm sections according to an established protocol (Grider et al., 2006). Profiles were quantified in binary images (threshold 140) using the "analyse particle" function of Image J with particle size 1 to infinity and circularity from 0.00 to 1.00. Comparing this method with manual counts indicated an average divergence of 6.25%. Images that contained fewer than approximately 100 profiles were manually counted.

For quantification of TH1/dopamine double-labeled axons we performed deconvolution of images taken with a 63x objective. Images were taken with Nyquist sampling rate (63x oil lens, 0.3µm z-step, zoom=3). The program Huygens Essential (Scientific Volume Imaging, Hilversum, Netherlands) was used for deconvolution. Subsequent image processing and analysis was performed in Image J.
4. Materials and Methods

4.2.13 Stereological counts of cells

Stereological counts were performed in confocal image stacks of three randomly selected vibratome sections from the region up to 750 μm rostral to the lesion site and three sections from the region up to 750 μm caudal to the lesion site. Depending on the addressed question, cell numbers were then calculated for the entire 1.5 mm surrounding the lesion site or separately for rostral and caudal. Variability of values is given as standard error of the mean.

4.2.14 Profile counts in spinal cord sections

Immunoreactive positive cellular profiles in the ventricular zone (up to one cell diameter away from the ventricular surface) were counted in vibratome sections (50 μm thickness) in the same region of spinal cord as described for stereological counts. At least 6 sections were analyzed per animal by fluorescence microscopy and values were expressed as profiles per 50 μm section. The observer was blinded to experimental treatments. Variability of values is given as standard error of the mean.

4.2.15 Statistical analysis

For statistical analysis of the data, the program Prism was used. The tests were chosen according to the experimental set up, respectively, and are stated there individually.

4.2.16 Transformation of plasmid DNA and DNA isolation

Plasmids were transformed into competent E. coli DH5 α. 10 ng of plasmid DNA was added to competent E. coli DH5 α or XL1-Blue cells and incubated for 30 mins on ice. After a heat shock (30 seconds, 42°C) and successive incubation on ice (5 mins), 500 µl of LB-medium were added to the bacteria and incubated at 37°C with 200 rpm shaking. Bacteria were plated on LB-Agar plates containing 50 μg/ml ampicilline or 30 μg/ml kanamycine and incubated overnight at 37°C.
4. Materials and Methods

4.2.17 Colony-PCR

Colonies received by transformation of bacteria with plasmids coding for vsx1 or GAD67 were checked by colony PCR. For this, with a pipette tip one single colony was tipped, dipped into a PCR tube and then plated on a new plate.

The standard PCR (Saiki et al., 1985), an amplification of DNA by in vitro enzymatic replication, was performed in an MJ minsi-gradient thermal cycler (Biorad, UK).

| Program |
|-----------------|-----------------|-----------------|
| Cycles | Time | Temperature |
| 1 | 5mins | 94°C |
| 25-40 | 30s | 94°C |
| 45 s | | Tm-1°C |
| 1 | 1 mins per kb | 72°C |
| 1 | 10 mins | 72°C |

4.2.18 DNA extraction

Colonies checked by colony PCR were picked to inoculate overnight cultures consisting of 5ml LB-Medium with 50μg/ml ampicilline. Bacteria were harvested by centrifugation at 8000 rpm and plasmid DNA was isolated using GFXTM Micro Plasmid Prep Kit27-9601-02 (GE Health Care). The plasmids were confirmed by DNA sequencing (Sequencing Service, College of Life Sciences, MSI/WTB Complex University of Dundee, UK).

4.2.19 Restriction enzyme digestion and agarose gel electrophoresis

Plasmids provided by various sources were analyzed with restriction enzyme digestion. Resulting DNA fragments were checked by subsequent agarose gel electrophoresis using a 1.5% agarose gel containing ethidium bromide was

| GoTaqM (Promega) | 5 µl |
| T3/T7 Primer pair | 1 µl |
| H2O | 4 µl |
4. Materials and Methods

performed. Potential clones were retransformed into chemically competent *E. coli* XL1-Blue or DH5α cells and one single clone each was incubated in 50ml LB medium overnight for subsequent DNA isolation using HiSpeed® Plasmid Midi Kit (Qiagen, UK). The plasmids were confirmed by DNA sequencing. For receiving linearized DNA as template for probe making, plasmids were digested with restriction endonucleases. The enzyme was chosen depending on the sequence of the plasmid, orientation of the template and restriction sites. Plasmids and corresponding restriction endonucleases are given in the table above.

In general the following reaction mix was used and incubated at 37°C overnight.

<table>
<thead>
<tr>
<th>Template (plasmid DNA)</th>
<th>1.6 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>2µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>Fill up to 100 µl</td>
</tr>
</tbody>
</table>

The reaction product was tested by agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Template (linearized DNA)</th>
<th>5 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O</td>
<td>5 µl ddH₂O</td>
</tr>
<tr>
<td>DNA Loading buffer</td>
<td>0.8 µl</td>
</tr>
</tbody>
</table>

4.2.20 DNA precipitation

To clean the linearized DNA, I used the following protocol:

Sodium acetate (3M, pH 4.9, 1:10 v/v) and 2.5x volumes cold (-20°C) ethanol were added to the DNA. After mixing, the reaction tubes were kept on ice for 30 mins and centrifuged for 15 mins at 16000x g (RT). Supernatant was removed and the pellet was washed with 800 µl 70% ethanol. After centrifugation and removal of the supernatant the pellet was washed repeatedly with ethanol 70% in 400 µl and 200 µl. The pellet was dried for 15 mins at RT and resuspended in ddH₂O. The concentration of linearized DNA was checked with a spectrophotometer (NanoDrop ND1000, Thermo Scientific, USA).
4. Materials and Methods

4.2.21  *In vitro* transcription

To generate DIG labeled probes for *in situ* hybridization, an *in vitro* transcription was performed using the MEGAscriptTM Kit (Ambion, Cambridge, UK). 10 µg of plasmid DNA containing the desired insert, flanked by a T3, T7 or SP6 promoter were digested with restriction endonucleases overnight. Thus only the promoter sequence and the desired DNA insert were transcribed. The digested plasmid DNA was precipitated as described in above (see 4.2.10). For the generation of DIG labeled RNAs, DIG-11-dUTP (Roche, UK) was used instead of UTP provided by Ambion.

To avoid contamination with RNAase, RNAse-free filter tips and eppendorf tubes and RNase-free reagents were used. Work surfaces were cleaned with RnaseZIP and all RNAse free were used. The Ambion MAXI-SCRIPT Kit was used. Reagents were thawed on ice 30 mins in advance; transcription buffer and ribonucleotides were mixed on a Vortext-Genie 2 and centrifuged briefly. All reagents were assembled at RT.

| DNA template (4 to 12µl, about 10 µg) | 4 to 12µl | 0 to 6µl |
| Total volume (DNA and H2O) | 12µl |
| ATP | 1µl |
| GTP | 1µl |
| CTP | 1µl |
| UTP | 0.6µl |
| Dig-Labeled UTP | 0.4µl |
| 10x transcription buffer | 2µl |
| RNA-Polymerase | 2µl |

4.2.22  Forced swim test

As previously described, we tested the endurance of unlesioned and lesioned fish by determining the time they were able to withstand a water current (Reimer et al., 2009). In a tunnel with a flat bottom (7cm width), 15 cm long compartments were divided off by wire mesh. A current of 7cm/s was induced using a pond pump (Nautilus 8000, Oase GmbH). The time was recorded that fish were able to hold their position in the water current. When a fish was not able to leave the wire mesh within
4. Materials and Methods

30s, the experiment was stopped. The time fish were able to withstand the water current was measured up to one hours, when full recovery was assumed.
Summary

Zebrafish, in contrast to mammals, are capable of functional regeneration after complete transection of the spinal cord. In this system I asked:

(1) To what extent do the dopaminergic and serotonergic system regenerate?

(2) Which spinal cell types regenerate in the lesioned spinal cord?

(3) Do dopaminergic axons from the brain influence cellular regeneration in the spinal cord?

(1) After spinal cord lesion, zebrafish completely recover locomotion within six weeks. 5-HT and dopaminergic axons cross the lesion site and re-innervate the caudal spinal cord. Here we show that changes in density of 5-HT and dopaminergic axon terminals in the caudal spinal cord during recovery correlate with functional recovery. After regeneration, the spinal dopaminergic and 5-HT system, consisting of neuronal somata in the spinal cord and descending axons, differ significantly from their unlesioned organization. Rostral to the lesion site axons sprout and innervation of the caudal spinal cord is limited: axonal density close to the lesion site is reduced and axons do not regrow into the far distal spinal cord.

(2) We have shown that spinal motor neurons that are lost after a spinal lesion are replaced by newly born motor neurons that mature and integrate into the spinal circuitry after a spinal lesion in adult zebrafish. In addition to motor neurons, ventrally located 5-HT neurons are generated after spinal lesion. Also, V2 interneurons that are absent in the unlesioned spinal cord, are newly generated in significant number after spinal lesion. In contrast dorsal parvalbumin positive cells are neither lost nor regenerated after lesion. Expression of ventral but not of dorsal embryonic markers was increased after spinal lesion.
(3) We hypothesized that signals released by descending axons are involved in cellular regeneration around the lesion site. Dopaminergic axons of supraspinal origin sprout rostral, but are almost completely absent caudal to the lesion site at two weeks post-lesion. Moreover, we observe that expression of the dopamine receptor drd4a is only increased rostral to the lesion site in the ventricular zone of progenitor cells, including olig2 expressing motor neuron progenitors. Correlated with these rostro-caudal differences, the number of regenerated motor neurons is almost two-fold higher rostral than caudal of the lesion site. Ablation of tyrosine hydroxylase positive axons reduced motor neuron numbers only rostral to the lesion site. In gain-of-function experiments, treatment with a dopamine agonist increased motor neuron numbers increased rostral but not caudal to the lesion site. Overall, during successful spinal cord regeneration in zebrafish various cell types are generated and plastic changes and regeneration of monoaminergic descending axonal projections occur. Dopamine released by descending axons is able to augment motor neuron regeneration, showing for the first time that signals from descending axons influence cellular regeneration in the lesioned spinal cord.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CiDi</td>
<td>Circumferential descending interneurons</td>
</tr>
<tr>
<td>ChAT</td>
<td>Cholin acetyl transferase</td>
</tr>
<tr>
<td>d0, d2, d4</td>
<td>Day zero, day two, day four etc.</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilisation</td>
</tr>
<tr>
<td>dpl</td>
<td>Days post lesion</td>
</tr>
<tr>
<td>ES</td>
<td>Electrical stimulation</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma aminobutyric acid</td>
</tr>
<tr>
<td>GAD67</td>
<td>Glutamate decarboxylase 1</td>
</tr>
<tr>
<td>GAP43</td>
<td>Growth associated protein 43</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>hh</td>
<td>Hedgehog</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>KA cells</td>
<td>Kolmer-Agdur cells</td>
</tr>
<tr>
<td>BWS</td>
<td>Hindlimb body weight support</td>
</tr>
<tr>
<td>fgf</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Ca2+</td>
<td>Calcium</td>
</tr>
<tr>
<td>nFLM</td>
<td>Medial longitudinal fasciculus</td>
</tr>
<tr>
<td>MAG</td>
<td>Myelin associated glycoprotein</td>
</tr>
<tr>
<td>NPA</td>
<td>R(-)-Propylnorapomorphine hydrochloride</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>Mash1</td>
<td>Mammalian atonal homolog 1</td>
</tr>
<tr>
<td>MCoD</td>
<td>Multipolar commissural descending interneuron</td>
</tr>
<tr>
<td>MND</td>
<td>Motor neuron disease</td>
</tr>
<tr>
<td>NgN</td>
<td>Neurogenin</td>
</tr>
<tr>
<td>OMgp</td>
<td>Oligodendrocyte myelin glycoprotein</td>
</tr>
<tr>
<td>Ptc1</td>
<td>Patched 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST 0.1</td>
<td>PBS/0.1% Tween</td>
</tr>
<tr>
<td>PBStx 0.1</td>
<td>PBS/Triton-x 100 0.1%</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAC</td>
<td>Stratum album centrale</td>
</tr>
<tr>
<td>SFGS</td>
<td>Stratum fibrosum and griseum superficiale</td>
</tr>
<tr>
<td>SGC</td>
<td>Stratum griseum centrale</td>
</tr>
<tr>
<td>SPV</td>
<td>Periventricular layer</td>
</tr>
<tr>
<td>shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Tpp</td>
<td>Periventricular nucleus of posterior tuberculum</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling</td>
</tr>
<tr>
<td>wpl</td>
<td>Weeks post lesion</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1.1: TH1+ axons in the spinal cord are mostly dopaminergic and originate in the brain.................................................................34
Figure 1.2: TH2+ neurons are found in the brain, but not the spinal cord........35
Figure 1.3: Spinal lesion induces plasticity of TH1+ axons.........................40/41
Figure 1.4: Recovery of swimming capability is variable and is abolished by re-transection of the spinal cord.........................43
Figure 1.5: Recovery of swimming capability correlates with regrowth of TH1+ axons beyond the lesion site.......................................45
Figure 1.6: Spinal 5-HT+ axons originate in the spinal cord and brainstem..................................................................................47
Figure 1.7: Few TH1+ varicosities are in close proximity to 5-HT+ interneurons or ChAT+ motorneurons.................................49
Figure 1.8: Few 5-HT+ varicosities are in close proximity to parvalbumin+ interneurons or ChAT+ motoneurons..................50
Figure 1.9: Spinal lesion induces plasticity of 5-HT+ axons.....................53/54
Figure 1.10: Recovery of swimming capability correlates with regrowth of descending 5-HT+ axons beyond the lesion site........56
Figure 1.11: 5-HT+ cells are newly generated in the vicinity of a spinal lesion..................................................................................60
Figure 1.12: 5-HT+ cells that contact the central canal in a ventral position are found in the lesioned spinal cord...........62
Figure 1.13: Cyclopamine treatment impairs regeneration of 5-HT+ neurons..................................................................................63
Figure 1.14: Numbers of 5-HT+ cells caudal to the lesion site correlate with recovery of swimming function at 6 weeks post-lesion....65
Figure 1.15: Principiles of the quantification method and characterization of the axonal profiles are shown................................66
Figure 2.0: Schematic presentation of transcription factor domains set up in the developing vertebral neural tube by ventral/dorsal gradient of Shh........................................................................77
Figure 2.1: Subpopulation of ChAT+ cells possess pax2+ nuclei................80
Figure 2.2: Subpopulation of Tg(HB9:GFP)+ cells express parvalbumin........81
Figure 2.3: ChAT+ motor neurons die after spinal lesion, are newly generated and integrated into the spinal circuit......................83
Figure 2.4: Small-diameter Tg(HB9:GFP)+ cells are ChAT-........................85
Figure 2.5: Evidence for a pMN-equivalent zone in the ependymal layer of the adult spinal cord......................................................89
Figure 2.6: Tg(shha:gfp)+ ependymo-radial glial cells co-express Nkx6.1, but not Pax6.................................................................90
Figure 2.7: shh pathway gene patched is upregulated in ependymo-radial glial cells of the lesioned spinal cord..................................92
Figure 2.8: shh agonist does not increase number of tg(HB9:GFP)+ motor neurons.................................................................94
Figure 2.9: Tg(vsx1:GFP)+ cells are only found after lesion...............96
Figure 2.10: Tg(vsx1:GFP)+ cells are newly generated after lesion........98
List of Figures

Figure 2.11: \textit{Tg(vsx1:GFP)}+ cells are distinct from HB9+ or islet-1/2+ cells. 100
Figure 2.12: GFP enhancement and \textit{in situ} hybridization for vsx1 mRNA in spinal cross sections of \textit{Tg(vsx1:GFP)} transgenic fish. 101
Figure 2.13: Numbers of \textit{Tg(vsx1:GFP)}+ cells are higher in the caudal than in the rostral half of the lesion site at two weeks post-lesion. 102
Figure 2.14: \textit{Tg(vsx1:GFP)}+ cells are distinct from pax2+ cells. 104
Figure 2.15: \textit{Tg(vsx1:GFP)}+ cells originate from a p2-like zone. 106
Figure 2.16: Pax2+ cells are newly generated after lesion but do not increase in cell number. 109
Figure 2.17: Double-labeling of GFP (green) and parvalbumin (red) in spinal cross section of transgenic \textit{Tg(pax2a:GFP)} fish. 111
Figure 2.18: Parvalbumin+ cells are rarely newly generated after lesion. 114
Figure 2.19: Central canal contacting GABA+ cells are not affected by spinal lesion. 117
Figure 2.20: \textit{Tg(GAD67:GFP)}+ cells in spinal cord and tectum are shown. 119
Figure 2.21: GAD67+ mRNA expressing cells in tectum and spinal cord are shown. 120
Figure 2.22: \textit{Tg(Olig2:GFP)}+ cells devoid of central canal contact are newly generated after spinal lesion. 122
Figure 2.23: Comparison of \textit{Tg(GAP43:GFP)} transgene expression driven by full length GfG43S/A or truncated GfG43-708 promoter after a spinal lesion. 125
Figure 2.24: Various cell types express \textit{Tg(GAP43:GFP)} transgene after spinal lesion. 128/129
Figure 2.25: Schematic overview about the distribution of various cell markers within the spinal cord of adult zebrafish. 132
Figure 2.26: Overview of quantification of numbers of mature cells (5-HT, ChAT, GABA, parvalbumin, pax2, \textit{Tg(pax2a:GFP)}) and undifferentiated cells (\textit{Tg(HB9:GFP)}, \textit{Tg(vsx1:GFP)}). 136
Figure 2.27: Overview of quantification of cell numbers of mature cell markers (5-HT, ChAT, GABA, parvalbumin, pax2, \textit{Tg(pax2a:GFP)}) and undifferentiated cell markers (\textit{Tg(HB9:GFP)}, \textit{Tg(vsx1:GFP)}). 141

Figure 3.1: TH1+ axons and drd4a \textit{in situ} hybridization signal are present rostral, but not caudal to the spinal lesion site. 155
Figure 3.2: Numbers of newly-generated motor neurons, but not of 5-HT+ cells, differ between the rostral and caudal part of the lesioned spinal cord. 157
Figure 3.3: 6-OHDA destroys TH1+ axons, but not 5-HT+ axons, and selectively reduces the number of newly-generated motor neurons rostral to the lesion site. 160
Figure 3.4: Summary scheme of a lateral view of a lesioned spinal cord showing how TH1+ axons and drd4a receptor are present only rostral to a spinal lesion site at 2 weeks post-lesion. 165
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Bibliography


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Topic: Regeneration of the injured spinal cord in zebrafish

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30/11/2010  Travel grant, Ninth Göttingen Meeting of the German Neuroscience Society (300 Euro)

28/08/2009  Poster prize at the Scottish Neuroscience Groups Conference in St. Andrews, Scotland, UK (competitive)

03/2009  Travel award of the Scottish Cell Stem Network (£800)

09/2007  The College of Medicine and Veterinary Medicine Scholarship, University of Edinburgh (£12,608 per annum)

06/2001  Karl-von-Frisch Award (Karl-von-Frisch Abiturienten-Preis), competitive, honours excellent performance in the area of Biology

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27/08/2010  The Scottish Neuroscience Group Meeting, Glasgow, UK

»Dopaminergic axons of supraspinal origin control motor neuron regeneration in the lesioned spinal cord of adult zebrafish«

28/05/2010  Invited speaker for a seminar in Dresden, BIOTEC,

»Regeneration of the injured spinal cord in adult zebrafish«

06/09–10/09 16th international Society of Developmental Biologists Congress 2009, Edinburgh, Scotland, UK, Poster presentation,

»Neuronal circuit in the spinal cord of zebrafish before and after lesion«

15/07–19/07  6th European Zebrafish Genetics and Development Meeting, Rome, Italy, Poster presentation

»Neuronal circuit in the spinal cord of zebrafish before and after lesion«

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October 2010  Demonstrator for student practical »Quantification in Life Sciences«

19/06/2010  Organising and supervising »Kick start program« for pupils

»Neuroscience: Adventures with Zebrafish«
Publication list


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