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REGENERATION AND NEURAL CIRCUITS IN THE SPINAL CORD: AN IMAGING STUDY ON ZEBRAFISH

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REGENERATION AND NEURAL CIRCUITS IN THE SPINAL CORD: AN IMAGING STUDY ON ZEBRAFISH

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To my beloved wife Hanna, Fiona, and Arthur

En del lär sig aldrig!

Stockholm 2019-02-27 05:09 Note to Ego

ABSTRACT

The reversal of spinal cord injury (SCI) and its devastating effect on voluntary control is one of the most provocative challenges in neuroscience research. Preclinical and clinical research has for a very long time tried to address this challenge, but there is still no effective treatment that leads to functional recovery. In simplified terms, the spinal cord resembles a highway, with outgoing commands from the brain and incoming feedback from the periphery. However, the spinal cord is an extremely complex apparatus with billions of cells, connections, and neuronal circuits. Injury to the spinal cord in humans has acute disastrous effects, followed by secondary pathophysiological events leading to a permanent loss of sensation and motor function corresponding to the site of injury. The spinal cord of humans, as far as we know, lacks the capacity to regenerate after an injury, in contrast to that of vertebrate fish, such as the zebrafish, which has an extraordinary ability to regenerate. Investigating the regenerative capacity of zebrafish can unveil mechanisms and features that may be translatable to the clinic.

In paper I, we identified V2a interneurons as an intrinsic source of excitation and a necessity for the zebrafish larvae's normal generation of locomotor rhythm.

In paper II, we scaled up and developed the technique used in paper I to a robust method to induce precise spatial and temporal SCI with minimal collateral damage in zebrafish larvae.

In paper III, we investigated the impact of several factors, including lesion size, hypothermia, and analgesic substances, on the functional recovery of zebrafish larvae following SCI. Furthermore, we examined intrinsic Ca^{2+} signaling before and after SCI.

In summary, this thesis paves the way for further investigations of the remarkable regenerative capacity zebrafish possess.

LIST OF SCIENTIFIC PAPERS

I. Eklöf-Ljunggren E, Haupt S, Ausborn J, **Dehnisch I,** Uhlén P, Higashijima S, El Manira A
Origin of excitation underlying locomotion in the spinal circuit of zebrafish
Proceedings of the National Academy of Sciences U S A, 2012 Apr 3;109(14):5511-16

II. **Dehnisch Ellström I**, Spulber S, Hultin S, Norlin N, Ceccatelli S, Hultling C, Uhlén P
Spinal cord injury in zebrafish induced by near-infrared femtosecond laser pulses
Journal of Neuroscience Methods, 2019; 31: 259-266

III. **Dehnisch Ellström I**, Louhivouri L, Norlin N, Hultling C, Uhlén P
Impact of lesion size, hypothermia, and analgesics on locomotor recovery in spinal cord injured zebrafish
Manuscript in preparation

Publications not included in this thesis

Niklasson M, Maddalo G, Sramkova Z, Mutlu E, Wee S, Sekyrova P, Schmidt L, Fritz N, **Dehnisch I**, Kyriatzis G, Krafcikova M, Carson BB, Feenstra JM, Marinescu VD, Segerman A, Haraldsson M, Gustavsson AL, Hammarström LG, Jenmalm Jensen A, Uhrbom L, Altelaar AF, Linnarsson S, Uhlén P, Trantirek L, Vincent CT, Nelander S, Enger PØ, Andäng M
Membrane-depolarizing Channel Blockers Induce Selective Glioma Cell Death by Impairing Nutrient Transport and Unfolded Protein/Amino Acid Responses
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Jungebluth P, Haag JC, Sjöqvist S, Gustafsson Y, Beltrán Rodríguez A, Del Gaudio C, Bianco A, **Dehnisch I**, Uhlén P, Baiguera S, Lemon G, Lim ML, Macchiarini P.
Tracheal tissue engineering in rats
Nature Protocols. 2014 Sep;9(9):2164-79.

Matti Lam, Mohsen Moslem*, Julien Bryois*, Robin J Pronk, Elias Uhlin, Jessica Olive, Rebecca Morse, **Ivar Dehnisch Ellström**, Lauri Louhivuori, Per Uhlén, Britt-Marie Anderlid, Malin Kele, Patrick F Sullivan, Anna Falk
Single cell RNA-seq analysis of autism patient with bi-allelic NRXN1-alpha deletion reveal skewed neuronal fate choice in neural Progenitors
Under Revision in Stem Cell Reports

CONTENTS

LIST OF ABBREVIATIONS

2PLSM	Two-photon laser scanning microscopy
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole
ASIA	American Spinal Injury Association
CNS	Central nervous system
CPG	Central pattern generator
CSPG	Chondroitin sulfate proteoglycans
Dpf	Days post fertilization
Fgf	Fibroblast growth factor
GAP	Growth associated proteins
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Hpf	Hours post fertilization
Hpl	Hours post lesion
LSM	Light sheet microscopy
MAG	Myelin-associated glycoprotein
MPSS	Methylprednisolone
NMDA	*N*-Methyl-D-aspartate
OMgp	Oligodendrocyte myelin glycoprotein
ON	Optical nerve
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PNS	Peripheral nervous system
RAG	Regenerative associated genes
RT	Room temperature
SCI	Spinal cord injury

1 INTRODUCTION

1.1 THE SPINAL CORD

The spinal cord resembles a multilane highway with stations of various kinds along the way. The highway carries impulses traveling in both directions: the ascending impulses send sensory information such as temperature, body position, pain, and touch through the spinal cord to the brain, and the descending impulses from the brain project motor commands through the spinal cord to muscles, viscera, and blood vessels.

The spinal cord is a relatively thin structure situated and protected within the vertebral column, stretching from the base of the skull (medulla) to the level of 11–12 thoracic vertebrae. The vertebral column grows faster than the spinal cord after the third fetal month during development, leaving the vertebral column longer than the spinal cord, such that only the cervical segments are approximately at the same level as their corresponding vertebrae (Barson, 1970). The spinal cord consists of 31 segments subdivided into 8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal segment. Each segment has its own pair (right/left) of spinal nerve roots innervating the corresponding myotome and dermatome (Watson, 2009). Cervical segments innervate muscles involved in respiration and head, neck, arm, and finger movements. Thoracic segments mainly provide motor control of the fingers, chest, back, and abdominal muscles, whereas the lumbar and sacral segments are generally associated with the control of muscles involved in locomotion (See Figure 1).

The spinal cord itself can be divided in the transverse plane into gray and white matter: the gray matter is mainly composed of unmyelinated cell bodies and resembles a butterfly, with the central canal as a hole in the middle, whereas the white matter surrounds the gray matter as a columned layer and is mainly composed of myelinated motor/sensory axons and glial cells. The spinal cord is wrapped in three layers of meninges, namely, the pia, arachnoid, and dura mater. In addition to resembling a highway with signals traveling to and from the brain through the spinal cord, there are also myriad spinal networks involved in micturition, bowel control, ejaculation, and locomotion (Callaghan et al., 2018; Fowler et al., 2008; Johnson, 2006; Kiehn, 2011).

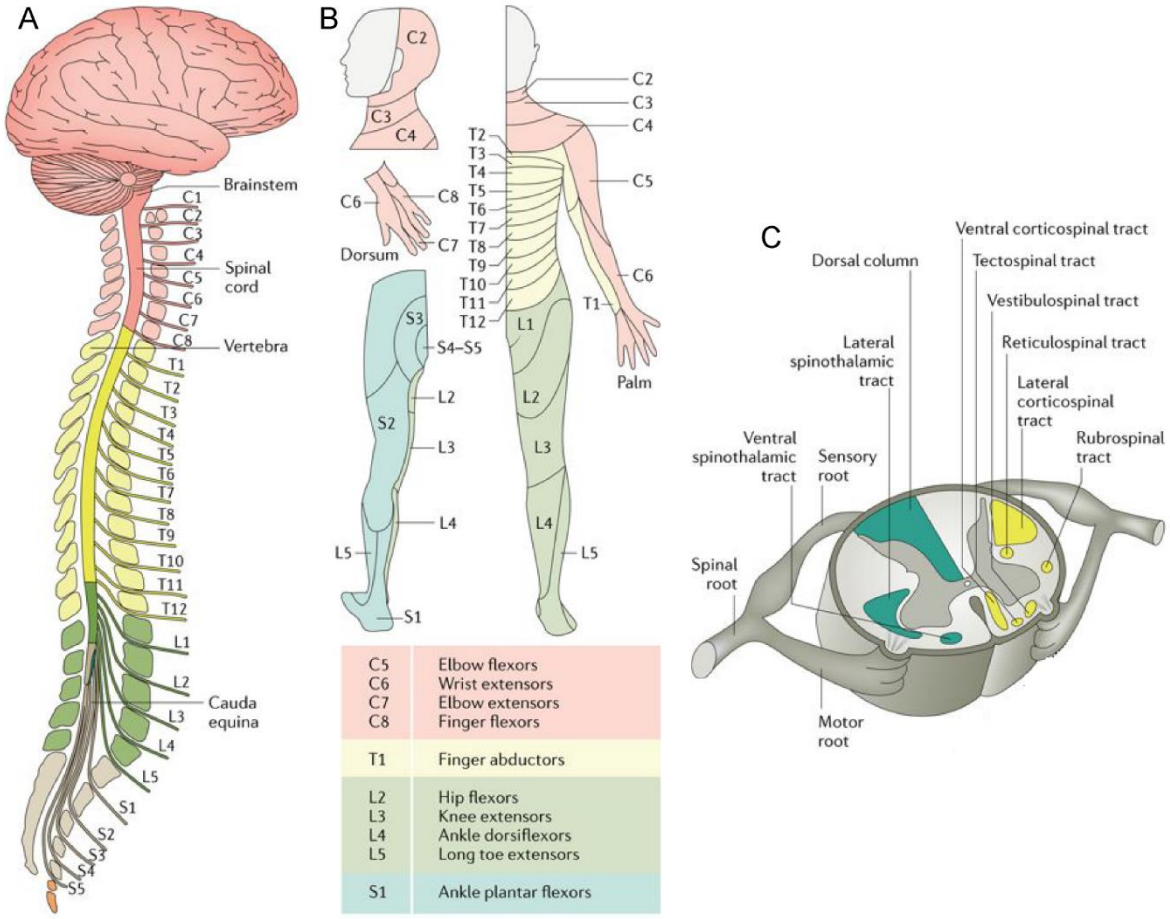

Figure 1. Anatomy of the spinal column. A-B) Each segmental region of the spinal cord innervates a specific region of the skin, muscle or organ group. Damage to the spinal cord can result in partial or complete loss of function below the level of the injury. C) The spinal cord is organized into grey and white matter. The white matter can be further subdivided into several ascending or descending tracts that transmit sensory or motor information. Adapted with permission from: Springer Nature, Nature reviews, Traumatic spinal cord injury, Ahuja CS et al., ©2019.

1.1.1 Locomotion

The ability to move from one location to another by any physiological means is termed locomotion (McNeill Alexander, 2003). One of the simplest motions is propulsion through liquid medium; this simple locomotive ability is crucial at the very start of human life, as sperm are propelled by flagella to the egg (Luconi and Baldi, 2003). However, locomotion evolved much earlier from propulsion into more complex behaviors such as swimming and walking. Swimming can be divided into many classes and characteristics depending on the species and developmental stage. The human fetus exhibits locomotion long before it is born (Prechtl, 1984), as does the zebrafish embryo, in which coiling and the touch response are observed as early as 17 h post fertilization (Kimmel et al., 1995). Zebrafish larvae typically hatch 48–72 h post fertilization. The very first locomotion outside the chorion consists of infrequent bursts; soon thereafter, at the age of 96–120 h post fertilization, a typical beat-and-glide pattern consisting of swimming episodes intermingled with gliding episodes can be observed (Budick and O'Malley, 2000; Saint-Amant and Drapeau, 1998). The exact time point and mechanism behind the development from the beat-and-glide pattern to continuous swimming in the

juvenile/adult zebrafish have not been fully investigated, though unpublished data from Abdel El Manira suggest that the change takes place 4–5 weeks post fertilization.

The first scientific report of studies investigating the neural mechanism underlying locomotion dates back to 1906 (Sherrington, 1906). Efforts have continued since then to identify the parts of the central nervous system (CNS) involved in locomotion. One study argued that the early spontaneous movements are evoked in the spinal cord without input from the brain, but at later stages, the hindbrain is involved in swimming and the touch-evoked response (Saint-Amant and Drapeau, 1998). Later reports suggest that this is incorrect and that the spinal cord in zebrafish can produce a reflex response and rhythmic motor output without the brain, just like that in other vertebrate species (Downes and Granato, 2006). Neuronal networks in the spinal cord can produce rhythmic locomotive outputs without sensory input (Marder and Bucher, 2001), they function as local control and command centers (Grillner, 2006; Kiehn, 2006). Such central pattern generators (CPGs) have been proven to control certain locomotor movements (Grillner, 1975; Grillner et al., 1976).

A locomotor circuit needs two components: a rhythm generator and a pattern generator (Kiehn, 2011). CPGs are composed of different classes of motor neurons and interneurons (Buchanan et al., 1989; Song et al., 2016) and are excellent experimental models for circuit analysis. The ventral neuronal patterning is generated by a ventral-to-dorsal secretion gradient of Sonic hedgehog from the notochord and floor plate and by bone morphogenetic proteins (BMP) activity (Liem et al., 2000). The interneurons located in the ventral spinal cord can be divided in to 4 classes (V0-V3) and further subdivided according to their unique transcription factor expression (Dougherty and Kiehn, 2010). V2a interneurons expressing the Chx 10 transcription factor have been thoroughly investigated and found to play an important function in generating locomotor rhythms (El Manira, 2014). Until recently, motor neurons were thought to be the end station of the common motor pathway; however, a recent investigation revealed that motor neurons in adult zebrafish are also involved in controlling the locomotor circuits via gap junctions and V2a interneurons (Song et al., 2016). Knowledge about the neural synaptic and molecular mechanisms behind neuronal circuits in the spinal cord is valuable information for the regenerative field, as these networks are disturbed and/or destroyed following spinal cord injury (SCI).

1.2 SCI

Etiologically, SCIs can be divided into traumatic and nontraumatic injuries (Noonan, 2013). The main cause of traumatic SCIs in Sweden and the United States alternates between transport-related events and fall accidents (Center, 2019; Joseph et al., 2017). The worldwide incidence estimations vary between 4 and 196 cases per million population (Jazayeri et al., 2015; Singh et al., 2014). In Sweden, the incidence is 20 cases per million (Joseph et al., 2017), and in the United States, 54 cases per million (Center, 2019).

The neurological level of the spinal lesion is defined as the level after which the caudal spinal segment has normal function. If the neurological level of the lesion results in a loss of function and sensation in both arms and legs, it is referred to as tetraplegic, and if the trunk and legs are affected, it is referred to as paraplegic (Maynard et al., 1997). The extent of injury affects the outcome as much as the level of injury and is classified according to the American Spinal Injury Association (ASIA) impairment scale, from A to E: A is complete, where no motor or sensory function is preserved; B is incomplete, where sensory but not motor function is preserved below the neurological level; C is incomplete, where motor function is preserved below the neurological level and more than half of the key muscles below the neurological level have a muscle grade of less than 3; D is incomplete, where motor function is preserved below the neurological level and at least half of the key muscles below the neurological level have a muscle grade of 3 or more; E is normal, where motor and sensory functions are normal (Kirshblum et al., 2011).

1.2.1 Pathophysiology

An injury to the spinal cord can be categorized as a primary injury or a secondary injury. The primary injury results from laceration, acute stretching, shearing, and sudden acceleration-deceleration (Baptiste and Fehlings, 2006). SCI can be further classified into four groups depending on the nature of injury: solid cord injury, contusion injury, laceration, and massive compression (Norenberg et al., 2004). These primary mechanisms rarely fully disrupt or transect the spinal cord completely (Bunge et al., 1993). The nonsevered axons crossing the lesion site are of high therapeutic value, as they represent the neural substrate. Animal studies have shown that as few as 5% of the original number of axons can be sufficient to sustain neurological function (Fehlings and Tator, 1995; Kakulas, 2004). The secondary injury can be subdivided into different phases: an immediate phase (0–2 h), an acute phase (2–48 h), a subacute phase (2–14 days), an intermediate phase (0.5–6 months), and a chronic phase (>6 months).

The immediate phase consists of the instantaneous results from the traumatic SCI itself, such as the severing of axons, cell death of neurons and glia, disruption of the blood-spinal cord barrier, and spinal shock (Ditunno et al., 2004; Norenberg et al., 2004; Whetstone et al., 2003). These events together lead to an instantaneous loss of function below the level of the injury. One of the first signs of pathology is the swelling of the spinal cord, edema, for which the secondary effect, ischemia, leads to necrotic cell death in the central gray matter. Microvascular disruption leads to hemorrhaging in the surrounding white matter, which combined with the swelling and ischemia, can extend several segments, both cranial and caudal, to further exacerbate the injury (Tator and Koyanagi, 1997). The disruption of the blood-spinal cord barrier permits an influx of vasoactive peptides, inflammatory cells, cytokines, and phagocytes (Mautes et al., 2000). Proinflammatory cytokines TNFα and IL-1β are upregulated immediately after injury and released from microglia, macrophages, T cells, and neutrophils, which later leads to intraparenchymal inflammation that can exacerbate the neuropathology (Donnelly and Popovich, 2008; Pineau and Lacroix, 2007). Excitotoxic levels

of glutamate can be attained within minutes after a lesion (Wrathall et al., 1996). These events lead to a sustained secondary injury cascade, which often is in excess of the primary injury.

During the acute phase, secondary injury processes take over and become the dominant force of the pathophysiology. Effects on cord tissue from glutamate-driven excitotoxicity, the production of reactive oxygen species, the loss of electrolyte homeostasis, and immune-mediated toxicity are seen at this stage, contributing to increased cell death and axonal damage (Tator and Fehlings, 1991). Hemorrhaging and vascular disruption lead to ischemia, which in turn results in cytotoxic cell and axonal swelling and an action potential blockade (Kakulas, 2004). The mechanism for prolonged ischemia is not fully understood, but factors such as the disruption of microvasculature, dysfunction of autoregulatory mechanisms, systemic hypotension, and increased interstitial pressure are thought to be involved (LaPlaca et al., 2007; Tator and Koyanagi, 1997). By-products released from necrotic cells, such as ATP, DNA, and K^+, create a cytotoxic postinjury milieu and recruit more phagocytes, which in turn further increase the levels of reactive oxygen species (Forman and Torres, 2002). Activated microglia, macrophages, and astrocytes release signaling molecules that induce the secretion of extracellular matrix proteins such as chondroitin sulfate proteoglycans (CSPG), which inhibit axonal growth, and NG2 proteoglycans, which combine with astrocytes to form the glial scar in the subacute-chronic phase (Ahuja et al., 2016; McKeon et al., 1991). The acute phase is the most amenable for neuroprotective therapies, and thus the phrase "time is spine!"

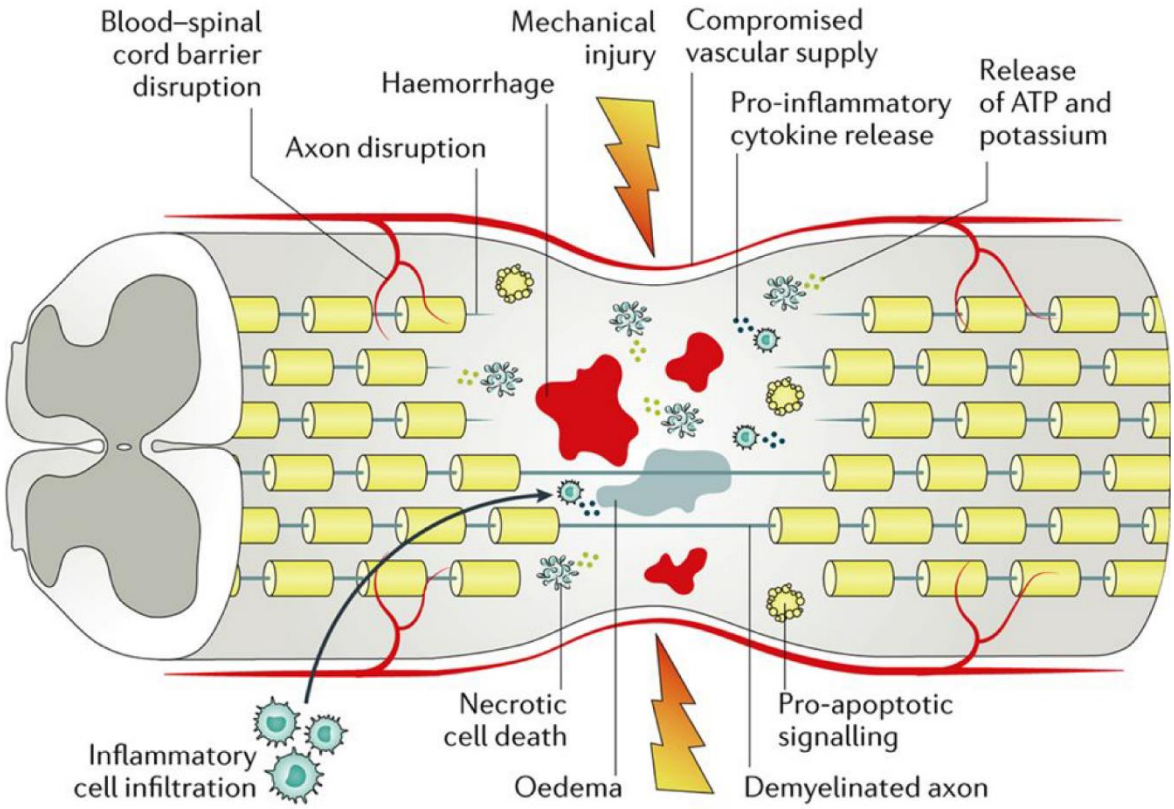

Figure 2. Acute phase (0-48h) pathophysiology of traumatic spinal cord injury. Adapted with permission from: Springer Nature, Nature reviews, Traumatic spinal cord injury, Ahuja CS et al., ©2019.

The subacute phase is characterized by the peak of phagocytic activity. As this is when debris and growth inhibitory components are removed, this phase might enable axonal regrowth (Donnelly and Popovich, 2008). In the periphery of the spinal cord lesion, astrocytes become hypertrophic and proliferate. These reactive astrocytes later generate a gliotic scar by producing large, cytoplasmic interweaving processes. The astrocytic scar tissue is both a physical and a chemical barrier to axonal regrowth. However, the reactive astrocytes restore homeostasis and the blood-spinal cord barrier, which limits the infiltration of immune cells and resolves the edema (Herrmann et al., 2008). At the same time, a further release of glutamate from dying neurons and glial cells increases the excitotoxicity initiated in the acute phase. Glutamate overload leads to over activation of NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole), and kainate receptors (Li et al., 1999;Li and Stys, 2000). This over activation leads to increased intracellular Ca^{2+} concentrations, which leads to neuronal cell death (Choi, 1988; Sattler and Tymianski, 2000; Tymianski and Tator, 1996).

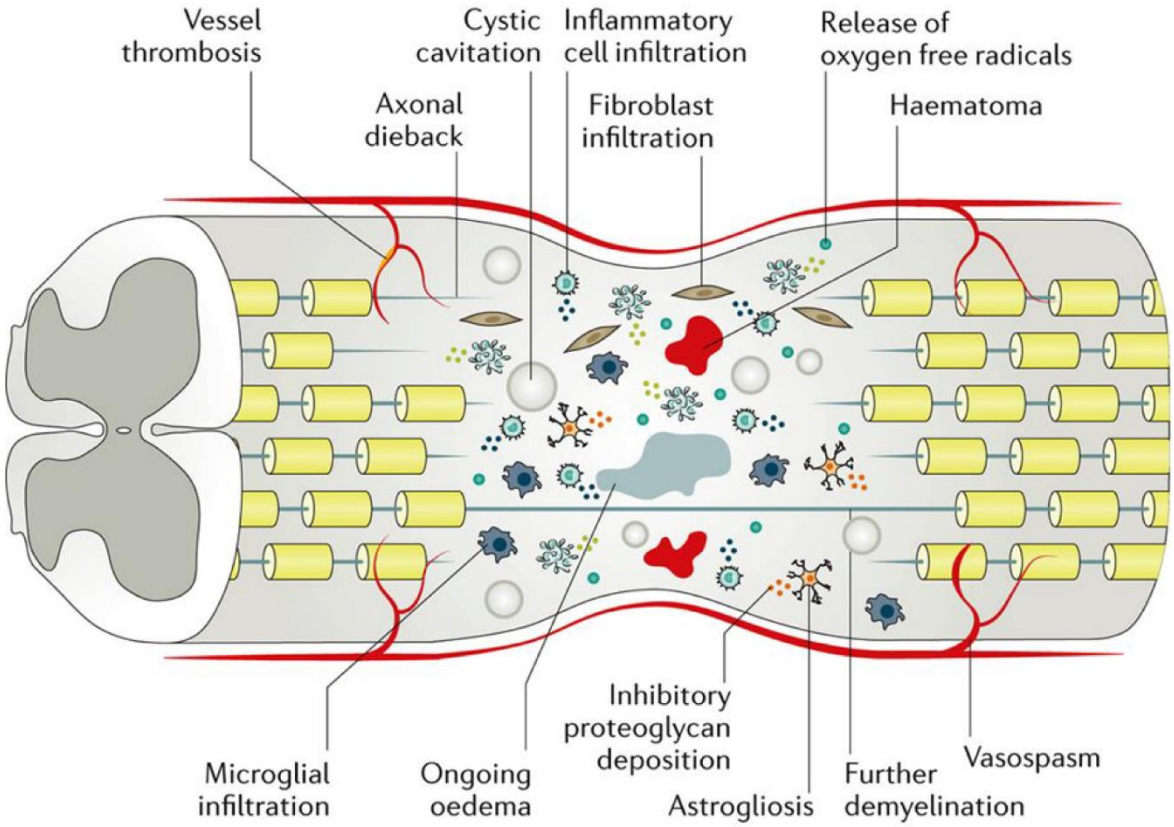

Figure 3. Sub-acute phase (2-14days) pathophysiology of traumatic spinal cord injury. Adapted with permission from: Springer Nature, Nature reviews, Traumatic spinal cord injury, Ahuja CS et al., ©2019.

During the intermediate phase, the astrocytic scar matures. In rat models, regenerative axonal sprouting (Hill et al., 2001) as well as the remodeling of neural circuits and remyelination (Kwon et al., 2004) have been observed during this phase.

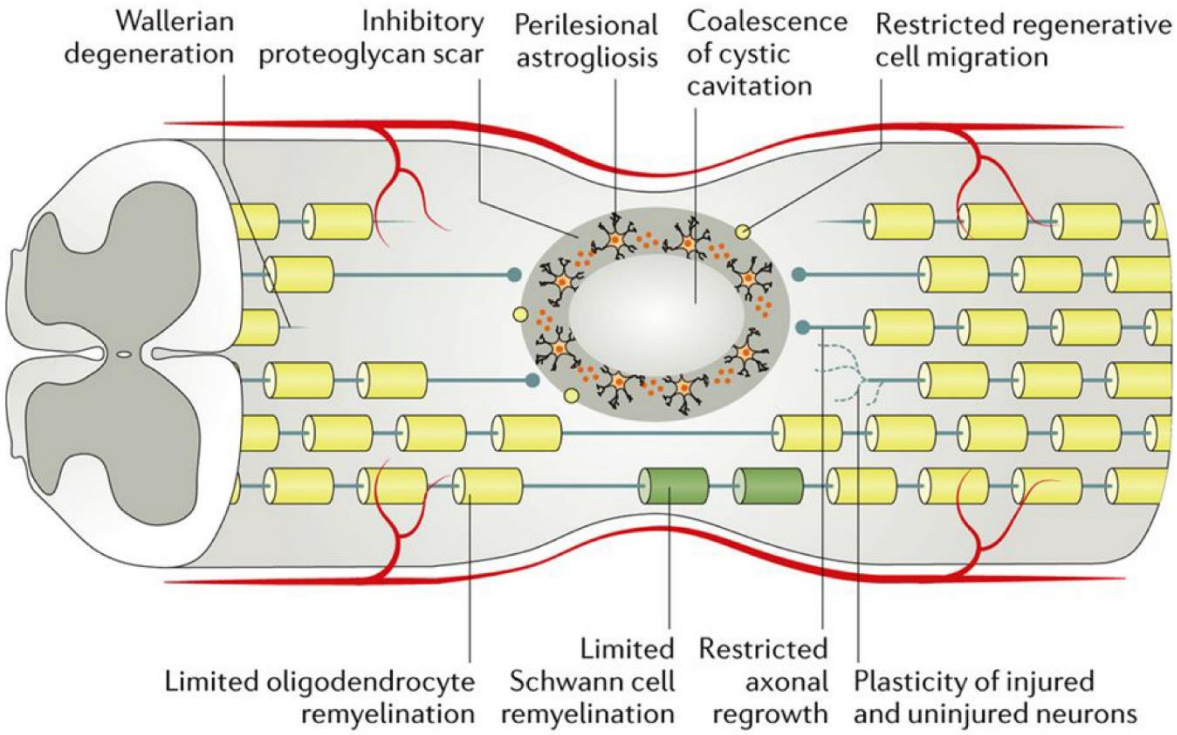

Figure 4. Intermediate – chronic phase (2w-6m->6m) pathophysiology of traumatic spinal cord injury. Adapted with permission from: Springer Nature, Nature reviews, Traumatic spinal cord injury, Ahuja CS et al., ©2019.

In the chronic phase, the reduced parenchymal volume leads to the coalescence of cystic cavities, creating a physical barrier for cell migration (Milhorat et al., 1995). The astrocytic scar continues to mature, thereby stabilizing the lesion and the neurological deficits are quite stable (McDonald et al., 2002). Nevertheless, in up to 30% of SCI patients, syrinxes form, which can result in ascending paralysis and neuropathic pain (Stoodley, 2000).

1.2.2 Treatment

Early surgical decompression. In many cases, SCI results in mechanical compression of the cord, which can cause ischemia due to the impaired blood flow and expand the zones of secondary injury, both cranial and caudal. Acute/early surgery to relieve the compression is performed to improve the vascular supply and thereby limit the extent of the secondary injury. Such treatments have shown positive effects in preclinical investigation (Batchelor et al., 2013).

Blood pressure augmentation. The enhancement of perfusion by blood pressure augmentation is a neuroprotective strategy that has emerged to compensate for ischemia in the injured area. Current American Association of Neurological Surgeons/CNS guidelines recommend maintaining mean arterial pressure at ≥85 to 90 mm Hg for 7 days postinjury, as this has been found to enhance long-term ASIA impairment scale grade outcomes for patients (Wilson et al., 2013).

Glucocorticoids. Methylprednisolone (MPSS) is a highly potent synthetic glucocorticoid that upregulates anti-inflammatory cytokines and reduces reactive oxygen species. A Cochrane review meta-analysis from 2012 found that patients receiving MPSS during the first 8 h postinjury had a 4-point ASIA motor score improvement, which may be of great functional motor significance for these patients (Bracken, 2012). However, MPSS is also associated with an increased risk of severe infections (e.g., sepsis and pneumonia) (Bracken et al., 1990). The usage of MPSS varies, but recommendations from an expert panel put together by AOSpine suggest that the beneficial effects outweigh the side effects if 24 h MPSS intravenous treatment is initiated within 8 h postinjury.

Pharmacological neuroprotective interventions

Riluzole is a benzothiazole sodium channel blocker currently approved by the European Medicines Agency for the treatment of amyotrophic lateral sclerosis (Bensimon et al., 2002; Bhatt and Gordon, 2007). The blockade of sodium influx to injured neurons and subsequent inhibition of presynaptic glutamate release are protective (Azbill et al., 2000). Animal studies have demonstrated that this results in reduced neuronal loss and cavity size together with improved sensory and motor electrophysiological outcomes (Nogradi et al., 2007; Simard et al., 2012).

Magnesium can be utilized for its antagonistic effect on the NMDA receptor to decrease excitotoxicity together with its anti-inflammatory properties. Stable magnesium levels in cerebrospinal fluid can be attained via delivery with an excipient such as polyethylene glycol (Kwon et al., 2009; Luo et al., 2002). In animal models, the magnesium-polyethylene glycol combination has been shown to enhance tissue sparing and lead to behavioral recovery (Kaptanoglu et al., 2003a; Kaptanoglu et al., 2003b).

Minocycline is a second-generation bacteriostatic tetracycline antibiotic that in animal studies of CNS disorders, such as multiples sclerosis and Huntington's disease, has demonstrated neuroprotective properties (Chen et al., 2000; Giuliani et al., 2005). The benefits are linked to the anti-inflammatory effect of minocycline, mediated by the suppression of microglial activation, IL-1β, TNFα, cyclooxygenase-2, and matrix metalloproteinases (Seabrook et al., 2006). One animal study of SCI has shown that minocycline treatment reduces lesion size and promotes tissue sparing (Wells et al., 2003).

Pharmacological neuroregenerative therapies

Rho-Rock inhibitor. SCI upregulates signaling through the Rho pathway, which leads to growth cone collapse that prevents axonal regeneration. Rho and Rock are further thought to be involved in the inhibitory effects of CSPGs on neuronal growth. Published preclinical studies suggest that inhibition of this pathway may boost neuroprotection and axonal regeneration after SCI (Forgione and Fehlings, 2014).

Anti-NOGO antibody. Anti-NOGO is a monoclonal antibody against NOGO-A, a major inhibitory component of adult CNS myelin. Anti-NOGO treatment delivered by intrathecal

injection has been shown to promote axonal sprouting and functional recovery in animal models by clearing this inhibitory signal (Bregman et al., 1995).

Nonpharmacological neuroprotective therapies

Therapeutic hypothermia (32–34°C) significantly reduces the basal metabolic rate of the CNS and decreases inflammatory cell activation (Kwon et al., 2008). It has been successfully applied in neonatal hypoxic-ischemic encephalopathy and after in-hospital cardiac arrest (2002; Dehaes et al., 2014). In preclinical SCI models, it has been shown to enhance tissue sparing and promote behavioral recovery (Lo et al., 2009).

1.3 FUNCTIONAL RECOVERY OF THE HUMAN SPINAL CORD

Significant functional recovery following trauma to the spinal cord has been reported in several patient cases; however, the SCI was incomplete in all these cases, with at least some spared axons in the white matter (Burns et al., 1997; Waters et al., 1995). These spared axons can lead to functional improvements in the later stages of SCI, which should not be confused with regeneration. In cases where SCI is complete, the spinal cord is completely destroyed with no intact axons at the level of the injury; there are no known cases of a significant regain of function below the injury with complete SCI. Nonetheless, there are reports indicating minimal functional recovery (Lee et al., 2016).

1.4 REGENERATION

The definition of regeneration in regenerative medicine is long and complex but can be briefly defined as the replacement of damaged or lost tissue with new healthy functional tissue. There are stories throughout history of creatures' abilities to regenerate. The northern Vikings had stories about Särimner, a boar that was eaten every night only to be found regenerated the following evening and then eaten again and again and again. Regeneration as a scientific subject was recognized for crustaceans by Aristotle. The first scientific paper in the field was published in 1712 by René-Antoine Ferchault de Réaumur, who reported the regenerative properties of crayfish limbs. Human liver, skin, and endometrium have the ability to regenerate. Understanding why and how regeneration occurs opens up an almost unlimited field of applications. To pinpoint the machinery, we need robust, adjustable, accessible, and reproducible model systems.

1.5 ZEBRAFISH

The zebrafish (*Danio rerio*) is a freshwater fish belonging to the minnow family (Cyprinidae) native to river basins in India and Pakistan. There are many reasons that zebrafish are used in science: they are small and robust, relatively inexpensive compared with mice, and exhibit

effortless mating triggering, and their genome has been sequenced and found to share roughly 70 percent of its genes with that of humans. Furthermore, thousands of transgenic lines already exist, and more are in the pipeline. The zebrafish can be chemically kept transparent to the age of 4 weeks (Whittaker, 1966), with available albino and mutated lines lacking pigmentation (Lister et al., 1999), making the zebrafish a top candidate for imaging investigations. Adult, juvenile, and larval zebrafish have well-defined neuronal circuits and a stereotypic behavioral repertoire that can be used to assess basic behaviors, such as spontaneous locomotion and escape reflexes (Blaser and Vira, 2014; Kalueff et al., 2013). Zebrafish can take up compounds through their gills into the bloodstream, which makes them an ideal vertebrate model for drug screening assays (Rombough, 2002).

Furthermore zebrafish are an astonishing creature that, unlike mammals, can regenerate their hearts, fins, and CNS; adult zebrafish are able to spontaneously regenerate axonal tracts after spinal cord lesioning (Becker and Becker, 2014; Becker et al., 1997). Following spinal cord transection, brain nuclei regrow their neuronal projections to the spinal cord, restoring the surgically induced loss of swimming ability (Bhatt et al., 2004). By contrast, mammals lose the ability to regenerate and regain function in the spinal cord after early development (Ferretti et al., 2003). Further knowledge is needed about why this potential for regeneration was lost during evolution and development and why it became so limited in higher vertebrates. Studies of the regenerative properties of the zebrafish might provide valuable insights into previously unknown ways to activate regenerative processes and deactivate inhibitory cues in the spinal cords of mammals.

1.5.1 Functional Recovery of the Zebrafish Spinal Cord

The spinal cord of a zebrafish is as fragile as ours, yet the outcomes after SCI completely diverge. Many of the molecular extracellular and intracellular pathways identified as playing a role in regeneration in zebrafish are evolutionarily conserved. There is no absolute answer as to why zebrafish regain function in the spinal cord after injury and mammals do not. The difference may lie in the neurons themselves and their intrinsic capacity for regeneration, the milieu in the spinal cord of a zebrafish that makes regeneration possible, neurogenesis, or perhaps a combination of these. Investigations have looked at axon growth *in vitro*, where mammalian and fish neurons were cultured with mammalian oligodendrocytes and myelin. The growth of both mammalian and zebrafish neurites was repelled by mammalian oligodendrocytes and myelin (Bandtlow et al., 1990; Bastmeyer et al., 1991; Fawcett et al., 1989). Interestingly, both mammalian and fish axons grew in fish-conditioned medium or in the presence of oligodendrocytes isolated from fish (Bastmeyer et al., 1993; Schwalb et al., 1995; Wanner et al., 1995). Thus, the milieu is of upmost importance for axonal growth, but what are the differences between these in mammals and fish?

Again, there are several proposed explanations for why axonal growth and regeneration occur in zebrafish but not in mammals:

1. Absence of inhibitory factors.
2. Presence of factors that block the inhibitory factors.
3. Absence of progrowth factors.

In the mammalian CNS, axon growth-inhibiting factors exist and have been identified as myelin proteins (Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein), extracellular matrix proteins (CSPG and tenascin), and chemorepulsive cues (semaphorins, ephrins, and netrins) (Giger et al., 2010). Nogo-A, MAG, and oligodendrocyte myelin glycoprotein have all been reported to have homologs in zebrafish (Lehmann et al., 2004; Shypitsyna et al., 2011). Mammalian Nogo and MAG inhibit axonal growth from zebrafish neurons; interestingly, purified Nogo and MAG from zebrafish do not inhibit axonal growth of mammalian or zebrafish axons (Abdesselem et al., 2009; Chen et al., 2013). A possible reason for this may be the evolutionarily diverged functions of these proteins.

Zebrafish do not form the characteristic glial scar produced by activated astrocytes following SCI in humans (Cregg et al., 2014). The main reason for this is suggested to be the lack of stellate astrocytes in zebrafish according to a review by Lyons and Talbot (Lyons and Talbot, 2014). In zebrafish, radial glial cells react to SCI by producing astroglia-like processes that extend over the lesion site and form a glial bridge for axons to attach to when transcending the lesion site (Goldshmit et al., 2012). By contrast, another investigation revealed that axons cross the lesion site independently of these astroglia-like processes (Wehner et al., 2017). The presence or absence of astrocytes and their involvement or noninvolvement in spinal cord regeneration in zebrafish have yet to be determined.

There is evidence of inhibitors of axonal regeneration in zebrafish. For example, tenascin-R has been reported to act as an inhibiting guidance molecule during optic nerve development (Becker et al., 2003). The inhibition of tenascin-C following SCI in adult zebrafish leads to improved functional recovery and axon regeneration (Yu et al., 2011).

The inhibition of factors that impede regeneration in the spinal cord represents another mechanism contributing to successful regeneration in zebrafish. The exposure of mammalian axons to conditioned medium from regenerating goldfish nerves promotes axonal growth; interestingly, the exposure to conditioned medium from nonregenerating goldfish nerves has no effect on axonal growth (Schwartz et al., 1985). Another example of a regenerative inhibitor is an IL-2-like molecule that is proposed to exert a cytotoxic effect on oligodendrocytes, thereby avoiding oligodendrocyte-derived inhibitory factors (Eitan and Schwartz, 1993; Eitan et al., 1992).

A third mechanism for regeneration in zebrafish may involve the secretion of proregenerative molecules from cells involved in SCI. The secretion of axogenesis factor 1

(mannose) and factor 2 promotes axon growth and the expression of growth-associated proteins (Petrausch et al., 2000; Schwalb et al., 1995). In addition, injury to the optic nerves results in an upregulation of purpurin, and the treatment of uninjured optic nerves with purpurin induces axonal growth (Matsukawa et al., 2004; Tanaka et al., 2007).

Interestingly, the intrinsic growth pathways identified in regeneration are relatively evolutionarily conserved, which leads to the idea that regeneration may be a quantitative rather than a qualitative matter. Axonal regeneration in central nervous neurons has been reported to occur after preconditioning, in which the injury to peripheral nerves triggers growth-associated proteins that promote regeneration; these findings add to the idea of quantity as a determining factor (Gutmann, 1942; Neumann and Woolf, 1999).

To regain full functional recovery after SCI, synaptic connections must be rewired and/or restored. Axonal regeneration is complex and far from fully understood. Not all neurons regenerate their axons following SCI in zebrafish; in fact, <50% of all dopaminergic and serotonergic synapses regenerate, also not all types of neurons regenerate their axons (Kuscha et al., 2012). The distance a regenerating axon travels appears to be limited to a fraction of its original length (Becker and Becker, 2001; Becker et al., 1997).

Nevertheless functional recovery is restored, this suggests that compensatory circuits contributes or that only a small fraction of the original synapses are sufficient to restore locomotion.

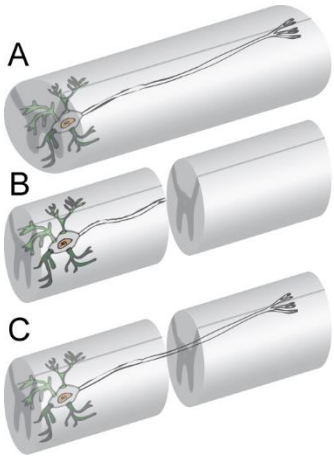

Figure 5. Illustration of axonal regeneration. A) Spinal cord with a neuron spanning its axon from gray to white matter. B) Lesioned axon. C) Regenerated axon.

Neurogenesis at the lesion site in adult zebrafish is reportedly mediated by fibroblast growth factor signaling (Goldshmit et al., 2018). Furthermore, several studies have found that radial glia persist as quiescent neural progenitors throughout the CNS in zebrafish and that these cells are involved in CNS regeneration (Briona and Dorsky, 2014; Kroehne et al., 2011; Wan et al., 2012). One study reports that radial glial neurogenesis following SCI requires Wnt/β-catenin signaling (Briona et al., 2015).

1.6 IN VIVO IMAGING

The word microscope is a combination of two greek words, mikrós translates to “small” and skopeîn translates into “to look” according to Wikipedia. The first compound microscope, that is a microscope with a combination of an objective lens near the specimen and an eyepiece that visualizes the same specimen is dated back to 1620 in Europe (Murphy, 2011). 400 years later the main function of a microscope is still the same, i.e to produce a magnified image, increase the resolution and present a visible image to the human eye. Nevertheless todays microscopes are quite more sophisticated than that.

There is a wide array of different microscopy techniques the choice depends on the question and specimen. In this thesis three projects, confocal microscopy, two photon laser scanning microscopy, light sheet microscopy and an array of conventional microscopes were used. Confocal microscopy is suitable for 3D-imaging of fixed fluorescently labeled specimens our superficial live 3-4D imaging. 1955 Marvin Minsky built the first confocal microscope although he did not called it confocal at that time being and he did not publish his creation. By definition all modern confocal microscopes are based on his principles, that can be read in his memoires (Minsky, 1987) and patent (M, 1957).
Cutting it short one major difference between conventional epifluorescence light microscopy and confocal microscopy is that in the first the entire field of view of the specimen is simultaneously bathed in the fluorescent light compare to the later were illumination of the specimen is achieved by scanning focused laser beams across the specimen point by point which renders so called optical sections (Conchello and Lichtman, 2005). Furthermore both techniques causes the entire specimen thickness to fluoresce this leads to out of focus fluorescence affecting the signal to noise ratio in a negative manner. In confocal microscopy this issue is solved by placing a pinhole aperture in front of the detector at a distance that conjugates to the in focus plane, by doing so only in-focus-light will reach the detector. Nonetheless, there are two major setbacks with confocal microscopy: i) scanning point by point is a relatively time consuming processes and ii) due to the scattering of light in tissue, which will have a negative effect on the signal to noise ratio, the penetration depth is limited to thin specimens (~100 µm) (Helmchen and Denk, 2005; Smithpeter et al., 1998).

With 2PLSM (Denk et al., 1990) thicker specimen can be imaged, the literature reports depth up to 1000µm (Theer et al., 2003). 2PLSM is a nonlinear process were two photons compared to one photon in confocal microscopy are needed to excite a fluorophore. The wavelength of these two photons are in the near infra-red spectrum (700-1100nm). Near-infrared photons scatter less in tissue compared to the visible spectrum used in one photon excitation, a simple way to visualize this is to shine a strong flashlight through the palm and look at the color of light that passes through (red). For the quantum event of excitation to happen two photons need to arrive simultaneously (within ~0,5fs), this requires a high flux of photons, therefor usually femtosecond lasers are used, these are powerful laser that can yield extremely high peak powers. Another benefit with working in the near infrared is the smaller phototoxic effect compared to visible light(Denk and Svoboda, 1997). 2PLSM don’t need a pinhole since no

background fluorescent signal is generated since the quantum event only occurs in the focal point were the flux is highest (Denk, 1995).

Light sheet microscopy (Huisken et al., 2004) overcomes the relative slow scan speed of laser scanning confocal microscopy and 2PLSM by a very elegant and relative simple manner. The specimen is illuminated from the perpendicular side of observation with a "sheet of light" that has a thickness of a few micrometers the entire section is illuminated at the same time and by so the acquisition speed is increased by 2-3 orders of magnitude (Huisken and Stainier, 2009).

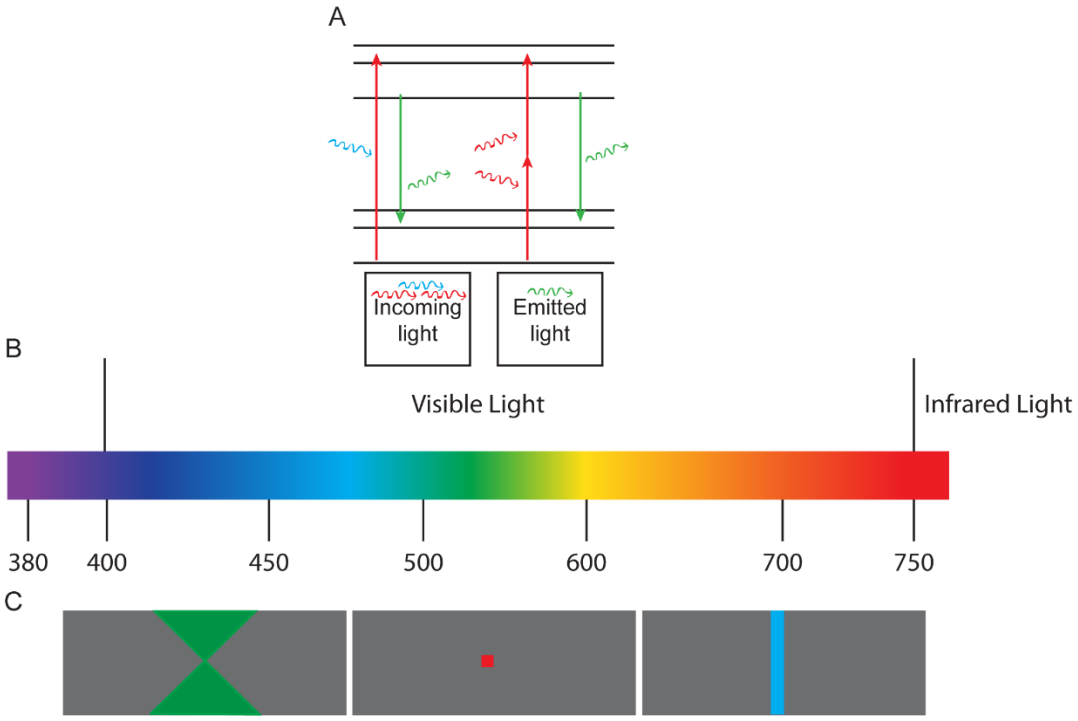

Figure 6. Jablonski diagram, visible – infrared light, illumination profiles in different modes of optical sectioning microscopy. A) Jablonski diagram of one respectively two photon excitation. B) Visible – infrared light spectrum. C) Illumination profile of laser scanning confocal microscopy (green), two photon laser scanning microscopy (red) and light sheet microscopy (turquois).

Calcium ($Ca^{2+)}$ is by far the most versatile ion in biological functions. Ca^{2+} is involved in nearly all aspects of neuronal development, neuronal induction, proliferation, migration and differentiation (Moreau and Leclerc, 2004; Rosenberg and Spitzer, 2011; Somasundaram et al., 2014; Spitzer, 2006; Zheng and Poo, 2007) furthermore Ca^{2+} is involved in neurogenesis (Toth et al., 2016). Ca^{2+} homeostasis is disturbed in the acute SCI phase and is exaggerated in the subacute phase; the main cause of this is NMDA, AMPA, and kainate receptor overactivation via glutamate overload (Ahuja et al., 2017a; Ahuja et al., 2017b). Additionally, evidence are emerging that the amplitude and frequency of Ca^{2+} oscillations code for a variety of downstream cytotoxic processes (Arundine and Tymianski, 2003). Thus, Ca^{2+} signaling can be used as a proxy to study the acute and subacute effects on neuronal circuitry as well as neuronal degeneration and regeneration following SCI.

In this thesis we combined the penetrable and powerful features of 2PLSM with high speed imaging feature of light sheet microscopy and functional whole volume 4D Ca^{2+} imaging to

induce SCI, and image the neuronal activity in the spinal cord of zebrafish larvae with single cell resolution.

2 AIMS

The overall aim of this thesis was to develop a robust and highly reproducible SCI method and to investigate spinal circuits involved in locomotion, and regenerative properties of the zebrafish spinal cord.

Paper I The main purpose of the project for paper I was to investigate if induced cell death in V2a interneurons affects the excitatory drive required for normal swimming activity in zebrafish larvae.

Paper II The studies in paper II were partly based on the method developed for paper I. In this project, we developed a robust and reproducible method to induce precise spatial and temporal SCI in zebrafish larvae.

Paper III The studies in paper III investigate and assess the regenerative capacity of the spinal cord in zebrafish larvae to identify clues about key processes and limiting factors for regeneration.

3 RESULTS AND DISCUSSION

3.1 PROJECT I: ORIGIN OF EXCITATION UNDERLYING LOCOMOTION IN THE SPINAL CIRCUIT OF ZEBRAFISH

Moving from one place to another by jumping from one cliff to another like the mounting goat, sprinting up the stairs as the PhD student or swimming through the water as a zebrafish larvae are all examples of locomotion controlled by an ensemble of neurons so called CPGs (Grillner, 2003). In vertebrates these CPGs are located in the spinal cord (Grillner, 2006). The complexity of these circuits are of varying degree and simplified one could argue that of these given examples the aquatic locomotion of the tiny zebrafish larvae is more simple (i.e. smallest amount of information exchange) then the other two. The components involved in the motor pattern for moving from point A to B can be divided in modules, one that is involved in the alternation of left and right body movement, and another that provides the excitatory drive and finally one that constitutes the output to the muscles from CNS. The left-right alternation module is composed of inhibitory commissural interneurons that projects to the contralateral side of the spinal cord (Buchanan, 2001; Cohen and Harris-Warrick, 1984).These connections ensures that axial muscles on one side of the body contracts when the other side relax. The muscle commands are committed via motor neurons projecting directly to the innervated muscle (Bagnall and McLean, 2014). The drive to both the inhibitory commissural interneurons and the motor neurons originate from ipsilateral monosynaptic projecting excitatory interneurons (Buchanan and Grillner, 1987). The literature suggest that V2a interneurons that are molecularly characterized by Chx10 expression, project ipsilateral and are glutamatergic (Kimura et al., 2006), this marks them as a good candidate for the underling excitable drive.

We tested the hypothesis that V2a interneurons are the origin of excitatory drive underlying locomotion in the spinal circuit of zebrafish. We started with selectively inducing cell death in 30% (15 out of 50) of the Chx10:GFP positive V2a interneuron population in 10 segments with 2PLSM. The 10 segments were located in the midbody region and the zebrafish larvae were 4-5 dpf. Thereafter we compared swimming activity in controls and treated zebrafish larvae. Interestingly the current needed to induce swimming via a glass stimulating electrode placed at the level of the otic vesicle had to be increased six fold higher in treated animals compared to controls. Furthermore the swimming bouts were significantly shorter, containing fewer burst and lower frequencies in treated animals compared to controls. We could not detect any difference in between the groups in left-right body movement alternation.
To rule out that the V2a interneurons are the excitatory drive and not a relay for descending input we prohibited descending input by spinalizing treated and control animals and induced swimming pharmacologically by NMDA application. In treated animals higher concentrations of NMDA were needed to induce swimming and rhythmic activity could only be detected in segments caudal of treated segments.

Next we assessed if the results were due to an unselective cytotoxic effect caused by the exposure to laser light. We targeted GFP expressing glycinergic interneurons with the same settings previously used. We could not detect any significant difference in excitability in treated and control animals. Furthermore we could not see any change in burst frequency nor duration of the bouts. These results indicates that the change of swimming pattern is not due to unspecific cytotoxic effects.
Finally we investigated if the intersegmental coordination was altered after elimination of V2a interneurons. The rostro-caudal phase lag was nearly increase two fold in treated animals compared to controls.

The literature suggests that genetic ablation of V2a interneurons in mammals affects left-right body movement alternation and that V2a elimination have very little effect on rhythm generation induced pharmacological (Crone et al., 2008; Crone et al., 2009). Our results suggest the excitatory V2a interneurons represents a source of excitation to basic swimming patterns in zebrafish larvae. The divergence in findings could be to the fact that in the mouse model nearly all V2a interneurons were genetically ablated by diphtheria toxin A without affecting V0, V1, V2b, V3 and motor neurons (Crone et al., 2008) and that in our model we ablated only ~30% of the V2a population in 10 midbody segments, it could be that the spared V2a interneurons were enough to maintain the left-right alternation of body movement. The spared V2a interneurons don't explain the divergence in effect on rhythm generation, it could be that the V2a interneurons role in rhythm generation is not evolutionary conserved and/or that a different subset of neuronal ensembles are used in over ground locomotion. Furthermore additional classes of interneurons could be involved in mammalian rhythm generation that compensates for the loss of V2a interneuron ablation. (Brownstone and Bui, 2010; Grillner and Jessell, 2009; Kiehn, 2006). We ablated ~30% of the V2a interneurons as a tradeoff in animal survival and observable results, it would be very interesting to ablate 100% of theV2a population with a more refined 2PLSM technique.

3.2 PROJECT II: SPINAL CORD INJURY IN ZEBRAFISH INDUCED BY NEAR-INFRARED FEMTOSECOND LASER PULSES

A spinal cord injury evokes a cascade of events, involving a myriad of cells, cell types and processes (Ahuja et al., 2017a). Functional recovery in mammals following SCI is very limited (Lee et al., 2016) in contrast to the zebrafish which regenerates its spinal cord after injury (Becker and Becker, 2014). The machinery behind neurogenesis and axonal regeneration in zebrafish have been investigated in many studies and both the milieu, extrinsic and intrinsic factors have been identified to play a crucial role (Bastmeyer et al., 1993; Becker et al., 2003; Cregg et al., 2014; Giger et al., 2010).

To investigate functional recovery following SCI and/or neuronal circuitry methods to inflict injury and cell death are needed, the more robust and reproducible a method is the more reliable the data will be. Various methods has been used to induce cellular and tissue injury such as glass capillary (Bhatt et al., 2004), metal needles (Wehner et al., 2017) and lasers (McLean et al., 2007; Muto and Kawakami, 2018; Sahu et al., 2018) in elegant studies. We strived to avoid unspecific cell death by identifying the optimal energy exposure settings. Our results suggest that an optimal power output shall be in the range of 310-470 mW and the exposure time per cell 2,6ms when using a wavelength of 800 nm. The energy levels delivered with our settings can be obtained by varying any of the parameters, for example extending the time and lowering the power output, this should be done carefully as resilience of tissue varies greatly depending on the nature of energy, the desired outcome may be absent if for example the exposure time is increased and output power lowered too much. An extreme metaphor could be that equal energy levels can be obtained by throwing tens of thousands ping pong balls against a brick wall or by throwing one gigantic kettlebell against a similar wall. The result will diverge from not a scratch to total destruction. To obtain and concrete these settings we argued to minimize the exposure time to avoid any movement of the specimen and to not use maximum output power of the laser since that would have reduced the number of applicable near infrared pump lasers.

We could not detect any difference in functional recovery in animals with a laser induced SCI compared to a metal needle induced SCI, this suggests that the laser can be used to investigate functional recovery in zebrafish larvae following a SCI and vice versa when the high specificity is not demanded. Furthermore with regards to the specificity of the presented method SCI can be carried out in one hemi section of the spinal cord sparing the other as an internal control.

3.3 PROJECT III: IMPACT OF LESION SIZE, HYPOTHERMIA, AND ANALGESICS ON LOCOMOTOR RECOVERY IN SPINAL CORD INJURED ZEBRAFISH

Zebrafish are an astonishing creature, after a complete lesion to the spinal cord most individuals almost fully recover their functional locomotor function in regards of swimming speed and distance compared to controls (Dehnisch Ellstrom et al., 2019). Hypothermia is reported to have cerebral sparing effects following a trauma (Berntman et al., 1981) furthermore there are reports of neuronal protective effects from hypothermia (Zhang et al., 2019) (Gao et al., 2019). A clinical study suggest that mild hypothermia improves the neurological outcome following acute SCI (Dididze et al., 2013). Our results didn't imply any detectable difference in functional improvement in zebrafish treated with hypothermia in the acute phase following a SCI compared to control animals. The reason could be due to the fact that zebrafish are a cold-blooded animal and the metabolic response to hypothermia might be different. Our results suggest that the regenerative machinery in zebrafish is not affected by modest hypothermia. Furthermore or experimental setup could be further optimized in regards of exposure time to treatment and temperature. We observed a 70% mortality rate in a group of animals exposed to 4˚C, this might have been avoided if gradually lowering the temperature, we moved SCI and sham animals directly from room temperature into the cooling chamber, this could have chocked the animals and led to death in the groups exposed to cooler settings.

The outcome of a SCI depends on several factors in which the severity of the injury is a contributing factor (Coleman and Geisler, 2004). We investigated if inducing a SCI over 3 segments in zebrafish larvae delayed the regeneration compared to animals with a SCI in 1 segment. Interestingly we didn't see a significant difference in functional recovery between the groups but merely a trend of delayed recovery. Our results suggests that the distance regenerating axons needs to re-extend plays a minor difference in the timing of functional recovery. To draw any statistical conclusions more experiments needs to be performed.

A traumatic SCI almost always entails severe pain to the patient. Pain is treated in the clinic by various medical drugs including morphine and non-steroid-anti-inflammatory-drugs (NSAIDs). Rodent experiments imply that opiate treatment following a SCI can adversely affect the functional recovery (Hook et al., 2007). We investigated the impact of morphine, diclofenac, naproxen and paracetamol on regained locomotor function after SCI in zebrafish larvae. Interestingly we noted reduced swimming distance in the SCI group treated with morphine and increased swimming pattern in the sham animal group treated with morphine. Morphine is reported to induce hyperactivity in fish (Chatigny et al., 2018), by so our administration of morphine were successful. The number of animals used in the study are too few to draw any conclusions. Moreover we saw a significant impairment in functional recovery in animals treated with diclofenac, interestingly in both the sham and SCI group suggesting that the diclofenac was toxic. Naproxen and paracetamol gave no notable effect on functional recovery, one possible explanation could be that the dosage was not therapeutic or that these medical drugs don't have any significant effect on regeneration. There are very few studies

available including fish and analgesic compounds, therefor the therapeutic window should be further optimized. More experiments are needed to draw any conclusions.

We developed a method for whole spinal cord volume calcium imaging. A clear abolishing of the calcium signaling and active cells was seen in both the rostral and caudal segment to the lesion site 4 h post lesion. Interestingly the activity and number of active cells returned to a certain degree already after 24h post lesion and fully 48 h post lesion. We can't make any statistical conclusions due to the low amount of experiments but our results implies that calcium signaling can be used to further investigate the neuronal recovery following a SCI in zebrafish larvae.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

The last decade several milestones have been achieved in both the biological and technical field. We now have an arsenal of tools that can be used to identify, manipulate and investigate specific genes, molecules and mechanisms involved in physiological and pathophysiological events, together with advanced live imaging technics.
In project I we conclude that V2a interneurons are a part of the excitatory drive needed to generate normal locomotive swimming behavior in zebrafish larvae. This project gave us new insight in the neuronal ensemble engaged in locomotion and that V2a interneurons role diverge in between zebrafish and mice. Knowledge as such is of importance to understand the components of the neuronal circuits.
In project II we scaled up the use of 2PLSM from single cell ablation and axotomy to be used as a highly spatial and temporal precise tool to induce reproducible SCI with minimized collateral damage. With a method as such we can now investigate specific cells involvement in various SCI settings. For example the microglial response following a SCI could locally and selectively be investigated by inducing cell death in activated microglia at site of lesion.
In project III we present a glimpse of how advanced imaging setups in combination with accessible and modifiable animal models can be used to identify various external factors involved in functional locomotor recovery. We conclude that zebrafish as a model system together with advanced imaging techniques can be used to address external factors involved in functional recovery after SCI. Furthermore 4D calcium imaging can be used as a proxy for identifying turning points in the degenerative-regenerative cascade.

Robust, temporal, spatial and reproducible methods are needed to dissect the myriad of processes involved in locomotion and regeneration. Identifying the components in neuronal circuits and the essence of locomotion is mandatory to come closer to understanding how to therapeutically restore functional recovery after a spinal cord injury. Revealing the machinery behind regeneration will revolutionize modern medicine, not only for spinal cord injured patients but for patients in all fields. Imagine if there is no longer a need for donors of organs and imagine how regenerative therapies can restore function to the blind, deaf, unwell, sick and injured. The zebrafish are a remarkable animal and indeed the benefits are many of using it as a laboratory animal. The path for the translation of the findings from these studies and others to the clinic will of course require several detours and involve thousands of small steps along the way. One possible breakthrough would be to identify the molecular components involved in degeneration and regeneration of zebrafish spinal cord with single cell sequencing combined with tissue clearing and expansion microscopy.
In the lifetime of mankind more questions have been raised then answered, development of tomorrows techniques can be used to answer yesterday's questions.

When I think of what the multidisciplinary field have managed to come up with the last ten years I get thrilled to see what the coming decade will reveal. Those who retain their health and lives shall see.

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