From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

Organization of the spinal locomotor network in zebrafish

Pattern of recruitment and origin of excitatory drive

Emma Eklöf Ljunggren

Karolinska Institutet

Stockholm 2012

Published by Karolinska Institutet
Printed by Larserics Digital Print AB

ISBN 978-91-7457-988-8

To my children Benjamin and Alma

ABSTRACT

Locomotion is of vital importance for animals to survive in a complex environment. Locomotor movements are generated by neuronal networks that reside in the spinal cord and are composed of motoneurons and interneurons. The aim of this thesis was to investigate the organization of the spinal circuitry with emphasis on the recruitment and origin of excitation therein.

We used an *in vitro* adult zebrafish preparation to investigate the recruitment pattern of the motoneurons and the underlying mechanisms. We found that the motoneurons in the adult zebrafish are organized into four pools with a specific topographic location in the spinal motor column. These motoneuron pools are recruited from ventral to dorsal with increasing swimming frequencies. In contrast to the larval zebrafish, the recruitment order of motoneurons in adult zebrafish is not set by their input resistance, but instead by a combination of their biophysical properties and the strength of the synaptic drive to motoneurons.

V2a interneurons constitute an important class of excitatory interneurons that make monosynaptic connections with motoneurons. To test if these interneurons provide the excitatory drive underlying locomotion, we used larval zebrafish. Photoablation of a part of the V2a interneuron population decreased the excitability of the spinal network. In zebrafish with ablated V2a interneurons the threshold for inducing locomotion was increased both pharmacologically and with electrical stimulation. These animals also showed an increase in the rostro-caudal delay. These results indicate that V2a interneurons represent an intrinsic source of excitation necessary for the normal expression of swimming activity.

Finally, we used optogenetic tools to determine if activation of V2a interneurons is sufficient to elicit swimming activity in larval zebrafish. For this transgenic zebrafish were used in which ChR2 was selectively expressed in V2a interneurons. Light activation of V2a interneurons induced synaptic excitatory inputs in other V2a interneurons that was sufficient to induce rhythmic locomotor activity. These results show that selective optogenetic activation of V2a interneurons is able to generate swimming activity in larval zebrafish.

The work presented in this thesis has provided new insights into the organization of the zebrafish spinal locomotor circuitry. We have gained novel insights into the mechanisms governing recruitment of motoneurons. In addition, we have uncovered the excitatory interneurons at the origin of the excitatory drive necessary for the generation of locomotion.

Key words: zebrafish, locomotion, motoneuron, recruitment order, V2a, Chx10, excitatory interneuron, ChR2

ISBN 978-91-7457-988-8

LIST OF PUBLICATIONS

I. Gabriel JP, Ausborn J, Ampatzis K, Mahmood R, **Eklöf Ljunggren E**, El Manira A. (2011) Principles governing recruitment of motoneurons during swimming in zebrafish. Nat Neurosci. 2011 Jan;14(1):93-9.

II. **Eklöf Ljunggren E**, Haupt S, Ausborn J, Dehnisch I, Uhlén P, Higashijima S, El Manira A. (2012) Origin of excitation underlying locomotion in the spinal circuit of zebrafish Proc Natl Acad Sci U S A. 2012 Apr 3;109(14):5511-6

III. **Eklöf Ljunggren E**, Haupt S, Ausborn J, El Manira A Optogenetic activation of V2a interneurons produces locomotion in vertebrate spinal cord (Manuscript)

CONTENTS

ABSTRACT ..... **V**

LIST OF PUBLICATIONS ..... **VI**

LIST OF ABBREVIATIONS ..... **VIII**

INTRODUCTION **1**

CENTRAL PATTERN GENERATORS 1

MOTOR CONTROL 1

THE SWIMMING CPG 3

SWIMMING IN THE ZEBRAFISH 4

TRANSCRIPTIONAL CODE UNDERLYING DEVELOPMENT 5

MOTONEURONS 6

EXCITATORY INTERNEURONS WITHIN THE SPINAL LOCOMOTOR CPGS 7

AIMS **11**

METHODS **13**

ZEBRAFISH AS A MODEL ORGANISM 13

Transgenic animals *13*

Larvae *13*

Adult *13*

PHOTO ABLATION OF NEURONS 14

ELECTROPHYSIOLOGY 14

Extracellular recordings *14*

Intracellular recordings *14*

BACKFILLING OF NEURONS 14

IMMUNOHISTOCHEMISTRY 15

RESULTS AND DISCUSSION **17**

PAPER I: PRINCIPLES GOVERNING RECRUITMENT OF MOTONEURONS DURING SWIMMING IN ZEBRAFISH. 17

PAPER II: ORIGIN OF EXCITATION UNDERLYING LOCOMOTION IN THE SPINAL CIRCUIT OF ZEBRAFISH. 20

PAPER III: OPTOGENETIC ACTIVATION OF V2A INTERNEURONS PRODUCES LOCOMOTION IN THE VERTEBRATE SPINAL CORD 23

CONCLUDING REMARKS AND FUTURE PERSPECTIVES **27**

REFERENCES **29**

ACKNOWLEDGEMENTS **35**

LIST OF ABBREVIATIONS

BMPs	Bone morphogenetic proteins
ChR2	Channelrhodopsin
CiDs	Circumferential descending interneurons
CPG	Central pattern generator
DLR	Diencephalic locomotor region
Hpf	Hours post fertilization
LMCs	Lateral motor columns
MLR	Mesopontine locomotor region
MCoDs	Multipolar commissural descending interneurons
MMC	median motor column
NMDA	N-methyl-d-aspartic acid
RS	Reticulospinal neurons
Shh	Sonic hedgehog

INTRODUCTION

CENTRAL PATTERN GENERATORS

Generation of motor behavior is the key component to survival of the animal in a complex environment. The ability to move allows animals and humans to seek food, interact and avoid dangers. Locomotor movements are generated by neuronal networks in the spinal cord producing the rhythmic pattern responsible for the sequential activation of muscles (Grillner, 1975). These networks are composed of interneurons and motoneurons and are called central pattern generators (CPGs) (Orlovsky, Deliagina and Grillner, 1999). These CPGs are capable of generating organized locomotor activity in the absence of rhythmic inputs from higher brain regions or sensory feedback (Grillner, 2003, 2006; Kiehn, 2006).

Animals display complex motor behaviors, some of which are repetitive such as locomotion, breathing, chewing and scratching making them easier to study than non-repetitive behaviors. In addition, the networks underlying these behaviors are well suited for a detailed cellular and molecular analysis due to their experimental accessibility both in vertebrate and invertebrate model animals (Ramirez and Richter, 1996; Marder and Bucher, 2001; Grillner 2003; McDearmid and Drapeau 2006; Fetcho et al 2008). Much effort has been put into unraveling the organization and function of the nervous system (Pearson, 1993; Marder 2000; Grillner 2003; Kiehn, 2006).

MOTOR CONTROL

The control structure for locomotion has a common organization in all vertebrates and is divided into three broad modules that perform selection, initiation and pattern generation (Grillner 2003; Goulding, 2009) (Fig. 1). While the selection of the motor programs takes place in the basal ganglia in the forebrain (Pombal et al, 1997a; Pombal et al, 1997b), the initiation is controlled in the brainstem, which contains descending command neurons that project to the spinal CPGs (El Manira et al, 1997). The mesopontine (MLR) and diencephalic (DLR) locomotor regions can induce well-organized locomotor activity in the spinal cord, when stimulated (Shik and Orlovsky, 1976; Orlovsky, Deliagina and Grillner, 1999; Sirota et al, 2000).

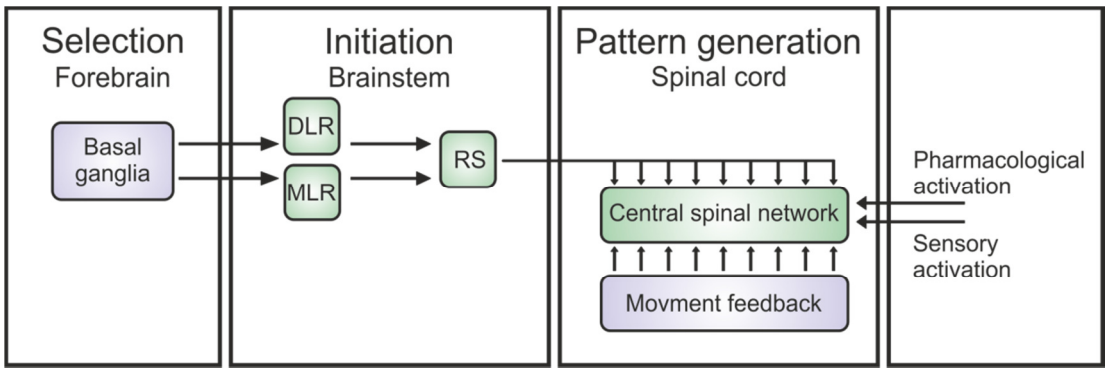

Figure 1: Schematic of the motor control structure common to all vertebrates. The selection of motor programs takes place in the basal ganglia in the forebrain. Locomotion is initiated by stimulation of diencephalic and mesopontine locomotor regions, which project independently to RS neurons that provide excitation to the CPG. Sensory feedback generated by the ongoing movement can help to fine-tune the different phases of locomotion. Experimentally locomotion can be induced by the application of pharmacological agents or activation of sensory input. (Modified from Grillner, 2003)

These two areas project independently to reticulospinal neurons (RS), which in turn activate the CPGs generating locomotor activity (Fig. 1).

Fine-tuning of motor control is of vital importance for the organism. Sensory feedback systems can help regulating the ongoing locomotion by adjustment of the different phases of the movement (Grillner and Rossignol, 1978; Duysens and Pearson, 1980; Lam and Pearson, 2002). Feedback from muscle and skin afferents as well as visual, vestibular and auditory input can help adapting the motor pattern to the environment (Rossignol, Dubuc and Gossard, 2006).

Under experimental conditions, the input from higher brain regions can be removed and replaced by the application of pharmacological agents or by electrical stimulation to induce locomotor activity. This was shown in the *in vitro* lamprey preparation (Cohen and Wallén, 1980; Poon, 1980; Wallén and Williams, 1984) and has since then been done in the cat (Deliagina et al, 1981), the *Xenopus* tadpole (Roberts et al, 1981), the neonatal mouse (Kiehn, 2006), and larval and adult zebrafish (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006; Gabriel et al, 2008), which are all model systems used to study fictive locomotion.

THE SWIMMING CPG

Studies in the lamprey have shown that the swimming CPG is made up of three main groups of neurons (Fig. 2). These have been shown to have functional and anatomical equivalents in other swimming species such as the zebrafish and the *Xenopus* tadpole (Grillner, 2006; Goulding, 2009).

- Motoneurons (MNs), which are segmentally organized and innervate the musculature. They represent the final stage of neuronal processing in the spinal cord.
- Inhibitory commissural interneurons (CINs), which project to the contralateral side of the spinal cord and provide inhibitory connection to ensure left-right alternation (Cohen and Harris-Warrick, 1984; Buchanan, 2001).
- Excitatory glutamatergic interneurons (EINs), which are ipsilaterally projecting and the major source of excitation within the locomotor CPG (Dale, 1986; Buchanan and Grillner, 1987).

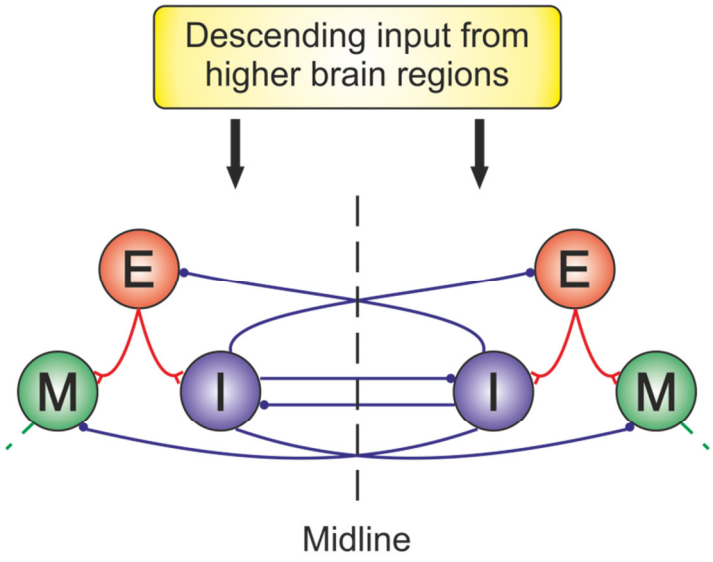

Figure 2: Schematic overview of the core elements of the lamprey CPG. E, excitatory interneurons (red); I, inhibitory commissural interneurons (blue); M, motoneurons (green). (Modified from Grillner, 2006)

Even though locomotion differs between swimming vertebrates and vertebrates that use limbs, the basic organization of the neuronal networks seems to be conserved across vertebrate species (Grillner, 2003; Kiehn, 2006). Model organisms such as the *Xenopus* tadpole, the lamprey and the zebrafish can, due to

their simplicity and experimental accessibility provide information about the organization of the locomotor networks of higher vertebrates.

SWIMMING IN THE ZEBRAFISH

Already at 17 hours post fertilization (hpf) the zebrafish embryo will show spontaneous behavior consisting of alternating side-to-side contractions of the trunk. After 26 hpf the embryo will respond with an escape movement to a touch of either the head or the tail (Saint-Amant and Drapeau, 1998). The embryo hatches at day 2 and at this stage the zebrafish is largely inactive (Buss and Drapeau, 2001). By approximately day four the larva is free swimming. At these early developmental stages the swimming pattern is characterized by episodes of swimming intermingled with episodes of non-swimming called "beat-and-glide" (van Raamsdonk et al, 1982; Buss and Drapeau, 2001, 2002). The episodic swimming pattern undergoes a developmental switch from long to short swimming episodes between 3 and 4 days post fertilization (Buss and Drapeau, 2001). It was shown that the orthopedia expressing dopaminergic population that constitutes the dopaminergic diencephalospinal tract mediates this developmental switch (Lambert et al, 2012).

The "beat-and-glide" pattern can be observed in the *in vitro* preparation of the larval zebrafish when fictive locomotion is induced by NMDA (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006). The rhythmic activity in the larval zebrafish is generated by a network of interneurons and motoneurons (Saint-Amant and Drapeau, 2001) which are recruited in a topographic manner with increasing swimming speed (McLean et al, 2007).

As the zebrafish grows older they develop an increasing number of red muscle fibers (van Raamsdonk et al, 1982) and at the same time the locomotor pattern switches from the high-frequency "beat-and-glide" swimming (up to 100 Hz) to a slower, continuous swimming pattern (Johnston, 1983; Müller and van Leeuwen, 2004; Gabriel et al, 2008). The swimming frequency of the freely moving adult zebrafish *in vivo* is in the range of 1-20 Hz (Kyriakatos et al. 2010). A continuous swimming pattern with a similar range of frequencies can be elicited in an *in vitro* preparation by electrical stimulation of descending excitatory axons.

TRANSCRIPTIONAL CODE UNDERLYING DEVELOPMENT

To be able to unravel the connectivity of the different interneurons in the spinal network, we have to be able to divide them into specific groups. Recently, investigation of the physiological and anatomical properties of the different interneuronal subclasses has been facilitated by the molecular labeling of different subclasses based on the transcription factors they express during early development (Jessell, 2000; Goulding, 2009; Grillner and Jessell, 2009). The molecular mechanisms underlying the diversity of interneurons forming the locomotor circuitry have begun to emerge. Two morphogen gradients impart dorso-ventral positional information to neural progenitors. Sonic hedgehog (Shh) is produced by the notochord and floor plate, while bone morphogenetic proteins (BMPs) are secreted dorsally from the roof plate. The opposing activities of Shh and BMPs generate different classes of embryonic neurons along the dorso-ventral axis of the spinal cord (Henneman et al, 1974; Lee and Jessell, 1999; Shirasaki and Pfaff, 2002). Neurons in the ventral part of the spinal cord are considered to be part of the circuitry controlling locomotor movement. These neurons belong to four different classes each expressing specific transcriptional factors. The interneurons in the ventral part of the spinal cord are derived from four progenitor populations and these progenitor populations give rise to interneuron classes termed V0, V1, V2 and V3 each expressing a specific transcription factor (Matise and Joyner, 1997; Saueressig et al, 1999; Jessell, 2000; Pierani et al, 2001; Shirasaki and Pfaff, 2002; Goulding et al, 2002; Peng et al, 2007; Lundfald et al, 2007; Al-Mosawie et al, 2007; Zhang et al, 2008).

The V0 interneurons are commissural interneurons that project 2-4 segments rostrally (Moran-Rivard et al, 2001; Pierani et al, 2001), the V1 neurons are a group of inhibitory interneurons that project ipsilaterally and rostrally (Saueressig et al, 1999; Higashijima et al, 2004), and the V3 neurons are excitatory commissural interneurons that project caudally (Zhang et al, 2008). The V2 interneurons are a group of interneurons that comprise of both excitatory V2a interneurons and inhibitory V2b interneurons. Both subgroups project ipsilaterally and caudally over several segments (Kimura et al 2006). The transcriptional coding of the interneuron populations has been shown to be conserved across vertebrate species (Lupo et al, 2006). The molecular characterization of the putative interneurons of the locomotor circuitry has fuelled new investigations aiming at determining their connectivity and function.

MOTONEURONS

Motoneurons are the final stage in the processing of motor control. The classification of the motoneurons is defined by the type of muscle fibers they innervate (slow, intermediate and fast), their position, axon trajectory, and spatial pattern of muscle innervation. Motoneurons are all derived from one single progenitor domain (Ericson et al, 1997; Briscoe et al, 1999). In higher vertebrates, it has been shown that motoneurons have three levels of organization. First, motoneurons are organized into different columnar groups, each column occupying a defined position along the rostrocaudal axis of the spinal cord (Landmesser, 1978a). The most prominent of these columnar groups are the lateral motor columns (LMCs) which are located at the limb levels of the spinal cord and innervate the limb muscles. In contrast to these segmentally restricted motor columns, motor neurons in the median motor column (MMC) are distributed along the spinal cord and innervate the axial musculature (Fetcho, 1987; Gutman et al., 1993). Second, motoneurons within one column are divided into medial and lateral motoneurons and their axonal projections are separated from each other. For example, within the lateral motor column the medial motoneurons will project to ventral limb muscles, whereas the lateral motoneurons will project to the dorsal ones (Landmesser, 1978b). Third, motoneurons are grouped into operational units called motor pools that each innervate a single muscle (Landmesser, 1978a). Each motoneuron pool will have a constant position along the rostro-caudal, medio-lateral and dorso-ventral axis of the spinal cord (Romanes, 1951).

In the zebrafish, motoneurons develop from dorsal to ventral with early born large primary motoneurons located dorsally and late born small secondary motoneurons located ventrally (van Raamsdonk et al, 1983; Myers, 1985; Westerfield, McMurray and Eisen, 1986; Lewis et al, 2003). In adult zebrafish, each myotomal muscle fiber is innervated by a single primary motoneuron and one or more secondary motoneurons (Westerfield, McMurray and Eisen, 1986). During slow swimming, secondary motoneurons are active whereas the primary motoneurons are mainly active during fast swimming, struggling and escape (Liu and Westerfield, 1987). The primary motoneurons within each segment can be distinguished by their position and by their muscle innervation pattern (Eisen et al, 1986). There is to date no anatomical or molecular technique to separate the different populations of secondary motoneurons.

The motoneurons within each pool have to be recruited in an orderly fashion to be able to produce smooth movements. Initially, it was thought that the order of recruitment was dependent on the input resistance of the neurons, with small neurons having high resistance and therefore reaching threshold before larger neurons with lower resistance (Haneman et al 1965; Kernell and Zwaagstra 1981; Mendell, 2005). This order of recruitment of motoneurons with increasing force or speed is called the "size principle" (Henneman, 1957; Henneman et al, 1965; Heneman and Mendell, 1981, Cope and Pinter, 1995; Mendell, 2005). Although the size principle has been very helpful to describe motor-unit recruitment, the importance of input resistance as the main factor defining the recruitment order of motoneurons has not been firmly demonstrated.

EXCITATORY INTERNEURONS WITHIN THE SPINAL LOCOMOTOR CPGS

The locomotor CPGs consist of networks of interconnected excitatory and inhibitory interneurons (Grillner, 2003). The inhibitory commissural interneurons ensure the alternating activity of the two sides of the spinal cord, and that between antagonistic muscles (Grillner, 2003; Kiehn, 2006; Jankowska, 2008). Blockade of inhibitory synaptic transmission switches the rhythmic alternating pattern into synchronous bursting in ventral roots on both sides of the spinal cord (Grillner, 2003; Kiehn, 2006). These results indicate that the locomotor circuitry consists of burst generating networks that can operate in the absence of inhibition which is further supported by the fact that when completely separated (hemi-cord), the two sides of the spinal cord continue to generate rhythmic activity (Cangiano and Grillner, 2002). For example, in both the lamprey and the *Xenopus* tadpole rhythmic activity can be generated in a completely hemisected spinal cord because each hemi-cord contains a network of excitatory interneurons with mutual excitation that is sufficient to sustain bursting (Soffe, 1989; Cangiano and Grillner, 2002).

In mammals different types of excitatory interneurons have been characterized that can play a role in the generation of locomotion (Kiehn, 2006). In cat, different presumably excitatory interneurons have been identified based on their activity pattern and the sensory inputs they receive (Angel et al., 2005; Cavallari et al. 1987; Edgley, Jankowska, 1987a, 1987b). Advances in the development of molecular tools have enabled the identification of specific classes of excitatory interneurons in mice

(Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2011) and the connectivity and role of these interneurons in the generation of locomotor activity is starting to emerge.

Electrophysiological analysis in the lamprey and the *Xenopus* tadpole has shown that the neurons generating rhythmic activity are glutamatergic interneurons with mostly descending axons that make monosynaptic connections with motoneurons and commissural interneurons (*Xenopus*: Roberts et al., 1998, 2010; lamprey: Grillner, 2003). In the lamprey spinal cord, the analysis of the synaptic connections between excitatory interneurons and motoneurons has shown that each motoneuron receives excitation from a small proportion of interneurons. In this preparation, stimulation of a single excitatory interneuron can elicit activity in the ventral root and change the frequency of the locomotor rhythm; however, it is unable to produce a sustained rhythm (Buchanan and Grillner, 1987, Buchanan et al., 1989). The excitatory interneurons are likely to be subdivided into groups which may enable different motor functions.

Similar interneurons with descending axonal projections (CiDs) also exist in the zebrafish. These interneurons make monosynaptic connections with motoneurons (McLean et al, 2008, Kimura et al, 2006) and express the transcription factor Alx, a homolog of the mammalian Chx10, corresponding to the V2a interneuron class.

To determine if the V2a interneurons are indeed providing the excitatory drive necessary for locomotor activity, they were completely eliminated during development using diphtheria toxin in transgenic mice (Crone et al, 2008). In these mice, rhythmic activity could still be induced pharmacologically *in vitro* in the isolated spinal cord and intact animals could walk on a treadmill (Crone et al, 2009). However, left-right alternation was altered leading to the conclusion that V2a interneurons are primarily excitatory commissural interneurons controlling left-right alternation (Crone et al, 2009). It has been difficult to characterize such synaptic connections electrophysiologically, possibly due to sparse interactions in the spinal cord (Crone et al, 2009). It should be noted that elimination or inactivation of a class of interneurons during early development can lead to homeostatic compensations that allow the remaining excitatory connections to fill the gap by scaling their synaptic gain enabling the circuitry to generate locomotor activity

In larval zebrafish, one class of contralaterally projecting excitatory interneurons (MCoDs) has been thought to be involved in producing slow swimming.

These interneurons make monosynaptic connections with contralateral motoneurons and are active at slow swimming speeds. Ablation of these interneurons prevented slow speed swimming (McLean et al, 2007), suggesting that the fast swimming activity is mediated by the recruitment of V2a (CiD) interneurons. In embryonic and larval zebrafish, the whole range of swimming frequencies is generated by different groups of excitatory interneurons. The recruitment follows a topographic order, with one group of neurons gradually switching off as another group is recruited, thus allowing for different groups to drive different swimming speeds (Mclean et al, 2007). The situation changes as the zebrafish develop towards adulthood. The pattern of recruitment of the V2a interneurons shifts from being topographic into a more distributed organization (Ausborn et al., 2012). In the adult zebrafish the recruitment of the V2a interneurons is determined by scaling of their excitatory synaptic drive with their input resistance. Results from the adult zebrafish suggest that the locomotor network is composed of multiple microcircuits with overlapping connectivity, recruited sequentially in a continuum to cover the full range of locomotor speeds displayed by the intact animals.

AIMS

The aim of this thesis has been to study the organization of the locomotor network in the larval and adult zebrafish. We have used complementary experimental techniques to address the following questions:

1. What are the principles that govern the recruitment order of motoneurons in adult zebrafish?

2. Does the excitatory drive from V2a interneurons contribute to the normal expression of swimming activity in larval zebrafish?

3. Is optogenetic activation of V2a interneurons sufficient as a source of excitation to drive the generation of swimming activity in the larval zebrafish?

METHODS

ZEBRAFISH AS A MODEL ORGANISM

Transgenic animals

For the work described in this thesis four different transgenic zebrafish lines have been used. The first line, Chx10:GFP expresses green fluorescent protein (GFP) under the Chx10 promoter and was used in paper II. In this line only neurons that express the transcription factor Chx10 will be expressing GFP, giving us the opportunity to selectively study this group of neurons. The second line, Et(-1.5hsp70I:Gal4-VP16)s1011t (referred to as s1011t) expresses Gal4-VP16 in V2a interneurons and has been used in paper III. The remaining two lines are the UAS:kaede and the UAS:ChR2-mCherry lines. These two UAS lines express their protein/channel only after being crossed with a Gal4-VP16 line. For studies in paper III, we crossed the s1011t with the UAS:kaede to obtain green fluorescing neurons and we crossed the s1011t with the UAS:ChR2-mCherry to express channelrhodopsin and mCherry in the V2a interneurons.

Larvae

Larval zebrafish were used for studying the contribution of the V2a interneurons to the generation of locomotion (papers II & III). For extracellular recordings the animals were anesthetized and the skin was removed from the animal. The fish was placed dorsal side up and treated with α-bungarotoxin to block the neuromuscular junctions. During locomotion left-right alternation and rostro-caudal delay were monitored.

Adult

Adult zebrafish were used to investigate the recruitment of motoneurons (paper I). The animals were anesthetized and dissected in slush of frozen saline. The skull was opened and the epaxial musculature (sparing the tail) of the animal was removed. The bones overlying the spinal cord were removed and the spinal cord, vertebral column and the tail muscles and the vertebra column were cut out from the surrounding muscles. The preparation was placed on the side in the recording chamber and treated with α-bungarotoxin to abolish muscle twitches.

PHOTO ABLATION OF NEURONS

Larval zebrafish were anesthetized and embedded in low melting point agarose. Targeted ablation of specific neurons was performed using a two-photon laser (paper II). After ablation of the desired number of neurons, the animals were left to recover from the anesthetics. In this way we could study the impact of the V2a interneurons on the generation of locomotion. A huge advantage with laser ablation of neurons is the possibility to study the acute effect of the ablation, before any compensatory mechanisms have started. The drawback is that the ablated neurons release toxins that will affect the surrounding neurons. This sets a limit to how many neurons can be ablated while leaving the phenotype unchanged.

ELECTROPHYSIOLOGY

Extracellular recordings

Extracellular recording electrodes were pulled from borosilicate glass and broken to the desired tip diameter after which they were fire polished. Extracellular recordings were made from motor nerves in the intermyotomal cleft and locomotion was induced with either NMDA application (paper II), electrical stimulation (paper I & II) or with channelrhodopsin driven light activation (paper III).

Intracellular recordings

Intracellular recordings were made from identified neurons in the spinal cord of adult (paper I) and larval zebrafish (paper III). Electrodes were pulled from borosilicate glass and filled with an intracellular solution. Neurons were visualized with a microscope equipped with infrared-DIC optics and a CCD camera. Intracellular recordings were amplified and low-pass filtered.

BACKFILLING OF NEURONS

Motoneurons were backfilled by dissolving rhodamine dextran in deionized water and applying it to the skin of the animal. An etched tungsten wire was then used to puncture the skin introducing the dye into the muscles and damaging the motor axons to allow uptake of the dye. The animal was then left to recover overnight (adult) or for 1 h (larvae) before dissection or fixation.

Long projecting interneurons were backfilled by cutting the axons in the caudal part of the animal with a dye-filled patch pipette. The animals were then left to recover for 1 h before fixation and imaging with confocal microscopy.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to detect colocalization of Chx10 and Gal4 in the larval zebrafish (paper III). Embryos were fixed and incubated with a Chx10 antibody and a fluorescent secondary antibody was used to image the neurons under the confocal microscope.

RESULTS AND DISCUSSION

PAPER I: PRINCIPLES GOVERNING RECRUITMENT OF MOTONEURONS DURING SWIMMING IN ZEBRAFISH.

During locomotion, motoneurons need to be recruited in an appropriate order to produce coordinated movements. It has been shown that motoneurons in the larval zebrafish are recruited according to the size principle, with small high-resistance motoneurons being recruited first at slow swimming speeds and larger, low-resistance motoneurons being added as the swimming speed increases (McLean et al, 2007). Zebrafish motoneurons develop from dorsal to ventral with early born large motoneurons situated dorsally and late born smaller motoneurons located ventrally (van Raamsdonk et al, 1983; Myers et al, 1985; Lewis et al 2003). Thus motoneurons in larval zebrafish are recruited from ventral to dorsal with increasing swimming frequencies. As the zebrafish develops from larval to adult stages, the swimming pattern changes from a beat-and-glide pattern to continuous swimming. This is associated with an increase in the amount of red muscle fibers and a lateral extension of the motor column (van Raamsdonk et al, 1978; van Raamsdonk et al, 1982; Buss et al, 2000). The question we asked in this first study of the thesis was if the maturation of the swimming pattern and muscle fiber composition is accompanied by a refinement of the recruitment of the motoneurons in the adult zebrafish.

To address this question we used an *in vitro* brainstem-spinal cord preparation of adult zebrafish. In this preparation, the spinal cord together with the vertebral column is dissected out from the surrounding tissue. The vertebral column was opened up by removing bones along 3-4 rostral segments to place a stimulating electrode at the junction between the brainstem and spinal cord and along 4-5 segments in the spinal cord to allow patch clamp recordings from motoneurons (Fig. 3). Rhodamine dextran was injected into muscles to backfill motoneurons and hence allow their identification in the *in vitro* preparation (Fig. 3). Intracellular patch-clamp recordings were made from the motoneurons while motor activity was simultaneously monitored by recording from motor nerves extracellularly.

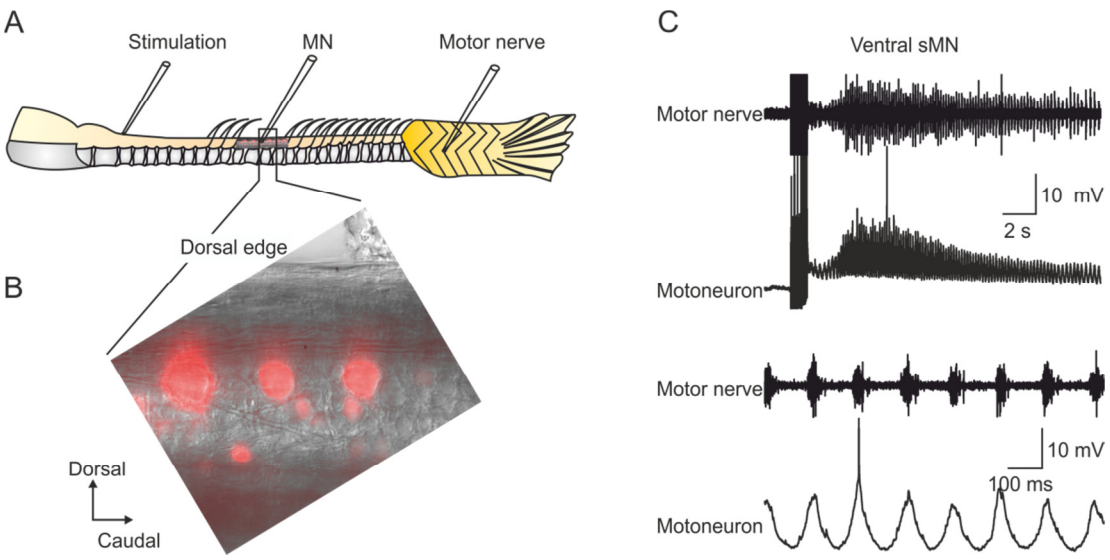

Figure 3: (A) Schematic drawing of the brainstem-spinal cord *In vitro* preparation. (B) Overlay of differential interference contrast and fluorescence image showing the location of rhodamine dextran backfilled motoneurons. (C) Example traces showing a locomotor episode (upper traces) and an enlarged section of the locomotor episode (lower traces) elicited by electrical stimulation and monitored intracellularly and extracellularly.

We found that motoneurons were organized into four different pools with a specific topographic location for each pool in the adult zebrafish spinal cord. These motoneuron pools were incrementally recruited with increasing swimming frequencies following a latero-medial and ventro-dorsal pattern. No correlation was found between the input resistance and the position of the motoneurons, or their oscillation amplitude. This indicates that the recruitment order is not a consequence of the input resistance, but is determined by the intrinsic and synaptic properties of the motoneurons. Indeed, the low frequency recruited ventrolateral motoneurons received large synaptic drive (Fig. 4) and displayed intrinsic pacemaker bursting. High frequency recruited ventromedial motoneurons received intermediate synaptic drive, but did not show any pacemaker properties. Rather, they fired continuously. In contrast, the non-recruited dorsal secondary and primary motoneurons received weak synaptic drive and displayed strong spike frequency adaptation (Fig. 4). Thus our results show that in adult zebrafish, motoneurons are organized into four different pools with distinct locations in the motor column. The ventrolateral motoneurons are recruited first, followed by the ventromedial ones, while the dorsal secondary motoneurons are recruited only at higher swimming frequencies (Fig. 4). In contrast, primary motoneurons do not participate in swimming and are only recruited during escape.

The order of recruitment of motoneurons is crucial for the generation of locomotion of the correct speed and force. The size principle has been suggested to explain the order of recruitment of motoneurons with the input resistance as the determining property (Mendel, 2005). The underlying assumption for this is that all motoneurons will receive the same synaptic input and thus small motoneurons with high input resistance will be recruited before large ones (Henneman and Mendell, 1981; Kernell and Zwaagstra, 1981; Gustafsson and Pinter, 1984; Cope and Pinter, 1995; Mendell, 2005). In the anesthetized cat, the recruitment of motoneurons in a given pool has been suggested to depend on both the input resistance and the synaptic current (Heckman and Binder, 1988). Our results indicate that the input resistance is not the primary factor setting the recruitment order of the motoneurons in the adult zebrafish spinal cord, but instead it is determined by the amplitudes of the synaptic drive they receive and their membrane properties.

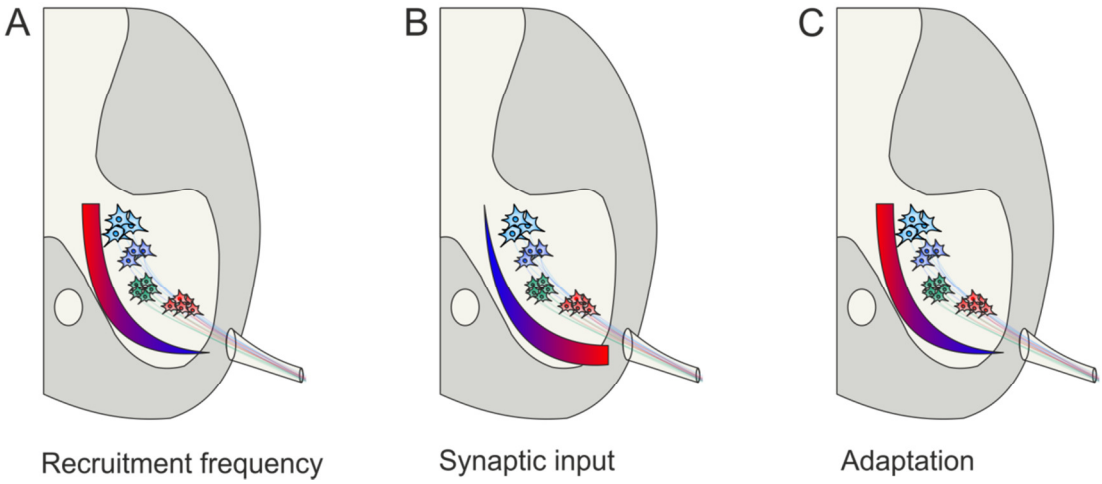

Figure 4: Summary of results. Gradients indicate high (red) to low (blue) parameters in all figures. (A) Motoneurons are recruited from ventral to dorsal with increasing locomotor frequencies. (B) Ventral motoneurons receive more synaptic input than dorsal motoneurons. (C) Spike frequency adaptation is stronger in dorsal motoneurons than in ventral motoneurons

The motor column of the larval zebrafish extends only in the dorso-ventral direction and not the lateral (van Raamsdonk et al, 1983; Lewis, Elsen, 2003; Myers, 1985). It consists of primary motoneurons and dorsal secondary motoneurons (Myers, 1985). Motoneurons in the larval zebrafish are recruited from ventral to dorsal according to the size principle (McLean et al, 2007). Our results indicate that the topographic organization of the motoneurons is maintained in the adult zebrafish, but that the rules for recruitment are changed during development. Therefore our results

provide novel information about the organization of motor pools in the adult zebrafish. In addition, we show that the mechanisms for setting the recruitment of motoneurons are refined as the zebrafish develop from larval to adult stages.

PAPER II: ORIGIN OF EXCITATION UNDERLYING LOCOMOTION IN THE SPINAL CIRCUIT OF ZEBRAFISH.

The generation of the basic locomotor pattern is mediated by a network of inhibitory and excitatory interneurons (Fig. 1). The inhibitory interneurons ensure the alternation of the two sides of the spinal cord and the excitatory interneurons are believed to provide the drive necessary for the generation of the rhythm (Pearson, 1993; Kiehn, 2006; Grillner, 2003; Berkowitz et al, 2010; Brownstone, Bui, 2010; Fetcho, Higashijima, McLean, 2008; Gabriel et al, 2010; Grillner, Jessell, 2009; Roberts et al, 2008; Wyart et al, 2009). These excitatory interneurons are considered to project ipsilaterally and drive the activity of motoneurons and inhibitory interneurons and have been characterized electrophysiologically and morphologically in the lamprey and in the *Xenopus* tadpole (Roberts et al, 2010; Berkowitz et al, 2010; Roberts et al, 2008; Buchanan and Grillner, 1987; Dale and Roberts, 1985). They provide monosynaptic excitation to motoneurons and interneurons and display rhythmic activity during locomotion (Kimura et al, 2006; McLean et al, 2007; McLean et al, 2008; McLean et al, 2009). These interneurons are homologous to the V2a interneurons and are defined molecularly in mice and zebrafish by the expression of the transcription factor Chx10 (Goulding, 2009; Jessell, 2000; Kimura et al, 2008; Ladle et al; 2007). The available information suggests that these ipsilaterally projecting V2a interneurons are a source of excitation underlying locomotion.

In this part of the thesis, we investigated if acute elimination of the V2a interneurons affected the generation of locomotion in the larval zebrafish. We used two-photon laser microscopy to specifically ablate ~30% of the V2a interneurons in 10 segments in the midbody of the animal (Fig. 5). We induced swimming, either with electrical stimulation or pharmacologically and compared the locomotor pattern between control animals and those with a V2a interneuron ablation. When electrical stimulation was used, the threshold stimulation for initiation of swimming in V2a ablated animals had to be was six fold increase compared to control and once locomotion was induced, the duration of the swimming bout was significantly shorter. To determine the contribution of the V2a interneurons to the excitability of the spinal

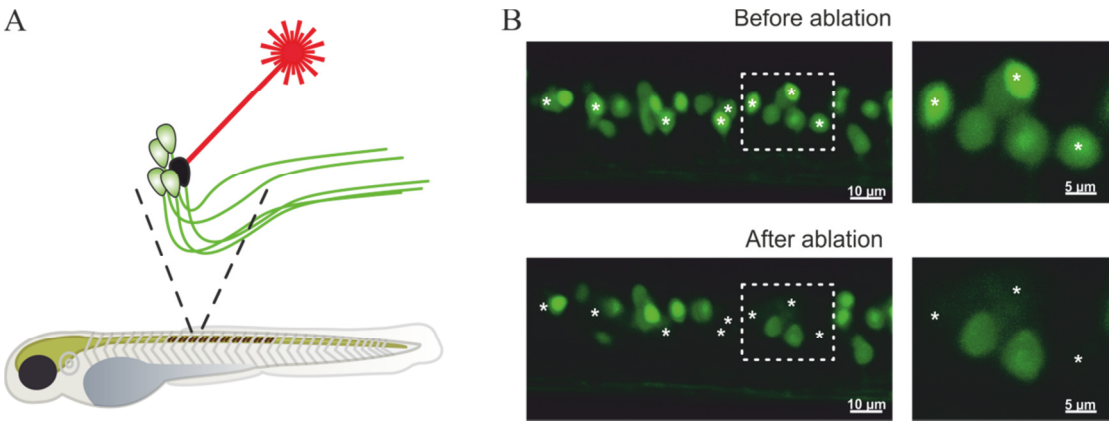

Figure 5: Ablation of V2a interneurons using two-photon laser. (A) V2a interneurons were ablated over 10 segments in the larval zebrafish. (B) Reconstruction of 1.5 segments in the spinal cord before and after photoablation of V2a interneurons (asterisks).

circuitry we analyzed the cumulative distribution of the swimming frequencies. We concluded that in the V2a ablated animals swimming frequencies were shifted towards lower values compared to control (Fig. 6). To test whether V2a interneurons act directly on the rhythm generation or if they act as relays for descending input we induced swimming by pharmacological activation with NMDA. To induce locomotion in V2a interneuron ablated animals a higher concentration of NMDA was needed compared to control animals. When the locomotion pattern was analyzed an increase in the rostro-caudal delay could be observed (Fig. 6), however the left-right alternation remained intact.

To ascertain that the changes in the swimming pattern were due to the loss of V2a interneurons and not due to loss of neurons per se, we laser-ablated the same number of glycinergic interneurons. This ablation did not have the same impact on the excitability of the spinal cord as the ablation of V2as.

For the spinal circuitry to be able to generate locomotor behavior an intrinsic source of excitation is needed. The identity of these excitatory interneurons has been unclear (Kiehn, 2006; Brownstone, Bui, 2010; Grillner, Jessell, 2009). We now propose that the V2a interneurons are a source of excitation since even partial ablation of this population causes considerable changes in the generation of swimming activity. It affects the threshold for locomotion induction, increases the rostro-caudal delay and decreases the swimming frequency.

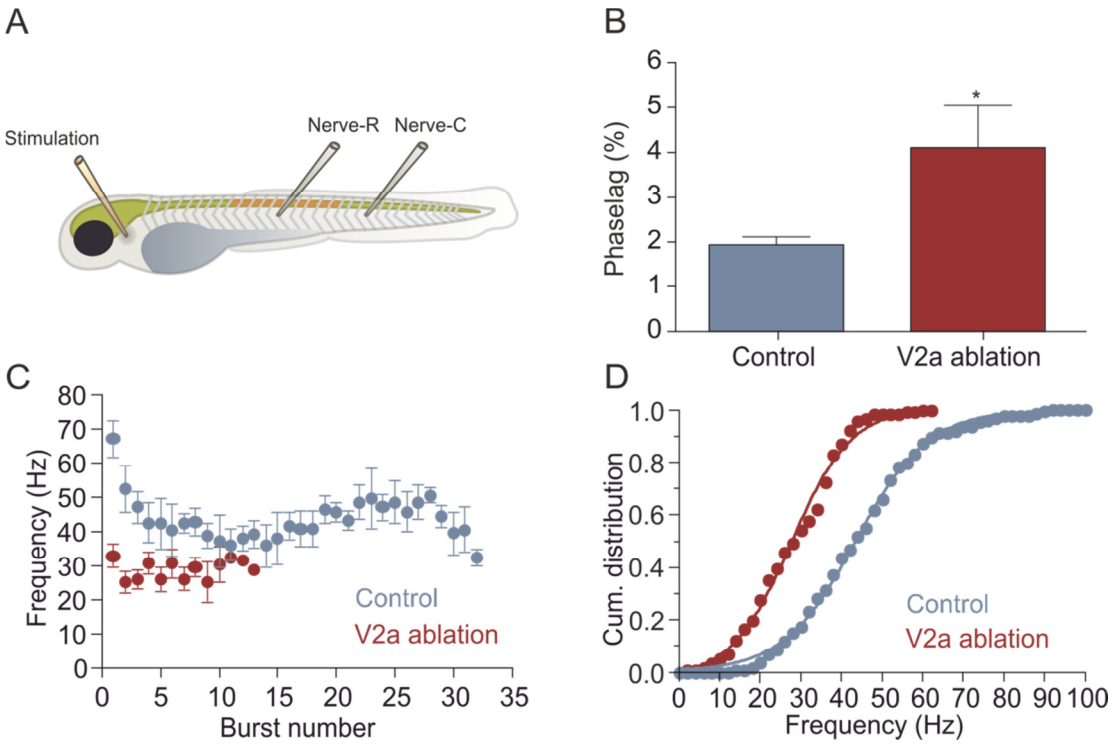

Figure 6: Ablation of V2a interneurons affects the swimming pattern. (A) Experimental setup showing the position of the stimulation and recording electrodes. (B) Phase lag is increased in V2a ablated animals. (C) Burst frequency and bout duration is decreased in V2a ablated animals. (D) Cumulative distribution of the burst frequencies

The connectivity of the V2a interneurons in the zebrafish shows similarities to what has been described for the swimming circuitry in the lamprey and the *Xenopus* tadpole (Roberts et al, 2010; Berkowitz et al, 2010; Roberts et al, 2008; Buchanan, Grillner, 1987; Buchanan et al, 1989; Dale, Roberts, 1985). There is also evidence for similar connections between V2a interneurons and motoneurons in rodents (Al-Mosawie et al, 2007; Lundfald et al, 2007; Stepien et al, 2008; Stepien et al, 2010). The V2a interneurons have been suggested to be the main candidate for supplying excitation at the core locomotor generating circuitry (Kiehn, 2006, Fetcho, Higashijima, McLean, 2008, Grillner, Jessell, 2009). When V2a interneurons were genetically eliminated in newborn mice, little effect on the generation of rhythmic output was observed (Crone et al, 2008; Crone et al, 2009; Kiehn et al, 2010). In mice it seems that the V2a interneurons are not essential for normal rhythm generation but are mostly involved in the coordination of left-right alternation at medium to high frequencies (Crone et al, 2008; Crone et al, 2009; Dougherty et al, 2010). An explanation for this could be that the V2a interneurons have a different role in the zebrafish spinal network than in the mouse. Another possibility is that the mouse has overlapping classes that are underlying the rhythm generation and that the elimination

of one of these classes is not sufficient to induce changes in the rhythm activity (Kiehn, 2006; Brownstone, Bui, 2010; Grillner, Jessell, 2009).

Our results indicate that the V2a interneurons contribute to the excitability of the spinal network in the zebrafish and that they are important for the generation of a normal locomotor pattern.

PAPER III: OPTOGENETIC ACTIVATION OF V2A INTERNEURONS PRODUCES LOCOMOTION IN THE VERTEBRATE SPINAL CORD

In paper II we showed that the V2a interneurons in the larval zebrafish are necessary for normal expression of the locomotor rhythm and that they contribute to the excitability of the spinal network. The question we wanted to address in this part of the thesis was if the activation of V2a interneurons can generate rhythmic activity in the spinal network of the larval zebrafish.

We started with characterizing a transgenic zebrafish line Gal4s1011t which was derived from an enhanced trap screen. Crossing this line with UAS-kaede transgenic fish showed that the expression of Gal4-VP16 was limited to interneurons with morphology and axonal projections similar to those of the V2a interneurons (Fig. 7; Bernhardt et al., 1990; Kuwada et al., 1990; Hale et al., 2001). To determine if the expression is limited to interneurons and does not leak to motoneurons, we backfilled motoneurons with rhodamine dextran in transgenic animals Gal4s1011t-UAS-GFP. There was no overlap between backfilled motoneurons and the neurons expressing GFP. To ensure that the expression of Gal4 is indeed limited to only V2a interneurons, we performed immunohistochemistry using an antibody against the transcription factor Chx10, commonly known to be expressed by V2a interneurons (Karunaratne et al., 2002; Kimura et al, 2006). For this we used transgenic zebrafish obtained by crossing Gal4s1011t and UAS-kaede. Results from these experiments show that almost all interneurons expressing kaede are double positive for Chx10 immunoreactivity (Fig. 7). These results show that in the Gal4s1011t transgenic line the expression of Gal4 is restricted to V2a interneurons.

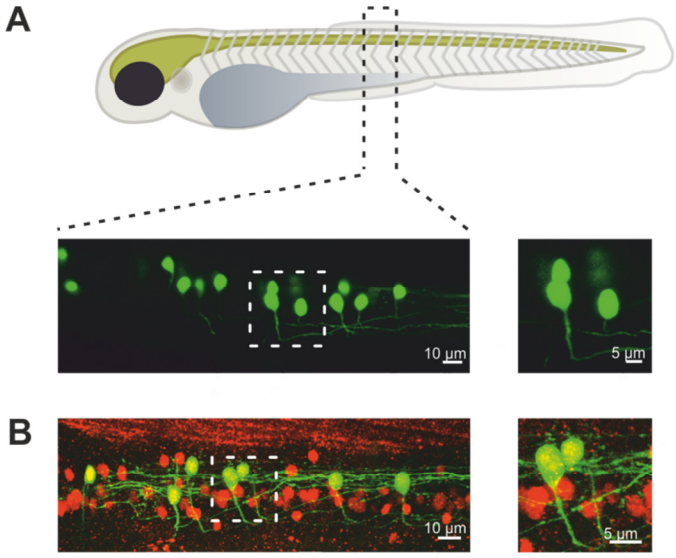

Figure 7: Imaging of Gal4-VP16 neurons in the spinal cord. (A) Reconstruction of 1.5 segments in the spinal cord of the larval zebrafish. (B) Immunohistochemistry against Chx10 shows double labeling with Gal4-VP16.

The Gal4s1011t zebrafish line was then crossed with a line carrying channelrhodopsin (ChR2) under an upstream activator sequence (UAS) together with mCherry. This way we could express ChR2 in the V2a interneurons while mCherry made it possible for us to visualize the ChR2 positive interneurons. We first determined if light activation could depolarize V2a interneurons using the patch-clamp technique. Application of a short blue light pulse (465nm) indeed depolarized ChR2-expressing V2a interneurons and the amplitude of the depolarization increased with increased light intensity, driving the interneurons to fire action potentials.

The ability of excitatory interneurons to produce rhythmic locomotor activity is considered to arise from their formation of a mutually excitatory network. To test if the V2a interneurons are receiving excitatory drive from other neighboring V2a interneurons, we recorded from mCherry-labeled V2a interneurons and applied short light pulses. Activation of ChR2 induced a direct depolarization of the recorded V2a interneurons followed by a delayed synaptically-mediated depolarization. This was also seen in voltage-clamp mode where light activation of ChR2 induced a fast direct inward current followed by a delayed synaptic excitatory current. These results show that activation of V2a interneurons can excite other V2a interneurons suggesting that they constitute an excitatory network.

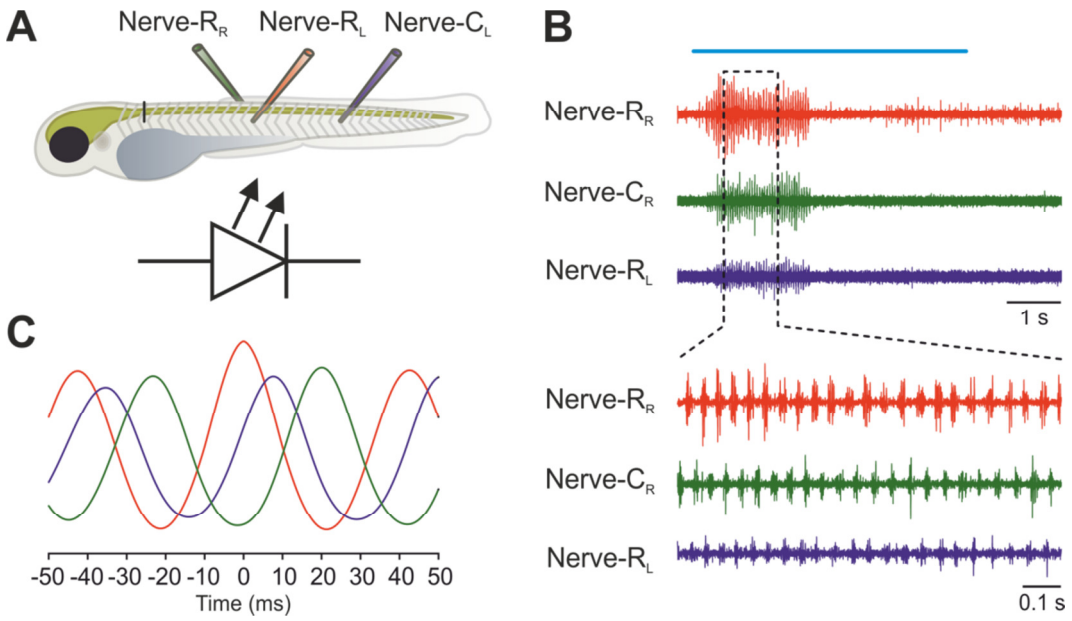

Figure 8: Fictive locomotion elicited in the spinalized preparation. (A) Schematic drawing showing the experimental setup and position for the recording electrodes. (B) Fictive locomotion induced by light stimulation. (C) Correlation analysis of the swimming trace shown in (B). Red line – autocorrelation, blue line – cross-correlation showing rostro-caudal delay of the locomotor activity and green line – cross-correlation showing left-right alternation of the locomotor activity

To test whether the activation of V2a interneurons could induce swimming in the zebrafish, we first tested if a long light pulse is able to induce rhythmic bursting in intracellular recording of V2a interneurons. Indeed, applications of long light pulses induced repetitive bursting that persisted after termination of the light pulse. This pattern of activity was similar to the swimming pattern generated by larval zebrafish. We then tested if the activation of V2a interneurons is able to produce coordinated swimming activity. For this we used spinalized zebrafish expressing ChR2 in V2a interneurons to avoid any unspecific activation of descending brainstem neurons. Application of light stimulation to the spinal cord induced fictive swimming in the *in vitro* preparation with the characteristic left-right alternation and rostro-caudal delay (Fig. 8). The swimming was induced when the light reached a specific intensity, as shown by a ramp increase in the intensity; however the duration of the swimming episodes and the swimming frequency were independent of the light intensity. These results show that the excitatory drive provided by the V2a interneurons is sufficient to induce locomotion in the spinal cord of larval zebrafish. Finally, we tested if the excitatory drive produced by the V2a interneurons was sufficient to generate

rhythmicity in the absence of crossed inhibition. For this we blocked glycinergic receptors with strychnine in a spinalized *in vitro* preparation. Application of light in this preparation could induce repetitive burst activity that occurred simultaneously in the recorded left and right motor nerves.

Lesion studies in lamprey have shown that a network of excitatory interneurons is able to produce the basic rhythmic activity (Cangiano et al., 2003; Cangiano et al., 2005). It has also been shown in *Xenopus* tadpoles that hindbrain excitatory descending neurons mutually excite each other and generate basic rhythmic activity (Li et al, 2006). In the mouse, light activation of excitatory interneurons evokes rhythmic activity (Hägglund et al, 2010). Results of this part of the thesis together with our previous results from paper II identify the V2a interneurons as necessary for the normal expression as well as sufficient to generate locomotor activity in the larval zebrafish. The identity of the excitatory interneurons responsible for the generation of locomotor activity has been elusive and our results have now identified the V2a interneurons as a source of excitation sufficient to produce a coordinated swimming pattern. It is likely that in other vertebrates the V2a interneurons play a similar role. A previous study has shown that optogenetic activation of the GABAergic Kolmer-Agduhr (KA) neurons located close to the central canal is able to produce swimming activity in the larval zebrafish (Wyart et al., 2009). The swimming activity induced by KA neurons appears as a rebound after terminating the light stimulation. It is possible that these GABAergic neurons elicit rebound activation of other interneurons that produce the swimming activity. V2a interneurons could be the downstream interneurons producing the rebound excitation necessary for the generation of the swimming activity following light activation of KA neurons. Further analysis is required to determine how KA neurons mediate their effects. It is still unclear how the V2a interneurons are activating each other and whether any functional heterogeneity in these interneurons exists that allows them to play different functional roles within the zebrafish spinal locomotor circuits.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

New technological advances are facilitating the characterization of the organization of the neural networks underlying motor behavior. The work presented in this thesis has provided novel insights into the rules governing the recruitment of motoneurons in the adult zebrafish as well as the identification of the interneurons underlying the excitatory drive necessary for the normal expression of swimming in the larval animal. The results of this thesis add to previous knowledge in the zebrafish, the lamprey, the *Xenopus* tadpole and the new-born mouse where some of the critical components of the locomotor networks have already been elucidated.

An important aspect of our work is the possibility to examine molecularly defined neurons in an adult model system. This allows us to determine how the mechanisms underlying locomotion are refined as the animal develops from early stages to adulthood. The zebrafish is one of the few vertebrate systems in which a detailed electrophysiological examination of the processing that is taking place within the locomotor network is possible from embryonic to adult stages. The analysis of the spinal networks in accessible adult vertebrates such as the zebrafish will allow a comparison of the organization of these networks with those of limbed animals such as mammals to discriminate between the conserved principles and those that have arisen during evolution.

Zebrafish have the ability to regenerate axonal tracts and cells, as well as entire tissues in the central nervous system. They can regrow one or several axons from a neuron that has been axotomized, and they can replace lost neurons from neuronal stem cells. This makes the zebrafish an attractive model organism for the study of regeneration after spinal cord injury (Becker and Becker, 2008).

Taking this ability to regenerate together with our results it would be appealing to investigate how the spinal network changes after a V2a ablation if the animals are left to recover and develop to adult stages. Will they acquire a normal locomotor pattern? If so is this due to replacement of the missing neurons or by rewiring of the existing spinal networks to compensate for the loss of V2a interneurons?

REFERENCES

Al‐Mosawie, A, Wilson, JM, Brownstone, RM. (2007) Heterogeneity of V2‐derived interneurons in the adult mouse spinal cord. Eur. J. Neurosci. 26:3003–3015.

Angel MJ, Jankowska E, McCrea DA. (2005). Candidate interneurones mediating group I disynaptic EPSPs in extensor motoneurones during fictive locomotion in the cat. J. Physiol. 563:597–610

Ausborn, J, Mahmood, R,El Manira, A. (2012) Decoding the rules of recruitment of excitatory interneurons in the adult zebrafish locomotor network. Proc Natl Acad Sci U S A, under revision

Becker CG, Becker T. (2008) Adult zebrafish as a model for successful central nervous system regeneration. Restor Neurol Neurosci. 26:71-80.

Berkowitz A, Roberts A, Soffe SR. (2010) Roles for multifunctional and specialized spinal interneurons during motor pattern generation in tadpoles, zebrafish larvae, and turtles. Front Behav Neurosci 4:36.

Bernhardt RR, Chitnis AB, Lindamer L, Kuwada JY. (1990) Identification of spinal neurons in the embryonic and larval zebrafish. J Comp Neurol 302:603–616.

Briscoe, J. et al. (1999) Homeobox gene Nkx2. 2 and specification of neuronal identity by graded Sonic Hedgehog signaling. Nature 398:622–627.

Brownstone RM, Bui TV. (2010) Spinal interneurons providing input to the final common path during locomotion. Prog Brain Res 187:81–95.

Buchanan JT. (2001) Contributions of identifiable neurons and neuron classes to lamprey vertebrate neurobiology. Prog Neurobiol 63:441-466.

Buchanan JT, Grillner S. (1987) Newly identified 'glutamate interneurons' and their role in locomotion in the lamprey spinal cord. Science 236:312-314.

Buchanan JT, Grillner S, Cullheim S, Risling M. (1989) Identification of excitatory interneurons contributing to generation of locomotion in lamprey: Structure, pharmacology, and function. J Neurophysiol 62:59–69.

Buss, RR, Drapeau, P. (2000) Physiological properties of zebrafish embryonic red and white muscle fibers during early development. J. Neurophysiol. 84, 1545–1557

Buss RR, Drapeau P. (2001) Synaptic drive to motoneurons during fictive swimming in the developing zebrafish. J Neurophysiol 86:197–210

Buss RR, Drapeau P. (2002)Activation of embryonic red and white muscle fibers during fictive swimming in the developing zebrafish. J Neurophysiol 87:1244–1251

Cangiano, L, Grillner, S. (2002) Fast and slow locomotor burst generation in the hemi-spinal cord of the lamprey. Soc. Neurosci. Abstr. 28, 65.10

Cangiano L, Grillner S (2003) Fast and slow locomotor burst generation in the hemi-spinal cord of the lamprey J Neurophysiol, 89, 2931–2942

Cangiano L, Grillner S (2005) Mechanisms of rhythm generation in a spinal locomotor network deprived of crossed connections: the lamprey hemicord. J Neurosci, 25, 923–935

Cavallari P, Edgley SA, Jankowska E. (1987). Post-synaptic actions of midlumbar interneurons on motoneurones of hind-limb muscles in the cat. J. Physiol. 389:675–89

Cohen AH, Harris-Warrick RM. (1984) Strychnine eliminates alternating motor output during fictive locomotion in the lamprey. Brain Res 293:164-167.

Cohen AH, Wallen P. (1980) The neuronal correlate of locomotion in fish. "Fictive swimming" induced in an in vitro preparation of the lamprey spinal cord. Exp Brain Res 41:11-18.
Cope, TC, Pinter, MJ. (1995) The size principle: still working after all these years. News Physiol. Sci. 10:280–286
Crone SA, et al. (2008) Genetic ablation of V2a ipsilateral interneurons disrupts leftright locomotor coordination in mammalian spinal cord. Neuron 60:70–83.
Crone SA, Zhong G, Harris-Warrick R, Sharma K. (2009) In mice lacking V2a interneurons, gait depends on speed of locomotion. J Neurosci 29:7098–7109.3.
Dale N. (1986) Excitatory synaptic drive for swimming mediated by amino acid receptors in the lamprey. J Neurosci 6:2662-2675.
Dale N, Roberts A. (1985) Dual-component amino-acid-mediated synaptic potentials: Excitatory drive for swimming in Xenopus embryos. J Physiol 363:35–59.
Deliagina TG, Orlovsky GN, Perret C. (1981) Efferent activity during fictitious scratchreflex in the cat. J Neurophysiol 45:595-604.
Duysens J, Pearson, KG. (1980) Inhibition of flexor burst generation by loading ankle extensor muscles in walking cats. Brain Res. 187:321–332.
Edgley SA, Jankowska E. (1987a). Field potentials generated by group II muscle afferents in the middle lumbar segments of the cat spinal cord. J. Physiol. 385:393–413
Edgley SA, Jankowska E. (1987b). An interneuronal relay for group I and II muscle afferents in the midlumbar segments of the cat spinal cord. J. Physiol. 389:647–74
El Manira A, Pombal MA, Grillner, S. (1997) Diencephalic projection to reticulospinal neurons involved in the initiation of locomotion in adult lampreys Lampetra fluviatilis. J. Comp. Neurol. 389:603–616
Ericson J. et al. (1997) Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell 90:169–180
Fetcho JR. (1987) A review of the organization and evolution of motoneurons innervating the axial musculature of vertebrates. Brain Res. 434:243-80.
Fetcho JR, Higashijima S, McLean DL. (2008) Zebrafish and motor control over the last decade. Brain Res Rev. 57:86-93.
Gabriel, J.P. et al. (2008) Locomotor pattern in the adult zebrafish spinal cord in vitro. J. Neurophysiol. 99:37–48
Goulding M, Lanuza G, Sapir T, Narayan S. (2002) The formation of sensorimotor circuits. Curr. Opin.Neurobiol. 12:505–515 .
Goulding M. (2009) Circuits controlling vertebrate locomotion: moving in a new direction. Nat Rev Neurosci. 10:507-18.
Grillner S. (1975) Locomotion in vertebrates: central mechanisms and reflex interaction. Physiol Rev. 55:247-304.
Grillner S. (2003) The motor infrastructure: from ion channels to neuronal networks.Nat Rev Neurosci.4:573-86
Grillner S. (2006) Biological pattern generation: the cellular and computational logic of networks in motion. Neuron. 52:751-66.
Grillner S, Jessell TM. (2009) Measured motion: searching for simplicity in spinal locomotor networks. Curr Opin Neurobiol. 19:572-86.
Grillner S, Rossignol S. (1978) On the initiation of the swing phase of locomotion in chronic spinal cats. Brain Res. 146:269–277.
Gustafsson B, Pinter MJ. (1984) An investigation of threshold properties among cat spinal alpha-motoneurones. J. Physiol. (Lond.) 357, 453–483.

Gutman CR, Ajmera MK, Hollyday M. (1993) Organization of motor pools supplying axial muscles in the chicken. Brain Res. 609:129-36.
Hale ME, Ritter DA, Fetcho JR. (2001) A confocal study of spinal interneurons in living larval zebrafish. J Comp Neurol 437:1–16
Henneman E. (1957) Relation between size of neurons and their susceptibility to discharge. Science 126, 1345–1347
Henneman, E., Somjen, G, Carpenter, D.O. (1965). Functional significance of cell size in spinal motoneurons. J. Neurophysiol. 28:560–580
Henneman E, Clamann HP, Gillies JD, Skinner RD. (1974) Rank order of motoneurons within a pool: law of combination. J. Neurophysiol 37:1338–1349
Henneman E, Mendell LM. (1981) Functional organization of motoneuron pool and its inputs. in Handbook of Physiology, Sect. 1, Vol. 2 (ed. Brooks, V.E.) 423–507 (American Physiological Society, Bethesda, Maryland, 1981).
Higashijima S, Masino MA, Mandel G, Fetcho JR. (2004) Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. J. Neurosci. 24, 5827–5839.
Hägglund M, Borgius L, Dougherty KJ, Kiehn O (2010) Activation of groups of excitatory neurons in the mammalian spinal cord or hindbrain evokes locomotion. Nat Neurosci 13:246–252
Jankowska E. (2008) Spinal interneuronal networks in the cat: elementary components. Brain Res Rev. 2008 Jan;57:46-55.
Jessell TM. (2000) Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. Nat Rev Genet 1:20–29.
Johnston IA. (1983) On the design of fish myotomal muscles. Mar. Freshw. Behav. Physiol. 9:83–98
Karunaratne A, Hargrave M, Poh A, Yamada T (2002) GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. Dev Biol 249:30–43
Kernell D, Zwaagstra B. (1981) Input conductance axonal conduction velocity and cell size among hindlimb motoneurones of the cat. Brain Res. 204, 311–326.
Kiehn O. (2006) Locomotor circuits in the mammalian spinal cord. Annu Rev Neurosci. 29:279-306
Kiehn O, et al. (2010) Probing spinal circuits controlling walking in mammals. Biochem Biophys Res Commun 396:11–18.
Kiehn O. (2011) Development and functional organization of spinal locomotor circuits. Curr Opin Neurobiol. 21:100-9.
Kimura Y, Okamura Y, Higashijima S (2006) alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. J Neurosci 26:5684–5697.
Kimura Y, Satou C, Higashijima S (2008) V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. Development 135:3001–3005.
Kuwada JY, Bernhardt RR, Nguyen N (1990) Development of spinal neurons and tracts in the zebrafish embryo. J Comp Neurol 302:617–628
Kyriakatos A, Mahmood R, Ausborn J, Porres CP, Büschges A, El Manira A. (2011) Initiation of locomotion in adult zebrafish. J Neurosci. 31:8422-31.
Ladle DR, Pecho-Vrieseling E, Arber S (2007) Assembly of motor circuits in the spinal cord: Driven to function by genetic and experience-dependent mechanisms. Neuron 56:270–28

Lam T, Pearson KG. (2002) The role of proprioceptive feedback in the regulation and adaptation of locomotor activity. Adv. Exp. Med. Biol. 508:343–355.
Lambert AM, Bonkowsky JL, Masino MA. (2012) The conserved dopaminergic diencephalospinal tract mediates vertebrate locomotor development in zebrafish larvae. J Neurosci. 32:13488-500.
Landmesser L. (1978a) The distribution of motoneurons supplying chick hind limb muscles. J. Physiol. (Lond.) 284.371–389
Landmesser L. (1978b) The development of motor projection patterns in the chick hind limb. J. Physiol. (Lond.) 284:391–414.
Lee K, Jessell TM. (1999) The specification of dorsal cell fates in the vertebrate central nervous system. Annu. Rev. Neurosci. 22:261–294.
Li W-C, Soffe, SR., Wolf E, Roberts A. (2006). Persistent Responses to Brief Stimuli: Feedback Excitation among Brainstem Neurons. J. Neurosci. 26, 4026–4035.
Liu DW, Westerfield M. (1987) Function of identified motoneurones and co-ordination of primary and secondary motor systems during zebra fish swimming. J. Physiol. (Lond.) 403, 73–89.
Lewis KE, Eisen JS. (2003) From cells to circuits: development of the zebrafish spinal cord. Prog. Neurobiol. 69, 419–449.
Lundfald L. et al. (2007) Phenotype of V2‐derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing spinal cord. Eur. J. Neurosci. 26:2989–3002.
Lupo G, HarrisWA, Lewis KE. (2006) Mechanisms of ventral patterning in the vertebrate nervous system. NatRevNeurosci 7:103-114.
Marder E. (2000) Motor pattern generation. Curr Opin Neurobiol. 10:691-8
Marder E, Bucher D. (2001) Central pattern generators and the control of rhythmic movements. Curr Biol.11:R986-96
Masino MA, Fetcho JR. (2005) Fictive swimming motor patterns in wild type and mutant larval zebrafish. J Neurophysiol 93: 3177–3188,.
Matise MP, Joyner AL. (1997) Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. J. Neurosci. 17:7805–7816.
McDearmid JR, Drapeau P.(2006) Rhythmic motor activity evoked by NMDA in the spinal zebrafish larva. J Neurophysiol. 95:401-17.
McLean DL, Fan J, Higashijima S, Hale ME, Fetcho JR (2007) A topographic map of recruitment in spinal cord. Nature 446:71–75.
McLean DL, Masino MA, Koh IY, Lindquist WB, Fetcho JR (2008) Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. Nat Neurosci 11:1419–1429.
McLean DL, Fetcho JR (2009) Spinal interneurons differentiate sequentially from those driving the fastest swimming movements in larval zebrafish to those driving the slowest ones. J Neurosci 29:13566–13577.
Mendell LM. (2005) The size principle: a rule describing the recruitment of motoneurons. J. Neurophysiol. 93:3024–3026
Müller UK, van Leeuwen JL. (2004) J Exp Biol. 207:853-68. Swimming of larval zebrafish: ontogeny of body waves and implications for locomotory development
Myers PZ. (1985) Spinal motoneurons of the larval zebrafish. J. Comp. Neurol. 236, 555–561
Orlovsky GN, Deliagina TG, Grillner S. (1999) Neuronal Control of Locomotion. From Mollusc to Man (Oxford Univ. Press, New York, 1999).

Pearson KG. (1993) Common principles of motor control in vertebrates and invertebrates. Annu Rev Neurosci. 16:265-97.
Peng C‐Y. et al. (2007) Notch and MAML signaling drives Scl‐dependent interneuron diversity in the spinal cord. Neuron 53:813–827
Pierani A. et al. (2001) Control of interneuron fate in the developing spinal cord by the progenitor homeodomain factor Dbx1. Neuron 29:367–384.
Pombal MA, El Manira A, Grillner S. (1997a) Afferents of the lamprey striatum with special reference to the dopaminergic system: a combined tracing and immunohistochemical study. J. Comp. Neurol. 386:71–91
Pombal, MA, El Manira A, Grillner S. (1997b) Organization of the lamprey striatum — transmitters and projections. Brain Res. 766:249–254
Poon MLT (1980) Induction of Swimming in Lamprey by L-Dopa and Amino-Acids. Journal of Comparative Physiology 136:337-344.
Ramirez JM, Richter DW.(1996) The neuronal mechanisms of respiratory rhythm generation. Curr Opin Neurobiol. 6:817-25
Roberts A, Kahn JA, Soffe SR, Clarke JD (1981) Neural control of swimming in a vertebrate. Science 213:1032-1034.
Roberts A, Soffe SR, Wolf ES, Yoshida M, Zhao FY (1998) Central circuits controlling locomotion in young frog tadpoles. Ann N Y Acad Sci 860:19-34.
Roberts A, Li WC, Soffe SR, Wolf E (2008) Origin of excitatory drive to a spinal locomotor network. Brain Res Brain Res Rev 57:22–28.
Roberts A, Li WC, Soffe SR (2010) How neurons generate behavior in a hatchling amphibian tadpole: an outline. Front Behav Neurosci 4:16.
Romanes GJ. (1951). The motor cell columns of the lumbo-sacral spinal cord of the cat. J. Comp. Neurol. 94:313–363.
Romanes GJ. (1964). The motor pools of the spinal cord. Prog. Brain Res. 11:93–119.
Rossignol S, Dubuc R, Gossard JP. (2006) Dynamic sensorimotor interactions in locomotion. Physiol Rev. 86:89-154.
Saint-Amant L, Drapeau P. (1998) Time course of the development of motor behaviors in the zebrafish embryo. J Neurobiol. 37:622-32.
Saueressig H, Burrill J, Goulding M. (1999) En1 and Netrin-1 control two distinct aspects of axon growth in association interneurons that project to motor neurons. Development 126:4201–4212.
Shik ML, Orlovsky GN. (1976) Neurophysiology of locomotor automatism. Physiol. Rev. 56:465–501.
Shirasaki R, Pfaff SL. (2002) Transcriptional codes and the control of neuronal identity. Annu. Rev. Neurosci. 25:251–281
Sirota MG, Di Prisco GV, Dubuc R. (2000) Stimulation of the mesencephalic locomotor region elicits controlled swimming in semi-intact lampreys. Eur. J. Neurosci. 12:4081–4092.
Soffe SR. (1989) Roles of Glycinergic Inhibition and N-Methyl-D-Aspartate Receptor Mediated Excitation in the Locomotor Rhythmicity of One Half of the Xenopus Embryo Central Nervous System. Eur J Neurosci.;1:561-571.
Stepien AE, Arber S (2008) Probing the locomotor conundrum: Descending the ‘V’ interneuron ladder. Neuron 60:1–4.
Stepien AE, Tripodi M, Arber S (2010) Monosynaptic rabies virus reveals premotor network organization and synaptic specificity of cholinergic partition cells. Neuron 68:456–472.
van Raamsdonk, W, Pool, CW, te Kronnie G. (1978) Differentiation of muscle fiber types in the teleost Brachydanio rerio. Anat. Embryol. (Berl.) 153, 137–155.

van Raamsdonk W, van't Veer L, Veeken K, Heyting C, Pool CW (1982) Differentiation of muscle fiber types in the teleost Brachydanio rerio, the zebrafish. Posthatching development. Anat Embryol (Berl) 164:51-62.
Wallen P, Williams TL (1984) Fictive locomotion in the lamprey spinal cord in vitrocompared with swimming in the intact and spinal animal. J Physiol 347:225-239.
Westerfield M, McMurray JV, Eisen JS. (1986) Identified motoneurons and their innervation of axial muscles in the zebrafish. J Neurosci. 6:2267-77.
Wyart C, et al. (2009) Optogenetic dissection of a behavioural module in the vertebrate spinal cord. Nature 461:407–410.

ACKNOWLEDGEMENTS

A thesis cannot be written without the help of a lot of wonderful people. I would like to thank them all.

My supervisor Abdel El Manira; thank you for all the help and encouragement I have received during the years. For laughter and discussions and for always giving me hope that there would be a thesis in the end.

My cosupervisor Per Uhlén and his lab members; thank you for always taking the time to help me. A special thanks to Ivar Dennish for teaching me the two-photon laser setup and Songbai Zhang for helping me with the molecular work.

My lab mates, past and present, Jessica, Sabine, Kostas, Song, Rebecka, Evanthia, Riyadh, Alexandros and Jens. Thank you for always having the time to answer questions and giving a helping hand, for laughter and discussions. Thank you Margareta for all the help in the lab.

I would also like to give thanks to the people at Södertörn University. Thank you Giselbert Hauptmann and Iris Söll for teaching me everything about zebrafish handling and techniques. Thank you Lars, Gilbert, Lasse and Janine for the fun time in the lab at Södertörn.

The zebrafish core facility at Karolinska Institutet for helping me with matings.

Sten Grillner for always taking the time to comment on papers and writing recommendation letters.

Russel Hill, Ole Kiehn and Kim Dougherty for comments and discussion of the manuscripts. Thanks to Peter Wallén for always helping me when the confocal microscope misbehaved.

Special thanks to Professor Anita Aperia for helping me find my way within research.

My mother and father who have always been there helping me and advising me during my not so straight way through university and PhD studies. Thank you for always believing in me and helping me find my own way.

Michael, the love of my life. For being there for me always. You complete me.