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Motor Control in Zebrafish

Excitatory Drive and Developmental Changes

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ABSTRACT

An essential characteristic of human and animal life is the ability to move from one place to another, in order to survive in a complex environment. All the different forms of locomotion, like walking, swimming, crawling and flying, have one common feature: rhythmic and alternating movements of the body. These movements are generated by neuronal networks in the spinal cord. The overall aim of this thesis is to investigate the mechanisms underlying locomotion in zebrafish, with particular focus on excitatory drive and developmental changes.

Excitatory interneurons are believed to represent the core components for the generation of the locomotor rhythm, since they drive both inhibitory interneurons and motoneurons. By ablating one specific group of interneurons, the V2a interneurons, we show that they represent an intrinsic source of excitation necessary for the normal expression of the locomotor rhythm. Ablation of V2a interneurons results in an increase in the threshold to induce swimming and a decrease in swimming frequency and episode duration.

To demonstrate that the excitatory drive from ipsilateral premotor V2a interneurons is also sufficient to drive swimming, we used optogenetics to activate the V2a interneurons specifically. Upon illumination, V2a interneurons displayed rhythmic oscillations that resembled the typical beat-and-glide swimming. Peripheral nerve recordings confirmed that the bursting activity in single neurons corresponds to swimming activity, which is characterized by left-right-alternation and rostrocaudal delay. This indicates that swimming activity emerges from the activity of an underlying V2a interneuron network.

The third aim of this thesis is to reveal the developmental changes of the swimming pattern and the motoneuron properties. By systematically recording peripheral nerve activity and primary motoneuron properties during different developmental stages, we were able to define the time frame of the switch in swimming behavior from larval episodic to adult continuous swimming to 4-5 weeks post fertilization. Primary motoneurons stop participating in swimming within the same time window and are from around 6 weeks onward only active during escape behavior.

In conclusion, we show that the excitatory V2a interneurons in zebrafish are necessary and sufficient for the rhythm generating network to generate a coordinated swimming motor pattern and that there is a major switch in the locomotor pattern and primary motoneuron recruitment around 4-5 weeks of development.

LIST OF SCIENTIFIC PAPERS

I.   Emma Eklöf Ljunggren, **Sabine Haupt**, Jessica Ausborn, Ivar Dehnisch, Per Uhlén, Shin-ishi Higashijima, and Abdeljabbar El Manira. 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.

II.   Emma Eklöf Ljunggren*, **Sabine Haupt***, Jessica Ausborn, Konstantinos Ampatzis, and Abdeljabbar El Manira. Optogenetic activation of excitatory premotor interneurons is sufficient to generate coordinated locomotor activity in larval zebrafish. J Neurosci. 2014 Jan 1;34(1):134-9.

* These authors contributed equally.

III.   **Sabine Görgens** and Abdeljabbar El Manira. Developmental Changes of Locomotor Activity in Zebrafish. *Manuscript*

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LIST OF ABBREVIATIONS

|	
BMP	Bone morphogenic protein
CaP	Caudal primary motoneuron
ChR2	Channelrhodopsin
CiD	Circumferential interneuron
CPG	Central pattern generator
Dpf	Days post fertilization
ER	Embryonic slow red muscle fibers
EW	Embryonic fast white muscle fibers
hpf	Hours post fertilization
IN	Interneuron
KA	Kolmer-Agduhr cell
MiP	Middle primary motoneuron
MN	Motoneuron
MS-222	Tricaine methanesulfonate
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pMN	Primary motoneuron
RoP	Rostral primary motoneuron
SHH	Sonic hedgehog
sMN	Secondary motoneuron
UAS	Upstream activation sequence
wpf	Weeks post fertilization

1 INTRODUCTION

1.1 CENTRAL PATTERN GENERATORS

To survive in a complex environment all animals need to perform a variety of motor tasks. Some of these behaviors are repetitive, such as locomotion, breathing, chewing and scratching and are therefore well suited to experimental studies. A lot of effort has been put into understanding how the nervous system is organized; starting with Sherringtons hypothesis that locomotion is an automatic result of successive activation of reflexes. Sherrington also introduced the concept of "reciprocal inhibition", which he defined as a reflex of "simultaneous double-sign", with excitation of one or more agonist muscles and simultaneous inhibition of the antagonist, acting at the same joint. This inhibition takes place in the spinal cord. His pioneering studies provided important information about the integration of neural pathways (Sherrington 1906; Burke 2007). Later on, Brown challenged his idea. He selectively removed all sensory input and showed that animals could still produce flexor-extensor-alternations. Browns "half-center" model argues that it is the interaction of two half-centers that mutually inhibit each other and thereby generate the locomotor rhythm (Brown 1914). His observations, that intrinsic networks in the spinal cord are able to generate rhythmic locomotor-like activity patterns, gave rise to the concept of central pattern generators (CPGs). They are defined as networks of neurons that autonomously - without sensory input - generate rhythmic patterns of activity (Grillner and Zangger 1979). Later on, it was shown that locomotor activity can be generated even in the absence of any inputs from higher brain regions (Grillner, Perret et al. 1976; Cohen and Wallen 1980; Poon 1980). This neural correlate of locomotion is called "fictive locomotion" (Cohen and Wallen 1980) and can amongst others be generated in larval (Masino and Fetcho 2005; McDearmid and Drapeau 2006) and adult zebrafish (Gabriel, Mahmood et al. 2008).

1.1.1 Swimming CPG

Important insights into the structure of the swimming CPG have been provided by studies in the lamprey and amphibian spinal cords (Roberts, Soffe et al. 1998; Grillner 2003). Terrestrial and aquatic vertebrates have a remarkably similar neuronal organization of the locomotor system, despite swimming movements differing from the more complex

locomotion in limbed animals (Dale and Kuenzi 1997; Grillner 2006; Kiehn 2006). The combination of a conserved underlying organization and the simplicity of the system make the swimming CPG an ideal model to study locomotion.
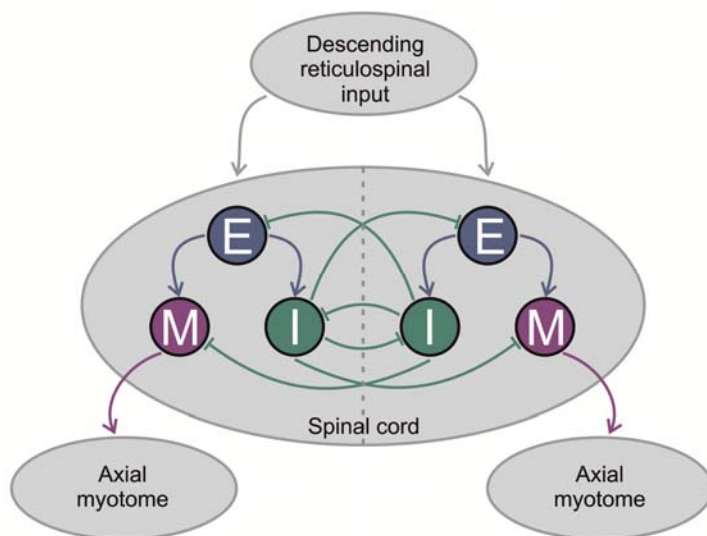
Figure 1. The swimming CPG

Schematic overview of the lamprey CPG. The spinal cord receives input from reticulospinal neurons. Excitatory interneurons (E) drive both inhibitory interneurons (I) and motoneurons (M). Inhibitory interneurons are responsible for reciprocal inhibition. Motoneurons project directly to the axial myotome and represent the output stage.

Modified from Grillner, 2006

Segmental models suggest that all spinal segments contain individual circuits that control coordination locally, while simultaneously sending long projecting axons to enable inter-segmental coordination. These segmental circuits consist of three classes of neurons, all of which are present on both the left and the right side of the individual segment (Fig.1). Activity in each segmental network is initiated by input from descending tracts from the reticular formation. Inhibitory commissural interneurons (I) cross the midline of the spinal cord and inhibit all neurons on the contralateral side. During swimming, these connections ensure that axial muscles on one side of the body contract out of phase with those on the opposite side. Excitatory interneurons (E) are rhythmically active and project ipsilaterally to both motoneurons and inhibitory interneurons. They are the main source of excitation and are believed to be the core components for the generation of the locomotor rhythm. Segmentally organized motoneurons (M) represent the output stage of neural processing in the spinal cord and project directly to the muscles on each side (Grillner and Wallen 1980; Buchanan 1982; Buchanan and Grillner 1987).

1.2 PATTERNING AND SPECIFICATION OF EARLY SPINAL CORD PROGENITORS

Neural circuits are built with great precision during embryonic development. In order to study the connectivity in the spinal cord it is necessary to classify certain groups of neurons. The differentiation of distinct classes of neurons from progenitor cells located at defined positions

within the neural tube is the starting point of the developmental program in the vertebrate central nervous system. The allocation of cell fate in the spinal cord depends on two signaling systems that are activated together with the more basic program of neural induction (Harland 2000). These two signaling systems intersect along the rostrocaudal and dorsoventral axes of the neural tube to establish a grid-like set of positional cues (Lumsden and Krumlauf 1996). Opposing gradients of morphogens provide instructive signals to the dividing progenitors. In the dorsoventral axis the most important developmental cues emanate from Sonic hedgehog (SHH) and bone morphogenic protein (BMP). BMP is secreted from the roof plate and its concentration gradient is responsible for the partitioning of the dorsal spinal cord (Lee and Jessell 1999), which is mostly involved in sensory processing. SHH is produced by the notochord and floor plate and controls ventral patterning (Jessell 2000). Progressive changes in SHH concentration generate five molecularly distinct classes of ventral neurons from progenitor cells (Ericson, Briscoe et al. 1997; Ericson, Rashbass et al. 1997). Neurons in the ventral part of the spinal cord are mainly controlling locomotion. All these five classes express specific transcription factors, which provide a basis for labeling different groups of neurons and altering them genetically. This thesis focuses on two of the ventral classes: V2a interneurons that express the transcription factor Chx10 and motoneurons that express Hb9 (Fig. 2).
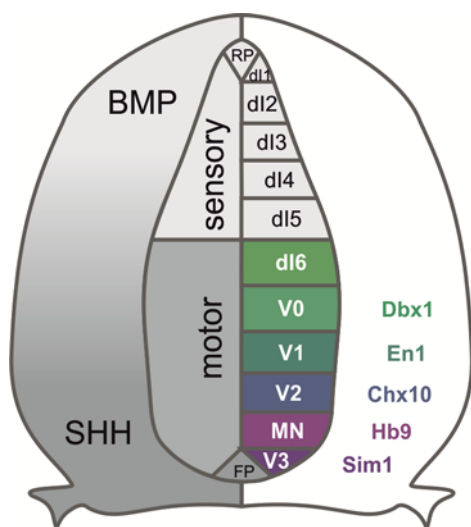
Figure 2: Early spinal cord progenitors

Schematic cross section of the embryonic zebrafish spinal cord. Illustrated are the dorsoventral postmitotic divisions that form during development. Opposing gradients of SHH and BMP provide instructive signals to dividing progenitors. dl1 to dl6 derive from dorsal progenitors. Those neurons contribute primarily to sensory pathways, while dl6, MN and V0-V3 neurons are involved in the locomotor circuitry. Some of the postmitotic transcription factors are indicated on the right side.

1.3   THE ZEBRAFISH MOTOR SYSTEM

The zebrafish as a model system in neuroscience research has been studied for many years. Emphasis has been placed on two discrete developmental stages: the embryo/young larvae and the juvenile/adult zebrafish. These are two distinct states of functional motor

systems that partially show differences in organization. In the following passage focus will lie on these two developmental stages and their similarities and differences in function and organization.

1.3.1 V2a interneurons

One important class of excitatory interneurons in the spinal cord is the V2a interneurons. V2a neurons arise from the p2 progenitor domain and are characterized by the expression of the transcription factor *alx*, which is a homologue of the mammalian *Chx10*. They project ipsilaterally over several segments and are excitatory glutamatergic (Kimura, Okamura et al. 2006). Paired recordings between *alx* neurons and motoneurons have shown that there are frequent monosynaptic excitatory connections onto motoneurons. Thus, V2a interneurons are likely premotor interneurons that regulate motoneuron activity during swimming (Kimura, Okamura et al. 2006). In zebrafish, V2a interneurons are recruited incrementally with increasing swimming speed. In larvae, the frequency of swimming at which a neuron is recruited is coupled to its position in the spinal cord. Ventral neurons are recruited first and subsequently more dorsal neurons are recruited during faster swimming (Fig. 3). While one set of interneurons is activated during strong, high frequency movements, those activated during weaker movements are silenced. This topographic map of function underlies the ability of zebrafish to produce movements over a wide range of speeds (McLean, Fan et al. 2007). In contrast, in juvenile/adult zebrafish, the order of recruitment is not imprinted by the topography or the input resistance of the V2a interneurons (Fig. 3). In fact, it is determined by scaling the effect of excitatory synaptic currents by the input resistance (Ausborn, Mahmood et al. 2012).

Embryonic zebrafish   fast / slow   Adult zebrafish
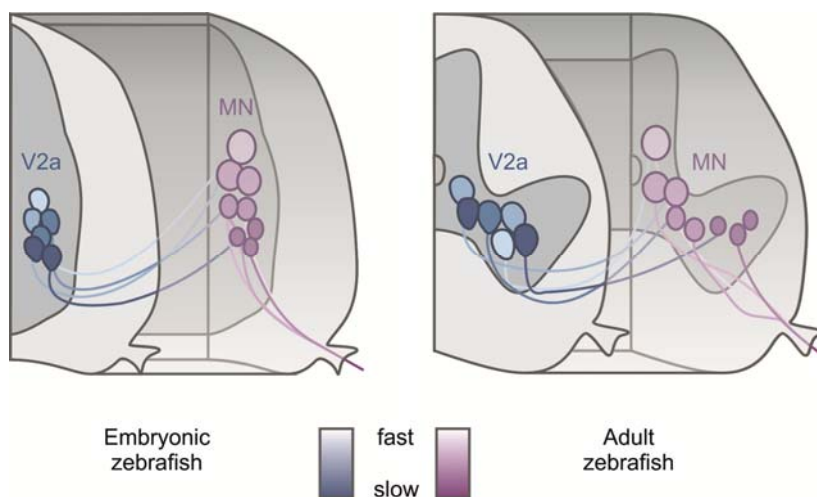
Figure 3: Recruitment order of V2a interneurons and motoneurons

Schematic overview of the recruitment order for V2a INs and MNs in embryonic and adult zebrafish. While in larvae V2a INs are topographically organized from ventral to dorsal depending on their recruitment frequency, they don't show a topographic correlation in adults. MNs, however are topographically organized in both developmental stages. Spinal neurons are organized in modules, where fast INs project to fast MNs and slow to slow accordingly.

In the lamprey and *Xenopus* tadpole spinal cords, a class of neurons has been described that are the source of ipsilateral excitatory drive necessary for the generation of swimming movements (Roberts, Soffe et al. 1998; Grillner 2003; Roberts, Li et al. 2010). These neurons were molecularly undefined, but are congruent with V2a interneurons in their morphological features: they are glutamatergic excitatory, rhythmically active, ipsilaterally projecting neurons that monosynaptically excite motoneurons and receive reticulospinal input (Buchanan and Grillner 1987). Based on this information, it can be concluded that V2a interneurons fulfill some criteria of being the source of ipsilateral excitatory drive necessary for the generation of swimming movements. The purpose of study 1 is to test this hypothesis in zebrafish.

1.3.2   Motoneurons

Motoneurons represent the final stage of neuronal processing. They project outside the spinal cord to innervate and control muscle fibers and produce movement. In zebrafish, motoneurons can be divided into two classes, primary and secondary, depending on the time of their differentiation, soma size and position.

Primary motoneurons (pMN) have large cell bodies and axons and are situated comparatively dorsal, in the middle third of the spinal cord. Their axons loop medially around the ipsilateral Mauthner axon and then pass laterally and caudally to exit the spinal cord at the ventral root (Fig.4). They have elaborate dendritic arborizations that extend to the lateral margin of the spinal cord (Myers 1985). The muscles on either side of each body segment of the zebrafish are innervated by three to four individually identifiable primary motoneurons. These MNs are recognizable from animal to animal based on their consistent morphology and are named according to their rostrocaudal position: RoP (rostral primary), MiP (middle primary) and CaP (caudal primary) (Westerfield, McMurray et al. 1986). A fourth pMN was initially thought to be an interneuron, since it lacked peripheral processes (Myers 1985). Later on, it was shown that the fourth big soma, which is only present in slightly less than half of the trunk hemisegments, has at least two different fates: it may become a pMN and arborize in an exclusive muscle territory, or it may die during embryonic development (Eisen, Pike et al. 1990). In the former case it would be named VaP (variable primary (Eisen, Pike et al. 1990)) or vRoP / dRoP (ventrally / dorsally projecting rostral primary (Menelaou and McLean 2012)). All primary motoneurons innervate cell-specific subsets of contiguous muscle fibers in mutually exclusive regions of their own body segment (Westerfield, McMurray et al. 1986) (Fig. 4).

Secondary motoneurons (sMN) have comparatively small axons and cell bodies that reside in the ventral part of the spinal cord. Their axons run directly caudally and laterally to the ventral root and are always lateral to the Mauthner axon (Fig. 4, left). Contrary to primary motoneurons, sMNs make no connections with the Mauthner axon and have only a few fine dorsally extending dendrites. With an approximate number of 20 sMNs per hemisegment they outnumber the pMN count by far and are not identifiable as individuals (Myers 1985).

Besides morphological differences in the spinal cord, pMNs and sMNs differ in the muscle type they innervate. While pMNs always innervate fast muscles, sMNs differ in their innervation and are classified as fast, intermediate and slow motoneurons, depending on if they innervate fast, intermediate or slow muscles, respectively. This classification results in four distinct pools of motoneurons (Ampatzis, Song et al. 2013). These pools are organized somatotopically with a defined location in the motor column that relates to the muscle type they innervate. In adult zebrafish, slow motoneurons are located ventro-laterally, intermediate motoneurons have a ventro-medial location and fast motoneurons are located medio-dorsally in the spinal column (Fig. 3). In larval zebrafish, however, all MNs lie in a continuum that is organized from ventral to dorsal, but without the medio-lateral extent. There is no clear segregation into distinct pools at this stage, since all neurons innervate embryonic fast muscles only.

During swimming in adult zebrafish, the different motoneuron pools are recruited in a stepwise manner from slow to fast to cover the full range of locomotor frequencies seen in intact animals (Ampatzis, Song et al. 2013). To understand the rules governing recruitment of motoneurons, the size principle has been discussed as one possibility. According to this concept, motor units are recruited in an orderly manner from small, high resistance MNs to large, low-resistance MNs (Henneman 1957). However, this framework has proven to provide only half of the answer. While primary (large) motoneurons are recruited last and

only at high locomotor frequencies during escape behavior, the order of recruitment of sMNs as a function of increased swimming speed in adult zebrafish is independent of their soma size or their input resistance. The recruitment order of sMNs is instead directly related to the innervated muscle type. Slow and intermediate MNs display almost identical morphological features and input resistance, but they are still not recruited at the same frequency during swimming (Gabriel, Ausborn et al. 2011; Ampatzis, Song et al. 2013).
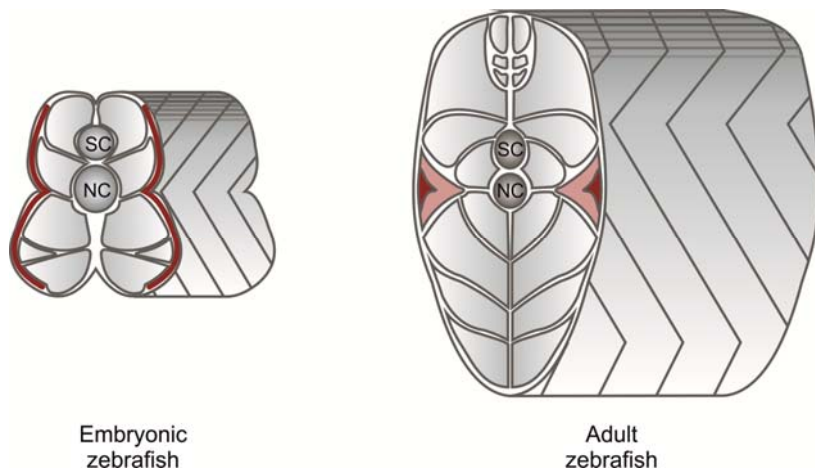
1.3.3 Muscles

In contrast to mammalian muscle, zebrafish muscle fibers are not intermingled within one muscle, but all fibers belonging to the same group are condensed to a certain area.

Newly hatched larvae possess two muscle fiber populations: a single superficial layer of embryonic red muscle fibers and white muscle fibers that constitute the majority of the myotome. In the adult zebrafish, three main types of muscle fibers have been described: superficial red, intermediate pink and deep white (Fig. 5).

Superficial red fibers are slow-contracting and fatigue-resistant with high oxidative enzyme activity. They occupy a small lateral area close to the midline of the fish. They have small round fibers with small blood capillaries and are separated from the rest of the musculature by a thin connective tissue septum (van Raamsdonk, Pool et al. 1978). Innervation to these slow fibers is provided by secondary motoneurons that lie in the most ventral part of the motor column (de Graaf, van Raamsdonk et al. 1990).

Intermediate fibers form a transitional zone between slow and fast fibers, in which the muscle fibers increase in diameter, while the mitochondrial content decreases. The intermediate zone is only a few cells thick and these fibers possess myofibrillar and metabolic properties of fast-contracting fibers with a resistance to fatigue. They are innervated by secondary motoneurons that reside in a dorsoventral position in between the fast and slow motoneurons (van Raamsdonk, Pool et al. 1978; de Graaf, van Raamsdonk et al. 1990).

The deep white fibers account for the vast majority of muscle fibers. They are fast-contracting and fatigue rapidly. These predominantly anaerobic fibers have a large polygonal cross section and very few mitochondria (van Raamsdonk, Pool et al. 1978). They are innervated by motoneurons grouped in the dorsal part of the motor column (de Graaf, van Raamsdonk et al. 1990).

There is general agreement that only the red fibers are employed at low sustained swimming speeds and that the white fibers are active during short bursts of maximum speed, which cannot be sustained for long time periods, such as the escape behavior.

1.3.4 Modular principle of recruitment

The prevailing concept of the recruitment order of motor units is based on the "size principle". This hypothesis emanates from the assumption that all motoneurons, both slow and fast, receive equal excitatory inputs from a common premotor source. The order of recruitment would then purely be a function of the input resistance. However, Ampatzis et al. have shown that the locomotor network does not consist of a uniform unit, but can be deconstructed into microcircuit modules, in which the excitatory drive to motoneurons is channeled in a specific and selective pattern according to a precise connectivity map (Gabriel, Ausborn et al. 2011; Ampatzis, Song et al. 2014). To produce locomotion of different speeds, different modules have to be activated. One module in this specific case consists of one set of V2a interneurons, motoneurons and muscles, respectively. During slow swimming, slow secondary motoneurons receive excitatory drive from slow V2a interneurons and project to slow red muscle fibers. As the swimming speed increases, the intermediate and fast microcircuit modules are recruited in a stepwise manner (Ampatzis, Song et al. 2014). Hence, the V2a interneurons are incrementally recruited during swimming and drive their corresponding motoneurons to increase the speed of locomotion. While in larvae, both the V2a INs and the MNs are organized somatotopically from ventral to dorsal with increasing speed, in adults, V2a INs are intermingled within the spinal column (McLean, Fan et al. 2007; Ausborn, Mahmood et al. 2012; Ampatzis, Song et al. 2013). This deconstruction of the locomotor network into three different microcircuit modules disproves

the previous assumption of a uniform unit that conveys equal excitatory inputs to all motoneurons. Overlapping mobilization of modules during swimming accounts for a smooth transition between different speeds by sequential activation or deactivation of the successive microcircuits (El Manira 2014).

Since all primary motoneurons exhibit equal recruitment patterns during swimming, current models would predict that they are activated in a synchronous manner. However, Bagnall et al. showed that pMNs that innervate the same muscle quadrant exhibit highly synchronous electrical postsynaptic potentials at a fine temporal scale (tens of µs), while out-of-quadrant-pairs show lower synchrony (Bagnall and McLean 2014). It was suggested that distinct premotor interneurons are the source of these gap junction potentials, however, they could arise from coupling between motoneurons themselves.

1.4 DEVELOPMENT OF THE ZEBRAFISH MOTOR SYSTEM

In paragraph 1.3 general aspects of zebrafish motor systems were discussed - with focus on two distinct developmental stages - larvae and adult. In this section the emphasis is placed on what is known about the developmental changes in between those stages.

1.4.1 Swimming development

Embryonic and larval zebrafish show a number of stereotypic motor behaviors. At 17 hpf the first movements consist of spontaneous side-to-side coiling contractions (Kimmel, Ballard et al. 1995). At that time, only a few primary motoneurons have extended their axons in the spinal cord (Kuwada and Bernhardt 1990). From 21 hpf and onwards larvae react to touch stimuli with vigorous coils and at 27 hpf they can swim at least one body length in response to touch (Saint-Amant and Drapeau 1998). At hatching, larvae swim in infrequent bursts and the typical beat-and-glide pattern begins when swim bladder and sensory systems are functional around day four to five. This beat-and-glide-pattern is characterized by discrete swimming episodes interposed between periods of passive gliding and can even be observed in the *in vitro* preparation, when fictive locomotion is induced by electrical stimulation or NMDA application (Granato, van Eeden et al. 1996; Drapeau, Saint-Amant et al. 2002; Masino and Fetcho 2005; Eklof-Ljunggren, Haupt et al. 2012).
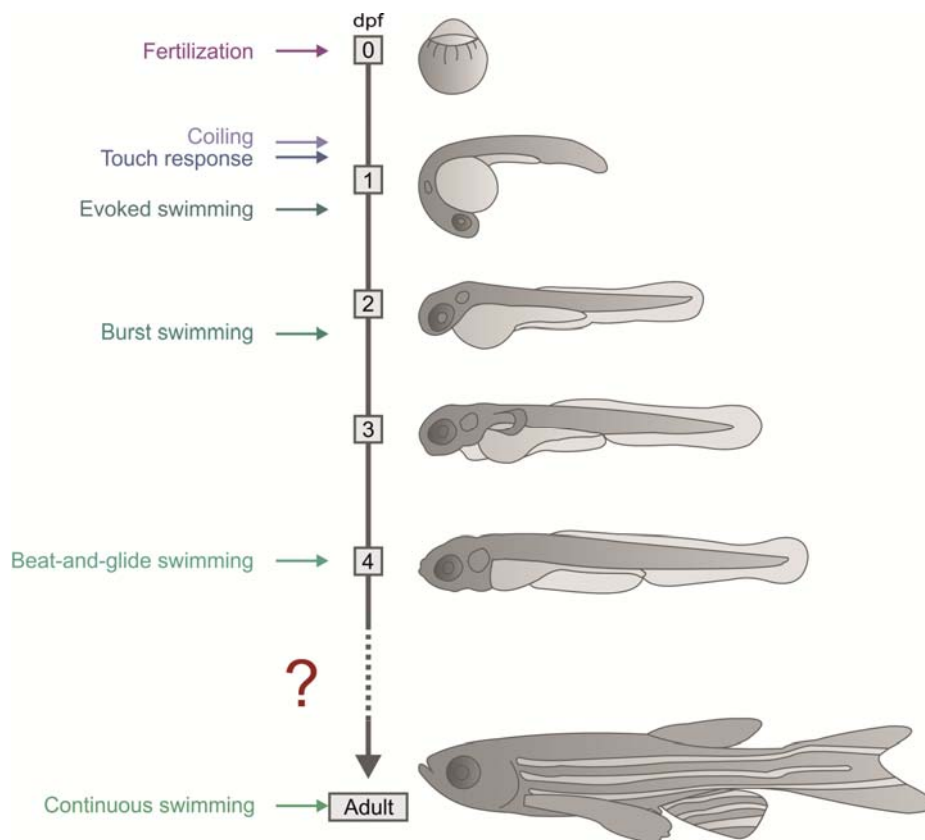
In contrast, adult zebrafish swim in a continuous way that consists of only one long bout of activity (van Raamsdonk, Mos et al. 1983; Ausborn, Mahmood et al. 2012; Ampatzis, Song et al. 2013). When the switch between those two behaviors occurs is shown in paper 3.

Each of these modifications is related to changes in the cellular mechanisms generating locomotion. Coiling is mediated by a limited electrically coupled spinal network, while the touch response occurs when chemical synaptic inputs are integrated. Integration of the premotor chemical synaptic drive is necessary for actual swimming movements (Drapeau, Saint-Amant et al. 2002). The change from beat-and-glide to continuous swimming is accompanied by the development of intermediate and adult slow red muscle fibers (see below).

1.4.2 Muscle development

All muscles derive from a structure called paraxial mesoderm, which is one part of the mesoderm during neurulation in the embryo that flanks and forms simultaneous with the neural tube. The paraxial mesoderm can be divided into medial and lateral paraxial mesoderm. The earliest stage of muscle development takes place around 11 hpf. Here, the medial paraxial mesoderm differentiates into adaxial muscle precursor cells (Devoto,

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Melancon et al. 1996; Barresi, Stickney et al. 2000; Norris, Neyt et al. 2000). One population of these adaxial cells maintains its medial position and develops into the first contractile myotomal fibers, the muscle pioneers. The other population migrates laterally and elongates to form the mononucleate superficial embryonic slow red muscle fibers (ER) (Devoto, Melancon et al. 1996). Embryonic fast white (EW) fibers are multinucleate and constitute the majority of the myotomal muscle. They differentiate from the lateral paraxial mesoderm (Coutelle, Blagden et al. 2001). When the first muscle contractions occur at 17 hpf the muscle fiber composition and organization is still very different from the adult structure, with only two fiber types present, compared to five (thereof three main) later in development (Drapeau, Saint-Amant et al. 2002). The morphology of the myotome also undergoes big changes: from the typical embryological chevron-shape (V) to the more complicated W-shape in adult fish (Fig. 5). This shape is optimal for producing the powerful undulating swimming movements (Mos and Vanderstelt 1982).

When the first coiling movements are observable around 17 hpf, the first primitive myofibrils occur and primary motoneurons transmit to muscle pioneers (Raamsdonk, van der Stelt et al. 1974). EW fibers are innervated soon after that and at 26 hpf secondary motoneurons start their innervations simultaneously with the appearance of the undulatory swimming behavior (Myers, Eisen et al. 1986).

None of the adult muscle fiber types (red, pink, white) are present in the embryonic stage; in fact the differentiation of these fibers occurs within the first four weeks after fertilization (van Raamsdonk, van't Veer et al. 1982). Just hatched larvae contain embryonic red (ER) superficial fibers as well as polygonal deep white. Both of them arise from an embryonic cell population. Most likely only the deep white fibers have a function for the motility of the early larvae (van Raamsdonk, van't Veer et al. 1982). Out of the muscle fiber types in the adult musculature, the white fast-fatigue fibers appear first. By the time the larvae have consumed their yolk they need to get active in search for food, which calls for a change in muscle fiber composition. At this point intermediate and adult red fibers are formed. While intermediate fibers originate from EW, adult red fibers arise as a new population (van Raamsdonk, van't Veer et al. 1982).

1.4.3 Motoneuron development

As the name suggests, primary motoneurons are the first to appear in development. They undergo their final round of DNA synthesis between 9 and 16 hpf and start being visible at 15 hpf. The first primary MN is always the CaP, followed by the other primaries (Myers, Eisen et al. 1986; Eisen, Pike et al. 1990). The first axon reaching the ventral root is also

send out from the CaP at 17 hpf and followed by axons from the MiP and the RoP within the next two hours (Myers, Eisen et al. 1986). All of the three pMN axons follow a common pathway on the medial surface of the myotome until they reach adaxial muscle pioneers at the horizontal myoseptum, where they pause before making their cell-specific divergent pathway choices and grow towards their specific muscle innervation targets (Fig. 4) (Drapeau, Saint-Amant et al. 2002). As the axons elongate, pMN somata migrate dorsally from their initial ventral position. At 2 dpf they stop actively moving and reach their temporal larval position, making more room for the ventrally located sMNs (Myers, Eisen et al. 1986). The Mauthner growth cone enters the spinal cord after all the pMNs of the trunk have begun their axonal outgrowth and passes by before the secondary motoneuron growth cones appear, which might explain why only pMNs have synaptic connections to the Mauthner axon (Myers, Eisen et al. 1986).

The first secondary motoneurons are born around five to six hours after the primary motoneurons and continue to be born until at least 25 hpf. Ten hours after the primary axogenesis, sMNs start their wave of axonal outgrowth towards the ventral root, before branching into the muscle mass (Myers, Eisen et al. 1986).

The adult number of motoneurons is reached in an early larval stage, even before the differentiation of muscle fiber types is completed (van Raamsdonk, Mos et al. 1983). At that time though, the motor column extends only dorsoventrally and is only one to two cell bodies thick. Despite the fact that even at early stages dorsally located motoneurons innervate fast muscles and ventrally located innervate slow muscles, all motoneurons lie in a continuum without clear borders between the groups. In the adult zebrafish these groups become more and more spatially segregated, as the motor column extends laterally (van Raamsdonk, Mos et al. 1983; Devoto, Melancon et al. 1996; Ampatzis, Song et al. 2013).

2 AIMS

The overall aim of this thesis is to investigate the mechanisms underlying locomotion in zebrafish, with emphasis on excitatory drive and development.

The specific aims addressed here are

1. To show that V2a interneurons represent an intrinsic source of excitation that is *necessary* for the normal expression of the locomotor rhythm.

2. To demonstrate that the excitatory drive from ipsilateral premotor V2a interneurons is *sufficient* to produce a swimming pattern that is chracterized by the typical left-right and rostrocaudal coordination.

3. To reveal the developmental changes in the swimming pattern and motoneuron properties.

3 METHODS

3.1 ZEBRAFISH PREPARATION FOR ELECTROPHYSIOLOGY

Larval zebrafish were used for most experiments herein. For extracellular recordings, animals were anaesthetized in MS-222 and pinned down in a Sylgard-lined chamber with two to three thin tungsten pins. The skin was removed from the trunk musculature to expose the intermyotomal clefts and the fish were treated with α-bungarotoxin to block neuromuscular junctions in order to immobilize the fish, before starting the experiment. For intracellular recordings, one to two epaxial muscle segments were carefully removed from the midbody region after the skin was separated to expose the spinal cord. Fish were then constantly perfused with curare instead of a short exposure to α-bungarotoxin.

In study III, juvenile fish were used in addition. Stimulation at the otic vesicle was not possible for larger fish due to ossification, so the skull was removed to be able to place the stimulation electrode directly on the nervous tissue. Skin and muscle were removed just like in larvae.

3.2 ELECTROPHYSIOLOGY

3.2.1 Extracellular Recordings

The recording chamber was continuously perfused with extracellular saline solution. In larvae, fictive swimming activity was induced by electrical stimulation of the otic vesicle, while in older animals the stimulation electrode was positioned directly on the nervous tissue at the border between the brainstem and the spinal cord. In addition to electrical stimulation, channelrhodopsin-driven light activation or bath application of NMDA were also used to induce fictive locomotion. Motor activity was recorded from the motor nerves running through the intermyotomal clefts.

3.2.2 Intracellular Recordings

Intracellular whole-cell recordings from identified neurons were performed in study II and III. For this, patch-clamp electrodes were pulled from borosilicate glass and filled with intracellular solution. Neurons were visualized with a microscope equipped with infrared-DIC optics and a camera.

3.3 TRANSGENIC ANIMALS

For study I, two transgenic lines were used. In line Tg[Chx10:GFP], GFP expression is driven by the Chx10 promotor, which selectively labels V2a interneurons with GFP (Kimura, Okamura et al. 2006). The Tg[GlyT2:GFP] line expresses GFP in glycinergic interneurons instead.

For study II, four transgenic zebrafish lines were used that were crossed based on the Gal4/UAS system. This system comprises of two parts: the driver line, which has the Gal4 gene under a tissue-specific promoter and the reporter line with UAS (upstream activation sequence), coupled to a reporter gene. When these two lines are crossed Gal4 binds to UAS and activates transcription of the reporter gene in the specified tissue (Fig. 7).
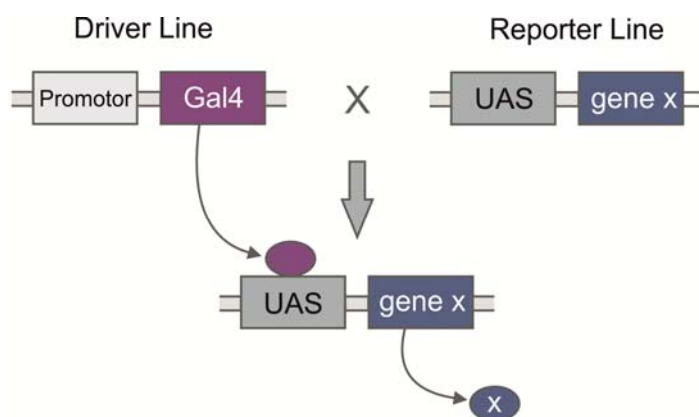
Figure 7. Gal4/UAS System

The driver line uses the *gal4* gene under a tissue-specific promoter. The reporter line has an upstream activation sequence, coupled to a reporter gene. Crossing of the lines results in Gal 4 binding to UAS and activation of the transcription of the reporter gene in the specific tissue.

We used the following lines: Gal4^{s1011t} (Et[-1.5hsp70I:Gal4-VP16]s1011t), in which Gal4-VP16 was expressed in circumferential ipsilateral descending (CiD)-like interneurons (Scott, Mason et al. 2007). This line was used together with three different UAS lines. Crossing the s1011t line with UAS:GFP yields in zebrafish expressing GFP in CiD (V2a) interneurons. Crossing with the UAS:Kaede^{s1999t} (Tg[UAS-E1b:Kaede]s1999t (Davison, Akitake et al.

2007)) and UAS:ChR2-mCherry (Tg[UAS:ChR2(H134R)-mCherry]s1986t (Schoonheim, Arrenberg et al. 2010)) lines, results in expression of the photoconvertible protein Kaede or Channelrhodopsin, respectively in CiD-like V2a interneurons.

3.4 PHOTO ABLATION

In order to ablate neurons in the spinal cord, the zebrafish larvae were anesthetized and subsequently embedded in low-melting agarose. A two-photon laser was then used to ablate a certain number of fluorescently marked neurons. This number was determined by a balance between sufficiency and necessity. Enough cells needed to be ablated to see an effect, without changing the phenotype by cytotoxic effects from the dead cells. After ablation the fish were released from the agarose and left to recover from the procedure.

3.5 OPTOGENETICS

In study II, swimming activity was elicited by using light stimulation (blue light; 465 nm) delivered by a custom-made LED system. For extracellular recordings, the LED was placed under the fish, illuminating the whole animal. For intracellular recordings, the LED was attached to the eye-piece of the microscope in order to only illuminate certain parts of the spinal cord. Illumination of channelrhodopsin triggers conformational changes in the ion channel that allows cations to travel through the membrane and depolarize the cell.

3.6 IMMUNOHISTOCHEMISTRY

Larval zebrafish were fixed in 4% PFA, washed in PBS containing 0.1% Triton X-100, and blocked with 1% Western Blocking Solution for 30 min before incubation with the primary antibody overnight at 4°C. After washing in PBS, larvae were incubated with the secondary antibody overnight at 4°C, washed 6 x 20 min with PBS, and mounted on glass slides in 75% glycerol solution (in PBS) before imaging with a confocal microscope.

3.7 NEUROBIOTIN STAINING

In order to reveal morphological characteristics of specific cells, single neurons were filled with 1 % neurobiotin tracer through patch-pipettes. After filling of the cell, the animals were fixed in 4 % PFA overnight at 4 °C and subsequently washed with PBS. After further dissection of epaxial and hypaxial muscles to expose the spinal cord, the fish were incubated with PBS containing 0.5 % Triton X-100 for 3 h at RT. Incubation with Streptavidin, coupled to a fluorescent dye was carried out overnight at 4 °C. After several washes in PBS, the fish were mounted with Vectashield on gelatinized slides and viewed with a laser scanning confocal microscope.

3.8 BACKFILLING OF NEURONS

In order to identify certain neurons in the spinal cord, fluorescently labeling them is essential. When not using transgenic lines, backfilling was used to mark either V2a interneurons or motoneurons. Small crystals of different dextrans were diluted in deionized water and taken up with the tip of custom-made tungsten pins or patch pipettes. Zebrafish were anaesthetized with MS-222. For filling of motoneurons the dye was applied to the lateral skin, which was then punctured with a sharp tungsten pin to damage the muscle and severe motor axons, which take up the dye. The fish were then left to recover for at least 1 hour (young larvae) or overnight (juveniles) before further processing. Long projecting interneurons were backfilled by severing axons in the caudal part of the larvae. For this purpose, skin and 1-2 muscle segments were removed, so that the spinal cord could be entered with a dye-filled patch-pipette. Larvae were then left to recover and to allow transportation of the dye into the rostral cell bodies for at least 1 hour.

4   RESULTS AND DISCUSSION

4.1   PAPER I: Origin of Excitation Underlying Locomotion in the Spinal Circuit of Zebrafish

In all animals, movements are controlled by central pattern generator networks that enable the appropriate activation of muscles in a precisely coordinated manner (Grillner 2003; Selverston 2005). The particular CPGs responsible for different rhythmic movements can be found in many different neural structures. The vertebrate CPG for locomotion is located in the spinal cord and is controlled by descending inputs from specific locomotor command regions in the brainstem (Grillner 2006). To produce swimming, three different modules need to interact with each other. One system that coordinates left-and-right-alternation, one that provides the excitatory drive and one that constitutes the output to the muscles (Fig. 1). Alternation between the left and right side of the body is ensured by crossed inhibition from glycinergic interneurons that inhibit all neurons on the contralateral side (Buchanan 1982). The drive is provided by excitatory interneurons that are rhythmically active and project ipsilaterally to both motoneurons and inhibitory interneurons. They are the main source of excitation and believed to be the core components for the generation of the locomotor rhythm (Buchanan and Grillner 1987; Grillner 2003; Kiehn 2006; Fetcho, Higashijima et al. 2008).

The molecular identity of these excitatory interneurons was unclear though. Reasonable candidates were the V2a interneurons that are characterized by Chx10 expression, since they fulfill some criteria of being the excitatory drive: They project ipsilaterally over several segments and are glutamatergic excitatory (Kimura, Okamura et al. 2006). They also make monosynaptic connections onto motoneurons. Thus, V2a interneurons are likely premotor interneurons that regulate motoneuron activity during swimming (Kimura, Okamura et al. 2006).

To understand the contribution of specific interneurons to locomotion, an important entry point is to remove them from the spinal circuits. Accordingly, in the first part of the thesis we acutely and selectively ablated these interneurons to determine their effect on the swimming behavior. To this end, we used a zebrafish line that expresses GFP specifically in the V2a interneurons (Chx10:GFP). These labeled neurons could now be selectively ablated using a two-photon laser microscope. We ablated around 30 % of the labeled neurons across 10 segments in the midbody region of 4 to 5 days old larvae, which corresponds to ~15 out of

~50 cells. This number was the result of an optimization of both effect and survival of the fish.
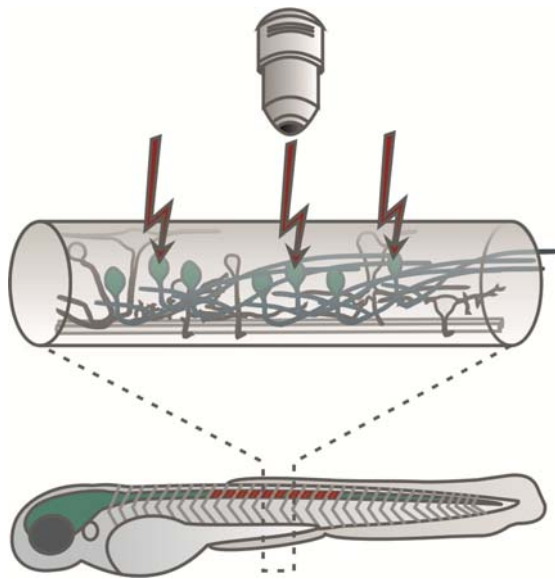
Figure 8: Laser ablation of V2a interneurons

Experimental setup for laser ablation of V2a interneurons.

Over 10 segments 30% of labeled V2a interneurons were ablated using a two-photon-laser.

By stimulating descending tracts, we then induced fictive locomotion and recorded from peripheral nerves in two areas along the rostrocaudal axis of the fish. In the ablated fish, the stimulation strength had to be increased six-fold compared to control fish in order to elicit locomotion. Also, the duration of the episodes produced by this stimulation was significantly shorter, containing fewer bursts and lower frequencies. This means that fish with ablated V2a interneurons are unable to swim with the high frequencies the control fish are capable of. We showed that V2a interneurons are important for normal swimming behavior, however, they might not be responsible for the effect we saw. Instead, they could also just relay descending information to other neurons, which would be deprived of their input upon V2a ablation.

To test this hypothesis, we substituted the descending input with NMDA and spinalized the larvae. Again, to induce locomotion we had to use higher concentrations of NMDA compared to control fish and in most cases rhythmic activity could only be elicited caudal to the ablated area. Also in NMDA the swimming frequencies were decreased. The fact that similar results were obtained with both methods indicates that V2a interneurons are not only relaying descending input, but are directly responsible for the observed changes.

To make sure that the effects are not due to unspecific cytotoxicity, we repeated the experiments, but ablated a different set of neurons. We used a zebrafish line expressing GFP in glycinergic interneurons and targeted those with the two-photon laser in the same way as the V2a interneurons. Ablating these neurons had only little effect on the excitability, indicating that the effects we saw when ablating V2a interneurons were specific and not a general cytotoxic effect.

After assessing the change in frequency, we investigated the effect on coordination. V2a interneurons send long descending axons that project over several segments, which makes them likely targets to participate in intersegmental coordination. Indeed, in fish with V2a ablations, the phase lag between rostral and caudal segments was increased from two to four percent per segment.

Based on our results, we propose that V2a interneurons contribute to the excitatory drive in the spinal locomotor circuit. Even partial loss of these neurons (1) increases the stimulation threshold needed to induce swimming, (2) decreases the swimming episode duration, (3) decreases the swimming frequency and (4) changes the timing of intersegmental coordination (Fig.9).

Figure 9: Changes in fish that have undergone V2a ablation

After ablation of V2a interneurons, larval zebrafish show alterations in their swimming network.

Purple upward arrow: increase

Blue downward arrow: decrease

Grey bar: no change

Threshold to induce swimming

Swimming episode duration

Swimming frequency

Cycle duration

Left-right-alternation
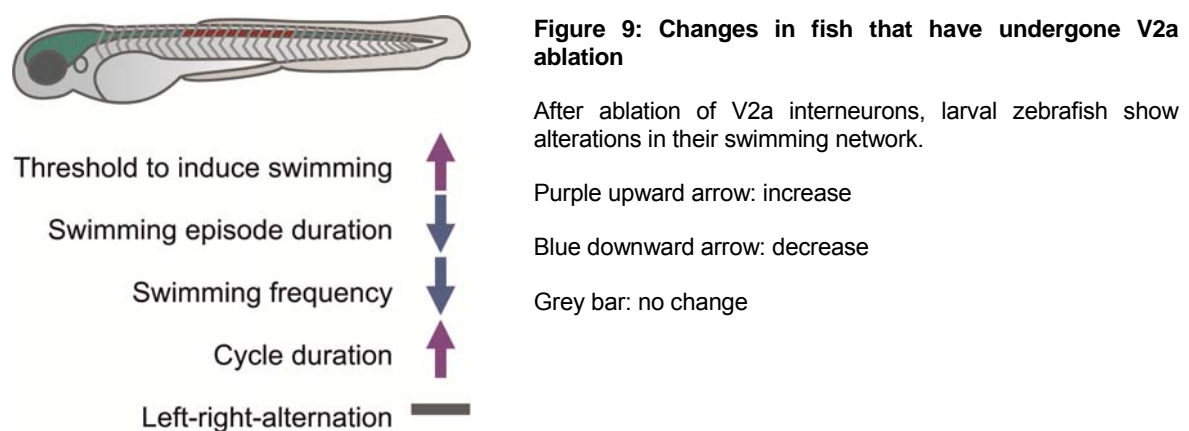
The connectivity of V2a interneurons has been shown to be similar to that in lamprey and tadpole swimming circuits (Buchanan and Grillner 1987; Buchanan, Grillner et al. 1989; Roberts, Li et al. 2008; Berkowitz, Roberts et al. 2010; Roberts, Li et al. 2010). In addition, anatomical substrates for connections between V2a interneurons and motoneurons have been found in rodents (Lundfald, Restrepo et al. 2007; Stepien and Arber 2008).

V2a interneurons are considered to serve as the main source of on-cycle excitation and represent important candidates for the excitatory interneurons driving the locomotor generating circuit (Kiehn 2006; Fetcho, Higashijima et al. 2008; Grillner and Jessell 2009). However, in newborn mice genetic elimination of V2a interneurons had little impact on drug-induced rhythm generation in the spinal cord (Crone, Quinlan et al. 2008; Crone, Zhong et al. 2009; Kiehn, Dougherty et al. 2010), but seemed to be more involved in left-right-coordination at medium to high locomotor frequencies (Crone, Quinlan et al. 2008; Dougherty and Kiehn 2010). These findings lead away from the theory that V2a interneurons are essential for normal rhythm generation and are quite contrary to our results, which show changes in coordination and frequency, but not in left-right-alternation. One possible explanation for the difference in the findings is that the locomotor organization might differ

between rodents and fish, which would not be a surprise, given the fact that rodents mostly use their limbs to locomote, while fish use their whole body. It is for example known that salamanders, which have both modes of locomotion, use a central pattern generator for swimming and later develop the limb CPG that is coupled to the body CPG (Ijspeert 2001; Bem, Cabelguen et al. 2003). V2a interneurons could thus play an important role in the swimming CPG, but not in limbed locomotion. Another possibility is that elimination of V2a interneurons early during development induces compensatory mechanisms. Other unknown neurons taking over parts of the duty of the V2a interneurons might mask the potential effect on locomotor frequency, when locomotion is induced pharmacologically. Furthermore, the generation of the rhythm underlying locomotion in mice could involve overlapping classes of interneurons. Recently it was found that Shox2 interneurons are important for the rhythmogenic activity of the locomotor network in mice. This group of interneurons includes several subclasses from different progenitor domains and shows major overlap with the V2a interneurons (Dougherty, Zagoraiou et al. 2013). Hence eliminating one of those overlapping classes might not be sufficient to prevent the expression of rhythmic activity in the spinal cord (Grillner and Jessell 2009; Brownstone and Bui 2010).

Overall, the effect of ablating V2a interneurons on the excitability of the swimming circuit argues that they are important and necessary for the generation of a normal locomotor pattern.

4.2 PAPER II: Optogenetic Activation of Excitatory Premotor Interneurons is Sufficient to Generate Coordinated Locomotor Activity in Larval Zebrafish

In the previous study (paper 1) we ablated V2a interneurons in the larval zebrafish and showed that they are an important source of excitation in the spinal cord and necessary for a normal expression of the locomotor pattern. In this study we sought to assess their sufficiency for the generation of locomotion using an optogenetic approach.

To determine if V2a interneurons could be specifically and directly activated, we required zebrafish that express channelrhodopsin in the V2a interneurons. Channelrhodopsin is a photoactivatable cation-selective ion channel that opens upon blue light illumination and depolarizes the cell. For our experiments, we used a line that expresses Gal4 in circumferential ipsilateral descending (CiD)-like interneurons, which has been obtained from an enhancer trap screening (Scott, Mason et al. 2007). In order to ensure that Gal4 was expressed only in V2a interneurons, we crossed this line with reporter lines having UAS coupled to GFP or Kaede, which both are green fluorescing. Marked interneurons resembled the typical morphology of V2a cells with their long descending axons (Bernhardt, Chitnis et al. 1990; Kuwada and Bernhardt 1990; Hale, Ritter et al. 2001). To further ensure the identity of the marked neurons, we backfilled motoneurons, but did not find any overlap, assuring that there is no leak of expression to motoneurons. Lastly, we performed immunohistochemistry with an antibody against the transcription factor Chx10, which is expressed in V2a interneurons exclusively (Kimura, Okamura et al. 2006) and found that all neurons expressing Kaede also expressed Chx10. These experiments assured that Gal4 was expressed in V2a interneurons exclusively.

To test if we could activate these cells, we crossed the Gal4-line with a line carrying Channelrhodopsin (ChR2) under an upstream activator sequence together with mCherry, which made it possible to visualize positive interneurons (Fig. 10).

We first determined if illumination with blue light (465 nm) could depolarize the interneurons in zebrafish larvae, using whole cell patch clamp recordings. Upon application of a short light pulse of increasing strength, the membrane potential depolarized gradually until an action potential was induced. In some cells, activation of ChR2 also elicited slow excitatory synaptic responses, suggesting that the V2a interneurons form an excitatory network.

Next, we investigated if rhythmic activity could be induced. When applying a longer light pulse, the V2a interneurons displayed rhythmic oscillations superimposed on a tonic depolarization, which resembled the typical beat-and-glide swimming. After blocking AMPA- and NMDA-receptors, these oscillations were completely abolished. These results indicate

that activation of V2a interneurons is sufficient to provide the necessary excitation that underlies rhythm generation.
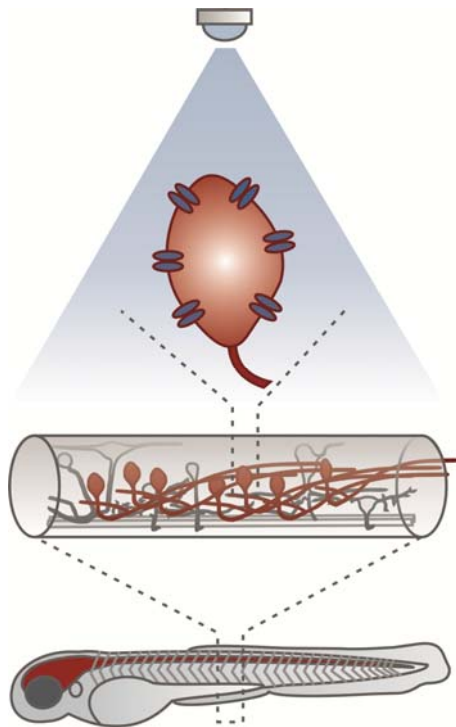
Figure 10. Optical activation of V2a interneurons

Experimental setup for optogenetic activation V2a interneurons.

V2a interneurons express Channelrhodopsin coupled to mCherry specifically. mCherry helps visualizing and targeting cells in the larval zebrafish spinal cord. Blue light activates Channelrhodopsin.

To investigate if the bursting activity in single neurons corresponds to swimming activity, we performed peripheral nerve recordings from spinalized larvae. Illumination of the whole fish induced an episode of fictive swimming, characterized by left-right-alternation and rostrocaudal delay, typical of a normal swimming behavior. This indicates that swimming activity indeed emerges from the activity of an underlying V2a interneuron network.

It has previously been shown that swimming activity can be induced by optical stimulation of Kolmer-Agduhr (KA)-cells, which are ciliated GABAergic neurons that contact the central canal of the spinal cord and have ipsilateral ascending axons (Wyart, Del Bene et al. 2009). To determine if optogenetically induced swimming activity was dependent on GABA$_A$ receptor activation, we bath-applied the specific antagonist gabazine, but could not detect any effect on the swimming. Both frequency and episode duration remained constant, indicating that GABA$_A$ receptor activation is not necessary for the swimming activity, induced by optical stimulation of V2a interneurons.

Locomotor activity is thought to not only be the result of ipsilateral glutamatergic excitation, but also crossed glycinergic inhibition, which ensures left-right-alternation. If activation of V2a interneurons is sufficient to produce basic rhythmicity, this should be possible even in the absence of inhibition. To test this, we blocked glycinergic transmission by strychnine application in spinalized animals, which lead to synchronous slow bursting that occurred simultaneously in left and right motor nerves. These results indicate that the excitatory drive

provided by V2a interneurons is sufficient to produce episodic motor activity even in the absence of crossed inhibition.

In paper 1, we showed that acute ablation of V2a interneurons decreases the excitability of the locomotor network and reduces swimming frequency, suggesting that they are necessary for a normal locomotor pattern. Here, we show that V2a interneurons are also sufficient to drive the activity of the whole locomotor circuit.

Swimming activity induced by optical stimulation is based on the activation of ionotropic glutamate receptors, but does not involve $GABA_AR$ activation. Wyart et al. showed that optical activation of Kolmer-Agduhr cells initiates swimming in larval zebrafish, depending on $GABA_AR$ activation. This might be explained by the fact that at early development the reversal potential for chloride is still more depolarized, leading to a depolarization of other neurons, for example V2a interneurons. They further showed that swimming appears as a rebound after termination of the light stimulation. V2a interneurons could therefore be inhibited by GABA from Kolmer-Agduhr cells and display rebound firing to activate the spinal network, suggesting that V2a interneurons work as a downstream targets for KA cells.

Excitatory interneurons within the locomotor network have been the target of many studies. In lamprey, lesion studies revealed that excitatory interneurons mediate a basic rhythmic activity (Cangiano and Grillner 2005). In *Xenopus* it has been shown that descending excitatory neurons in the brainstem make reciprocal synapses onto each other (Li, Soffe et al. 2006; Li 2011). It has also been shown in zebrafish that optical activation of these neurons is able to elicit locomotion (Kimura, Satou et al. 2013). Furthermore, in the mouse, optical activation of excitatory interneurons in the spinal cord or hindbrain induced a locomotor rhythm (Hagglund, Borgius et al. 2010). However, only now have we been able to identify these excitatory interneurons as V2a interneurons. Taking into account how conserved these neurons are across species it is possible that their function is similar in other vertebrates.

The identification of V2a interneurons as necessary and sufficient for initiating locomotor activity contributes importantly to our understanding of the neuronal processing underlying motor behavior.

4.3   PAPER III: Developmental Changes in Locomotor Activity in Zebrafish

In zebrafish, swimming is produced by left-right-alternation of axial muscles that are activated incrementally by pools of motoneurons. While larvae swim in a beat-and glide-pattern that is characterized by episodes of swimming intermingled with gliding episodes, adult zebrafish swim in a continuous fashion (Fig.11) (Bone, Kiceniuk et al. 1978; van Raamsdonk, Mos et al. 1983; Granato, van Eeden et al. 1996; Buss and Drapeau 2001; Masino and Fetcho 2005; Ausborn, Mahmood et al. 2012; Eklof-Ljunggren, Haupt et al. 2012). The muscles on either side of each body segment are innervated by three to four primary motoneurons. In larvae, these motoneurons fire action potentials during swimming, while in adults they only contribute to escape behavior (Westerfield, McMurray et al. 1986; McLean, Fan et al. 2007; Ampatzis, Song et al. 2013). Overall, a lot is known about swimming and motoneuron properties in very young larvae (3-7 dpf) and young adults (> 6 wpf). What remains unclear is when and how the swimming pattern and primary motoneuron recruitment change from the larval to the adult mode (Fig. 11). By means of systematic electrophysiological recordings we aim to define the time point of the switch in the swimming pattern and motoneuron recruitment.
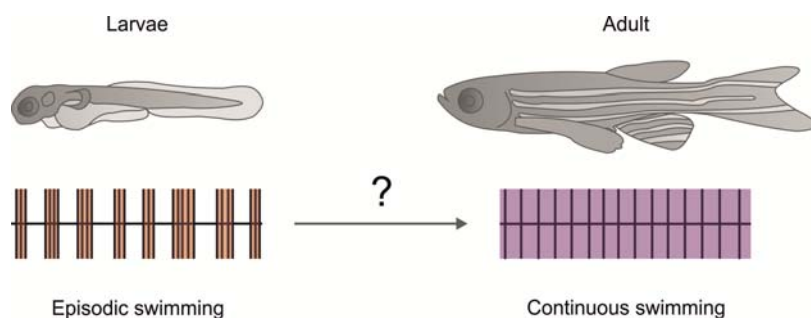
Figure 11. Swimming during development

While zebrafish larvae swim in a beat-and-glide-pattern, adults swim continuously without inter-episode breaks. When the switch between the 2 modes occurs needs to be determined.

To explore the timeline of the change in swimming pattern, we recorded from motor nerves at different stages of development, starting from 3 dpf up to > 6 wpf in order to span the whole range of swimming behaviors. Until 3 weeks of development, all fish were swimming in the typical larval beat-and-glide pattern. At 4 weeks of development, some fish started swimming in a continuous pattern. This number increased to ~80% at 5 weeks and at 6 weeks, all of the recorded fish were swimming in the adult pattern that consists of a single long swimming bout. These results suggest that the switch from larval episodic to adult continuous swimming takes place around 4 to 5 weeks of age.
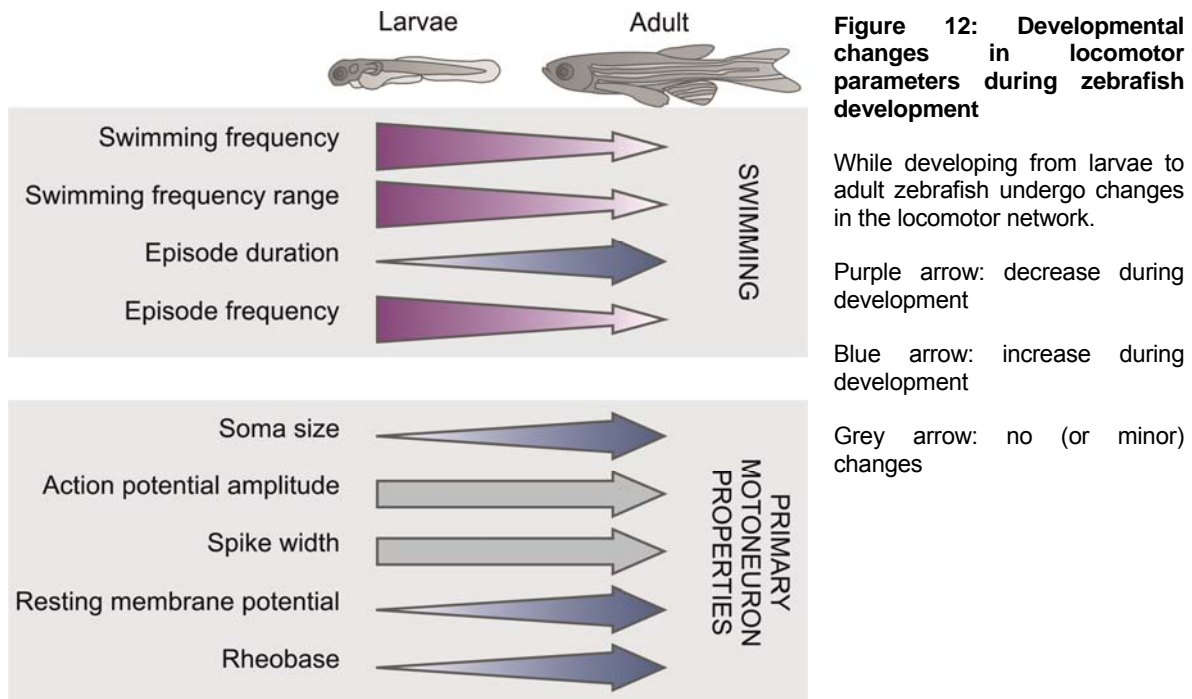
While the actual pattern of swimming changed drastically within a short time period, other swimming-related parameters undergo more smooth changes. The frequency decreases gradually from a mean of 42 Hz at 3 dpf to 5 Hz at 6 wpf. Additionally, the range of frequencies that the fish are able to swim decreases. When changing from an episodic to a

continuous swimming pattern, one parameter that might play a role is the duration of single swimming episodes. While it could be expected that swimming episodes increase in favor of gliding episodes, until a single long swimming bout is generated, this is not the case. Both, the swimming and gliding increase in length and are around 4-5 weeks abruptly replaced by the adult long swimming bout.

Next, we examined the primary motoneurons, as they could give a hint as to why the swimming pattern changes. Morphological characterization revealed that soma size increased steadily with development and is roughly 3 times as big in 6 week old fish, compared to 4 dpf old fish. The dendritic trees of the examined pMNs undergo similar developmental changes and grow in proportion to the soma. These changes are accompanied by a general hypertrophy of the whole spinal cord. During development, pMNs alter their relative position within the spinal cord: from being more dorsal and lateral at 3 and 4 dpf, to more ventral and medial at older stages. It is, however, unclear if it is the neurons per se that relocate or an (uneven) hypertrophy and reorganization of the whole spinal cord that is the reason for these differences.

To assess the intrinsic properties of primary motoneurons, we analyzed the action potential waveform and firing properties in quiescent preparations. Action potential amplitude and spike width did not change significantly during development, however, the resting membrane potential became more depolarized and the rheobase increased drastically, especially after 5 dpf.

After examination of morphology and neuronal properties in quiescent preparations, we investigated if primary motoneurons fire action potentials during swimming throughout development. As previously shown in larval fish, action potentials occur during both stimulated and spontaneous swimming at all stages from 4 dpf until at least 3 wpf. At all stages, action potentials could also be elicited during escape behavior. Recruitment frequency decreased during that period in accordance with the general decrease in swimming frequency, while the amplitude of phasic oscillations increased. Since it has been shown before that pMNs in 6 wpf old fish only fire action potentials during escape (Ampatzis, Song et al. 2013) it is likely that they stop participating in normal swimming around 4 to 5 weeks of age, which corresponds to the time point of the switch in swimming mode. Zebrafish larvae and adults differ in numerous parameters defining locomotion. Both stages are well studied in terms of swimming parameters and motoneuron properties, however, it was unclear when and how they change during development. Here, we show by systematically recording extra- and intracellularly from various developmental stages that drastic alterations take place between 4 and 5 weeks of development. At this time the adult muscle composition has reached their final adult state.

Figure 12: Developmental changes in locomotor parameters during zebrafish development

While developing from larvae to adult zebrafish undergo changes in the locomotor network.

Purple arrow: decrease during development

Blue arrow: increase during development

Grey arrow: no (or minor) changes

During development, the types of muscle fibers in fish always reflect and respond to the different needs of the individual. A newly hatched larva lives on the content of its yolk sac and has no need to move in search for nutrition. Accordingly, its muscle fibers consist of superficial embryonic red and a majority of deep polygonal white fibers (van Raamsdonk, van't Veer et al. 1982). By two weeks, most of the yolk is consumed and in order to survive, the larvae need to become more active and hunt for food. This change in behavior is accompanied by alterations in the muscle fiber type composition. After 2 to 2,5 wpf adult red and intermediate fibers appear (van Raamsdonk, van't Veer et al. 1982). From 4 weeks onward, there is a rapid growth of the musculature through both hyperplasia and hypertrophy mechanisms (Rossi and Messina 2014). Around the age of four weeks, the adult muscle composition is basically complete, which seems to enable the fish to change the swimming pattern from episodic to continuous. It has also been shown in other fish that the change in swimming correlates with a gradual increase of the red-like character of the superficial cells (Nag and Nursall 1972; Proctor, Mosse et al. 1980). Developmental changes in muscle composition are also associated with a refinement of motoneuron organization. The continuous arrangement of motoneuron pools in larvae changes towards clearly segregated pools that show distinct anatomical and physiological properties with a clear somatotopic organization, related to the different muscle types they innervate (Ampatzis, Song et al. 2013).

To determine the possible mechanisms underlying these developmental changes, we tested two different hypotheses. (1) When removing magnesium from the extracellular solution, the general excitability of the system increases due to the loss of the magnesium-block in NMDA

receptors, which could in turn lead to a change in the swimming pattern from episodic to continuous. (2) Hyperpolarization-activated currents (I_h) depolarize the cell upon hyperpolarization. Blocking this current in continuously swimming fish could change the swimming pattern towards episodic swimming. However, both of these hypotheses have not proven to be involved in the switch in swimming. The exact underlying mechanism for the profound transition between episodic and continuous swimming remains an open question.

5 CONCLUSION

The expression of the locomotor rhythm requires an intrinsic excitatory drive from interneurons within the locomotor circuits (Grillner 2003; Kiehn 2006; Roberts, Li et al. 2008; Grillner and Jessell 2009). However, the molecular identity of these interneurons has been unclear.

By means of ablation and optogenetical studies in the larval zebrafish, we revealed that the V2a interneurons are both necessary and sufficient to generate a meaningful locomotor pattern that is characterized by left-right-alternation and rostrocaudal delay. We showed that the V2a interneurons form an excitatory network and identified the V2a interneurons as the excitatory module within the spinal circuit that provides the excitatory tone to initiate and maintain a coordinated locomotor rhythm.

When ablating V2a interneurons in the larval zebrafish, we investigated acute effects on the locomotor pattern and found changes in threshold, episode duration, swimming frequency and cycle duration. It would, however, be interesting to examine swimming in these fish at the adult stage to see if the impairments persist, or if there are compensatory mechanisms that "repair" the larval loss of V2a interneurons, either by replacing the lost neurons or by innervation of their targets by other neurons. Since adult fish are capable of regenerating axonal tracts, cells and even tissues within the nervous system (Becker and Becker 2008), it seems plausible that plastic changes would make up for the early loss of excitatory interneurons. Because zebrafish and mammals share many molecular pathways, insights gained from work in zebrafish could possibly contribute to therapeutic approaches in central nervous system recovery in humans.

Zebrafish larvae and adults have been extensively studied in terms of their motor networks. What has not been investigated so far is when and how the swimming pattern changes from the larval beat-and-glide to the adult continuous swimming. Systematic recordings of motor nerves and primary motoneurons revealed that the switch in swimming pattern occurs around 4-5 weeks, the same time window in which primary motoneurons stop participating in normal swimming, in order to be active only during strong escape movements. Although we attempted to uncover the molecular mechanisms underlying this behavioral switch by investigating the involvement of I_h currents and magnesium, the actual cause is still unclear and needs further investigation.

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