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Generation and selection of motor behaviors

Neural circuits and endocannabinoid modulation

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To my parents

献给我的父母

ABSTRACT

The generation of basic motor behavior relies on the activation of spinal neuronal networks called central pattern generators (CPGs). Descending drive from higher brain areas activates the spinal CPG to generate motor behavior. In the face of changes in the environment, animals make constant motor behavioral selection. Both the generation and selection of motor behavior are performed by activating hard-wired neural circuits, whose activity is further refined by neuromodulation. In this thesis, we use the spinal locomotor networks in lamprey and zebrafish to explore the neural mechanisms underlying the generation and selection of motor behaviors. Firstly, in lamprey spinal locomotor circuits, we show that the release of endocannabinoids increases the excitability of the spinal locomotor network by depressing inhibitory synaptic transmission and potentiating excitatory synaptic transmission. Secondly, we show that the behavioral selection between escape and swimming in zebrafish is mediated by a hard-wired circuit. This circuit is supplemented with endocannabinoid modulation that promotes escape and suppresses swimming. Thirdly, we uncover a novel modular organization of the spinal locomotor circuit. We demonstrate a selective pattern of connectivity between excitatory V2a interneurons and motoneurons segregating them into slow, intermediate and fast sub-circuit modules. Fourthly, we show the existence of electrical coupling between motoneurons and excitatory V2a interneurons, which extends motoneurons influence onto premotor V2a interneurons. This enables motoneurons to become embedded in the spinal circuits generating the locomotor rhythm. Overall this thesis provides insight into the mechanism of generation and selection of motor behaviors.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which in the text will be referred to by their Roman numerals:

I. Song, J., Kyriakatos, A., and El Manira, A. (2012). Gating the polarity of endocannabinoid-mediated synaptic plasticity by nitric oxide in the spinal locomotor network. J. Neurosci. 32, 5097-5105.

II. Song, J., Ampatzis, K., Ausborn, J., and El Manira, A. Endocannabinoids supplement a hardwired circuit to encode behavioral choice in zebrafish. *Manuscript*

III. Ampatzis, K., **Song, J. (co-first author)**, Ausborn, J., and El Manira, A. (2014). Separate microcircuit modules of distinct V2a interneurons and motoneurons control the speed of locomotion. Neuron 83, 934-943.

IV. Song, J., Ampatzis, K., and El Manira, A. Motoneurons control of the rhythm generating circuit in the spinal cord. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
5-HT	5-Hydroxytryptamine
ACh	Acetylcholine
AEA	Anandamide
AHP	After-hyperpolarization
AM-251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-1-piperidinyl-1H-pyrazole-3-carboxamide
AP	Anterior pagoda
BMPs	Bone morphogenetic proteins
BAPTA	1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis
Carboxy-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
CB1 receptors	Cannabinoid receptors 1
Cx	Connexin
CNS	Central nervous system
CPG	Central pattern generator
C. elegans	Caenorhabditis elegans
CoLo INs	Commissural local interneurons
DAG	Diacylglycerol
DGL	Diacylglycerol lipase
DLR	Diencephalic locomotor region
E-Ins	Excitatory interneurons
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
FF	Fast fatigable
FR	Fast fatigue resistant
GPCR	G-protein coupled receptor
JZL184	4-[Bis(1,3-benzodioxol- 5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 4-nitrophenylester
I-INs	Inhibitory interneurons
IN	Interneuron

K_{Ca} channel	Calcium-activated potassium channel
IPSC	Inhibitory postsynaptic current
IPSP	Inhibitory postsynaptic potential
M cell	Mauthner cell
MGL	Monoacylglycerol lipase
mGluR1	Metabotropic glutamate receptors 1
MLR	Mesencephalic locomotor region
MN	Motoneuron
M-series	Mauthner series
NMDA	N-Methyl-D-aspartic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
PDF	Pigment-dispersing factor
PLCβ	Phospholipase Cβ
preBötC	PreBötzinger complex
RS	Reticulospinal
Shh	Sonic hedgehog
THL	Tetrahydrolipstatin
UBG	Unit burst generator
UAS	Upstream activation sequence

1 INTRODUCTION

Throughout the history of neuroscience research, scientists have put great effort into searching for suitable preparations to study important questions. These preparations range from invertebrates (molluscs, worms, insects, and crustaceans) to vertebrates (fishes, birds, amphibians, reptiles, and mammals). Interestingly, a high conservation of neural construction and a commonality of the operating mechanism in the nervous system have been observed across most of these species (Marder, 2002). Studies in animals with fewer neuronal types, smaller neural networks and more accessible nervous systems have revealed numerous general principles that can be applied to the nervous systems of other, less accessible species.

Hodgkin and Huxley used the giant axon of squid to describe the mechanisms underlying action potential (Hodgkin *et al.*, 1952). Furshpan and Potter was the first to discover electrical synapses in crayfish (Furshpan *et al.*, 1959). Dudel and Kuffler used quantal analysis to demonstrate presynaptic inhibition at the neuromuscular junction of crustaceans for the first time (Dudel *et al.*, 1961). Nicholls and Wallace first discovered that presynaptic depolarization facilitated synaptic transmission in leech heart interneurons (Nicholls *et al.*, 1978). These are just a few examples of fundamental principles, which were first discovered in the accessible neural circuits and later demonstrated in more complicated nervous systems, for example the mammalian nervous system.

Today, the advantage of studying small neural circuits in accessible preparations (e.g. C. elegans, Drosophila and zebrafish) is that these nervous systems contain few neurons and are amenable to genetic manipulation. These properties allow scientists to study the details of the neural components in neural circuits as well as the detailed neuromodulation during certain behaviors. In C. elegans, the components of neural circuits underlying certain behaviors have been well mapped with respect to neuronal connectomics, neuromodulators, and calcium signaling (White *et al.*, 1986; Bargmann *et al.*, 2013). In Drosophila, by using molecular markers or genetic identification, almost every neuron can be identified with respect to its morphology, location and connectivity, as well as its function in the network (Dickson, 2008; Bidaye *et al.*, 2014). In zebrafish, the transparency of the fish makes them advantageous for optical studies of neuronal circuits and behavior. Neuronal activity can be imaged or recorded with single-cell resolution in the brain or spinal cord (Ahrens *et al.*, 2013; Portugues *et al.*, 2014). Laser ablations of individual neurons in intact fish help in finding causal links between neurons and behavior (Fetcho *et al.*, 1998; Liu *et al.*, 1999; Eklöf-Ljunggren *et al.*, 2012). Moreover, combinations of genetic approaches employing mutant and transgenic lines promise to make the zebrafish an attractive and powerful vertebrate model.

1.1 CENTRAL PATTERN GENERATORS – NEURAL CIRCUITS OF RHYTHMIC MOVEMENTS

The concept of the central pattern generator (CPG) refers to the neuronal circuits that are able to generate an organized pattern of rhythmic motor activity in the absence of sensory inputs and was first described in invertebrates (Bullock, 1961). Later, many invertebrate and vertebrate preparations have been developed to study the neural circuits and the neuromodulation in the CPG network (Stein, 1971; Delcomyn, 1980; Grillner, 1981; Sten, 1981; Kristan *et al.*, 1983; Smith *et al.*, 1991; Hooper *et al.*, 2004; Büschges *et al.*, 2008). These extensive studies have led to a greater understanding of the basic cellular and circuit computations that underlie the rhythmic motor patterns.

Rhythmic movements, such as breathing, swallowing and locomotion, are driven by distinct CPG networks, which are located in different nuclei of the brainstem or different parts of the spinal cord (Holstege *et al.*, 1983; Smith *et al.*, 1991; Grillner, 2003). Efforts have been made to establish the appropriate preparations each with their own unique accessibility and advantages to understand the CPG networks at the cellular and circuit levels. One of the most prominent preparations is the *in vitro* brainstem slice (the medullary slice) in rodents to study the CPG network underlying breathing (Smith *et al.*, 1991). The medullary slice enables both recording of the breathing motor burst from the hypoglossal nerve roots as well as single cell recording from the rhythmically active neurons. By controlling the exact cutting sites and pharmacological treatment, the preBötzinger complex (preBötC) in the brainstem was shown to be responsible for the generation of the breathing rhythm (Smith *et al.*, 1991; Feldman *et al.*, 2003). The mechanisms underlying the rhythm generation in the preBötC were revealed by further studies in this preparation and are dependent on the synchronization of the activity of a group of excitatory interneurons, which display bursting pacemaker properties, while the inhibition in preBötC and BötC is responsible only for the pattern formation (Feldman *et al.*, 2013).

There are many other excellent preparations suitable for studies of the CPG network (Delcomyn, 1980; Shafer *et al.*, 1981; Harris-Warrick *et al.*, 1991). From this point onwards, my thesis will focus on the spinal locomotor CPG networks.

1.1.1 The spinal locomotor CPG network

The spinal locomotor CPG network is embedded in the spinal cord and generates locomotion in the absence of sensory inputs. This network contains different neuronal types, which are organized so as to produce rhythmic activity.

As early as in 1910, Brown found that in the absence of sensory feedback, spinal networks are capable of generating flexor-extensor alternating activity. Later, this finding led to the ‘half-center’ hypothesis. This hypothesis postulates that tonic descending excitation drives locomotor activity, while the alternation of flexors and extensors is based on the reciprocal inhibition between the two half-center networks

(Brown, 1914). However, flexor and extensor muscles are not always bursting antagonistically and the speed of locomotion also varies. Later, Sten Grillner further developed the concept of network function by introducing the 'Unit Burst Generator' UBG, a modular concept. Synergistic muscles of the same joint share the same UBG, which is composed of interconnected premotor interneurons and generates locomotion locally without any sensory input. The network in the UBG concept allows the motoneuron pools and their innervated muscles to change their relative activity during movement in an antagonistic or synergistic manner like walking forward and backward. It also provides the basic understanding of the coordination of limb muscles during movement (Grillner, 1981; Grillner, 1985).

To study the basic neural structure of the spinal locomotor CPG network, several different preparations have been developed. In a few segments of lamprey spinal cord (Cohen *et al.*, 1980; Poon, 1980), which precludes any sensory or afferent input, locomotor activity can be generated by appropriate pharmacological manipulation or electrical stimulation. Activation of the spinal CPG in the *in vitro* lamprey spinal cord generates rhythmic, left-right alternating locomotor activity, which can be recorded from the ventral roots as fictive locomotion (Cohen *et al.*, 1980). In the *Xenopus* tadpole (Roberts *et al.*, 1981), periodic swimming is induced by electrical stimulation or light dimming and left-right alternating swimming activity can be recorded from the motor nerves on both sides of the trunk in the presence of curare. In mammals, the *in vitro* lumbar spinal cord of neonatal rat was first developed to study the monosynaptic sensory reflex by stimulating the dorsal roots (Kudo *et al.*, 1987) and was then induced to perform locomotor-like activity by the application of 5-HT and NMDA to study the spinal CPG network (Cazalets *et al.*, 1992). Use of the *in vitro* lumbar spinal cord of neonatal mouse reveals role of specific neuronal populations in control of rhythm generation and left-right alternation through combining molecular and physiological approaches (Dougherty *et al.*, 2010; Arber, 2012; Talpalar *et al.*, 2013; Goulding *et al.*, 2014; Goetz *et al.*, 2015). Later, both larva and adult zebrafish model systems were also developed to study the organization and function of locomotor circuits (Masino *et al.*, 2005; McDearmid *et al.*, 2006; Gabriel *et al.*, 2011; Kyriakatos *et al.*, 2011).

In this thesis, the lamprey and zebrafish spinal locomotor systems are employed to answer specific questions. Therefore, I will focus on these two preparations in the following part of the introduction.

1.1.2 The lamprey swimming CPG network

Spinal swimming CPG network in fish produce a rhythmic and coordinated left-right alternating locomotor pattern, which makes these preparations a mini version of flexor-extensor pattern in limbed animals (Dale *et al.*, 1997; Grillner, 2006a; Kiehn, 2006). There are three big advantages for the utilization of these models:

1. Highly conserved spinal neuronal network composition with limbed animals;
2. Intact neural networks with intersegmental coordination in the preparation;

3. Excellent accessibility of the preparations for electrophysiology and other investigative methods.

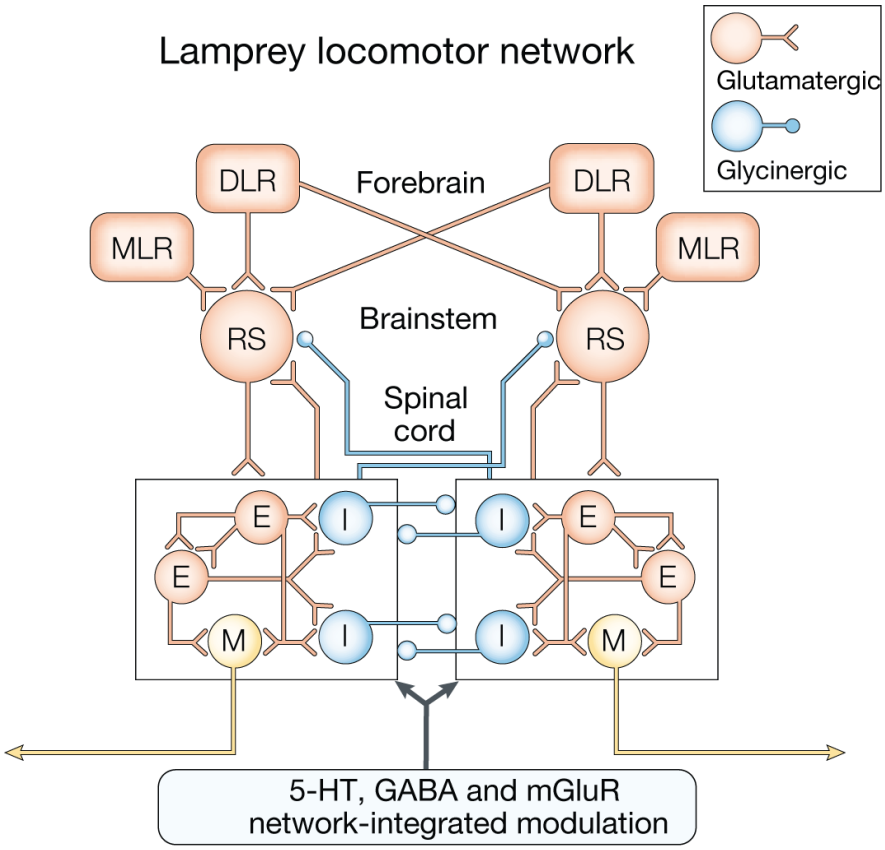

Figure 1. Schematic of lamprey locomotor network. Schematic representation of brainstem and spinal neural circuits that generate rhythmic locomotion. All neuron symbols represent populations rather than single cells. RS neurons receive excitatory synaptic inputs from the diencephalic and mesencephalic locomotor regions (DLR and MLR). Activation of RS neurons drives spinal interneurons and motoneurons. The excitatory interneurons (E) excite all types of spinal neurons — the excitatory interneurons (E), the inhibitory commissural glycinergic interneurons (I) and motoneurons (M). Neuromodulators are the integral part of the CPG. (Modified from Grillner, 2003)

The lamprey spinal cord preparation offers all of these advantages and each segment of the spinal cord has its own CPG circuit (Grillner, 2003). The descending inputs, which are initiated from the mesencephalic locomotor region (MLR) or the diencephalic locomotor region (DLR) and are relayed in the reticulospinal (RS) neurons (command neurons or system) to drive the swimming CPG network that generates locomotor activity. The frequency range is similar between the swimming *in vivo* animals and *in vitro* preparations. The fictive swimming can be induced by brainstem stimulation or pharmacological application of glutamate agonists in the isolated spinal cord. Experiments have been performed to reveal the neuronal components of the swimming CPG. Hemicords, in which the left and right hemi spinal cord are separated from the midline, can still generate slow and fast rhythmic activities (Grillner, 2003). The machinery of locomotor generation in the hemicord relies mainly on the activation of a group of ipsilateral excitatory interneurons (E-INs), which make excitatory synapses with other E-INs. They also excite motoneurons and commissural inhibitory interneurons, which ensure the left-right alternation. Application of strychnine, a glycinergic synaptic transmission blocker, does not change the swimming frequency

induced in the hemicords, which suggests that inhibitory synaptic transmission is not involved in the generation of locomotor rhythmicity. However, blockage of glycinergic synaptic transmission in whole lamprey spinal cord dramatically increases the swimming frequency. This effect was associated with a left-right burst synchrony. These experiments highlight the role of the inhibitory interneurons (I-INs) in the intact spinal CPG network. These interneurons receive excitation from E-INs and project contralaterally to provide inhibition to the neurons on the other side of the spinal cord. These properties allow I-INs to coordinate the activity between the two hemicords and participate in the generation of locomotor pattern (Ohta *et al.*, 1991; Parker *et al.*, 2000).

The foundation of the spinal swimming CPG therefore consists of ipsilateral-projecting glutamatergic interneurons and contralateral-projecting glycinergic interneurons. The descending input from higher brain areas drive E-INs, I-INs and MNs through excitatory synaptic transmission and the swimming CPG transforms the drive into swimming activity. Evidence from other preparations (*Xenopus* tadpole, mouse, zebrafish) also suggests that the spinal swimming CPG network is built on the combination of glutamatergic and glycinergic interneurons (Dale, 1998; Kiehn *et al.*, 1998; Stein, 1999; Pearson, 2000; Drapeau *et al.*, 2002).

The understanding of the neural construction in the lamprey swimming CPG network enables further studies on ion channels and neuromodulation of swimming activity. K_{Ca} channels in the lamprey spinal CPG neurons are activated following a transient increase of intracellular Ca^{2+} driven by action potentials. This activation induces an after-hyperpolarization (AHP) after the action potential. Apamine, a partial blocker of K_{Ca} channels, decreases the AHP and also spike frequency adaption, which potentiates the burst duration and depresses swimming frequency (El Manira *et al.*, 1994). Activation of G-protein coupled receptors (metabotropic glutamate receptors, 5-HT receptors, dopamine receptors and endocannabinoids receptors) by neurotransmitters or neuromodulators (glutamate, 5-HT, dopamine and endocannabinoids) significantly alters the locomotor frequency through different mechanisms (Wallén *et al.*, 1989; Krieger *et al.*, 2000; Hess *et al.*, 2001; Kettunen *et al.*, 2005; Pérez *et al.*, 2013).

1.1.3 Zebrafish as a model to study locomotion

Across all the developmental stages until adulthood, zebrafish are suitable for studying the locomotor network. In zebrafish, the transparency of fish body makes them advantageous for optical studies of neuronal circuits and behavior. Neuronal activity can be imaged or recorded with single-cell resolution in both the brain and the spinal cord (McLean *et al.*, 2007; Gabriel *et al.*, 2011; Ahrens *et al.*, 2013; Portugues *et al.*, 2014).

1.1.3.1 Zebrafish as a model system for swimming

At the larval stage, electrical stimulation of the otic vesicle triggers episodic swimming at frequencies up to 100 Hz (McLean *et al.*, 2007; Eklöf-Ljunggren *et al.*, 2012). In the adult zebrafish, stimulation of descending tracts induces continuous and long-lasting swimming bout in the *in vitro* preparation at frequencies up to 25 Hz (Gabriel *et al.*, 2011; Kyriakatos *et al.*, 2011; Ampatzis *et al.*, 2013; El Manira, 2014). The descending inputs drive the swimming CPG network that generates rhythmic left-right locomotor activity in both the larval and adult preparations. Both preparations allow for the accessibility to study the electrophysiological properties of network neurons during ongoing swimming activity. In larval zebrafish, the myotome contains mainly fast muscle fibers (Waterman, 1969; van Raamsdonk *et al.*, 1982; Devoto *et al.*, 1996; Stellabotte *et al.*, 2007) and swimming activity consists of a fast 'beat and glide' pattern (Drapeau *et al.*, 2002; Lewis *et al.*, 2003). However, at the adult stage, the muscle fibers are very well segregated into slow, intermediate and fast types and swimming switches to a continuous pattern with lower swimming frequency (25 Hz) (Ampatzis *et al.*, 2013). The locomotor pattern and frequency range display considerable differences between the larval and the adult stages, and these differences are associated with the lateral extension of the motor column.

The adult zebrafish spinal cord preparation that have been developed keep the intact and functional spinal swimming network and allow accessibility for whole-cell patch clamp recordings, detection of the activity of populations of neurons by calcium imaging, and detection of activity in populations of neurons by laser ablation and optogenetics. Furthermore, they are also amenable to genetic manipulation. By using these preparations, we could obtain novel insights into the organization of neural circuits and their modulation that mediate the generation of locomotion and the selection between motor behaviors

1.1.3.2 Zebrafish as an escape model system

Escape is a defensive response to sudden or threatening stimuli in vertebrates. When facing a dangerous situation, fish perform C-shape or S-shape escape behavior to relocate or move away from danger or predators. In zebrafish, head stimulation triggers C-shape escape, however, tail stimulation induces both C-shape and S-shape escape. It is worth noting though, that S-shape escape starts with stimulation-triggered local response, and is then followed by C-shape escape behavior (Liu *et al.*, 2012).

In zebrafish, the command system for escape behavior is located in the brainstem, and is called the Mauthner series (M-series). The M-series contains three bilateral pairs of identified reticulospinal neurons in the mid-dorsal region of the hindbrain segments, Mauthner (M) cells (4th segment), MiD cm (5th segment) and MiD3cm (6th segment) (Lee *et al.*, 1991). The M-series neurons are considered to be segmental homologs (Metcalfe *et al.*, 1986), since all of these neurons have similar morphology, each having two major dendrites and an axon that crosses in the midbrain and projects along the

contralateral spinal cord. (Liu *et al.*, 1999; Nakayama *et al.*, 2004; Kohashi *et al.*, 2008). The dendrites of the M-cells receive excitatory inputs from the auditory nerve, the posterior lateral line nerve and the optic tectum (Faber *et al.*, 1991). Hence, escape can be induced by sound, touch and light. Calcium imaging and patch-clamp recording in zebrafish have shown that sensory stimulation, head tap or sound, activates the neurons in the M-series, which initiates the fast C-starting escape behavior (O'Malley *et al.*, 1996; Nakayama *et al.*, 2004; Kohashi *et al.*, 2008). Even though the M-series acts as a functional unit, different sensory stimuli may activate different neurons in the M-series and induce different types of escape behaviors. Escape initiated by M cell activation has faster kinetics than that started by non-M cells (O'Malley *et al.*, 1996; Kohashi *et al.*, 2008).

Spikes in the M-cell excite contralateral spinal excitatory interneurons and fast motoneurons (Fetcho *et al.*, 1988; Ampatzis *et al.*, 2013), which induce contraction of the muscle trunk to perform the C-start bending, while simultaneously inhibiting ipsilateral spinal fast motoneurons via a group of identified inhibitory interneurons, the commissural local interneurons (CoLo INs) (Fetcho *et al.*, 1988; Liao *et al.*, 2008; Satou *et al.*, 2009). Furthermore, escape behavior has been shown to stop on-going swimming for a few cycles in freely swimming zebrafish (Svoboda *et al.*, 1996; Kyriakatos *et al.*, 2011). This suggests that the zebrafish is a fruitful model to study the neural mechanisms underlying behavioral selection between escape and swimming activity.

1.2 THE COMPONENTS OF SPINAL LOCOMOTOR NEURAL CIRCUITS

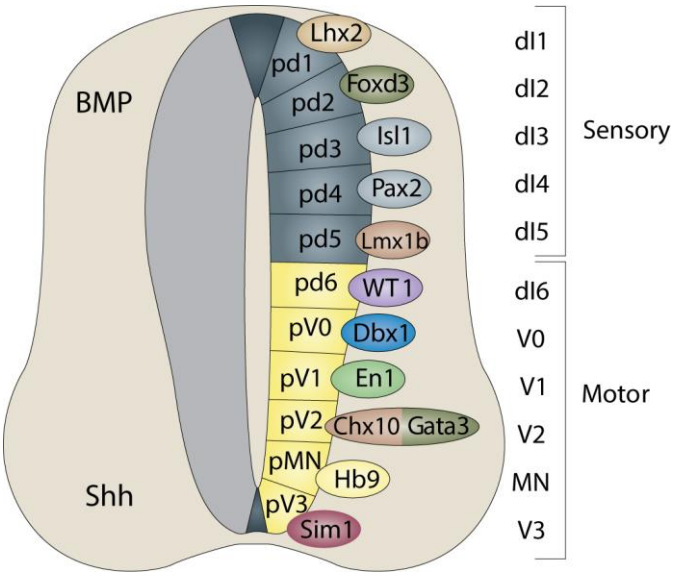

Figure 2. Schematic cross sections of the developing spinal cord showing the patterning and specification of early spinal cord progenitors and their neuronal progeny. Opposing gradients of sonic hedgehog (Shh) (ventrally) and bone morphogenetic proteins (BMPs) (dorsally) instruct differentiation of progenitors. DI1-dI5 neurons derived from dorsal progenitors contribute to sensory spinal pathways, whereas dI6, MN and V0–V3 neurons arising from ventral progenitors are involved in the locomotor circuitry. (Modified from Goulding, 2009)

Neuronal identity in the spinal cord is mainly determined by the interaction of two morphogen gradients that allocate dorsoventral position information to divide neural progenitors. Sonic hedgehog (Shh) is produced by the notochord and the floor plate and controls ventral patterning (Jessell, 2000; Shirasaki *et al.*, 2002), while bone morphogenetic proteins (BMPs) are secreted by the epidermis overlying the neural tube and roof plate and are responsible for the partitioning of the spinal dorsal zone (Lee *et al.*, 1999). The opposing effects of Shh and BMPs restrict the expression of patterning factors to spatially limit subsets of spinal progenitors. In the dorsal spinal cord, sensory neurons (dI1, dI2, dI3, dI4, and dI5) are generated from six progenitor domains and are mostly involved in sensory processing (Goulding *et al.*, 2002). There are six groups of spinal CPG interneurons and motoneurons (dl6, V0, V1, V2, MN and V3) that are originally derived from six progenitor domains (named pd6, p0, p1, p2, pMN and p3) in the spinal ventricular zone (Ericson *et al.*, 1997a; Ericson *et al.*, 1997b). This thesis focuses on the activity pattern and connectivity of the excitatory V2a interneurons and motoneurons.

1.2.1 Excitatory drive within the locomotor network

The foundation of the swimming CPG is built on the combination of glutamatergic and glycinergic interneurons. However, spinal excitatory drive in the spinal locomotor network mainly relies on the activation of a group of ipsilateral excitatory interneurons (E-INs), which make excitatory synapses with other E-INs, motoneurons and commissural inhibitory interneurons. The descending inputs from higher brain areas drive E-INs, which activate the spinal CPG network to transform the drive into locomotor activity in lamprey, *Xenopus* tadpole, mouse and zebrafish (Dale, 1998; Kiehn *et al.*, 1998; Stein, 1999; Pearson, 2000; Drapeau *et al.*, 2002; Grillner, 2006b). The genetic identification of ipsilateral excitatory V2a interneurons in mouse and zebrafish allows detailed studies of their function in locomotion.

The morphology of V2a interneurons in both mouse and zebrafish spinal cord is similar (ipsilateral projecting) (Al-Mosawie *et al.*, 2007; Lundfald *et al.*, 2007; Kimura *et al.*, 2008), however, they display different functions in the two locomotor systems.

In the zebrafish, selective laser ablation of V2a interneurons (~ 30% of V2a interneurons in each of 10 segments) strongly affects the generation of the swimming activity, indicating that they are the main source of excitatory drive underlying the locomotor rhythm. Moreover, optogenetic activation of V2a interneurons shows that V2a interneurons are interconnected and form an excitatory network, which produces sufficient input to drive rhythmic swimming activity (Eklöf-Ljunggren *et al.*, 2012). These studies reveal that V2a interneurons are a vital component of spinal CPG network and are necessary and sufficient in themselves for generating the locomotor rhythm.

In larval zebrafish spinal cord, ventral V2a interneurons are recruited at slow swimming speed, while dorsal V2a interneurons are engaged at faster speeds (McLean *et al.*, 2007). Moreover, the recruitment of the V2a interneurons in larval zebrafish is

dependent on their input resistance. By contrast, in adult zebrafish, the topographic organization of V2a interneurons is blurred. These interneurons are recruited in a incremental manner during the increase in swimming frequency. Their order of recruitment is determined by the scaling of their synaptic drive by the input resistance (Ausborn *et al.*, 2012).

In the mouse, most of the V2a interneurons are rhythmically active and display membrane potential oscillations related to either flexor or extensor activity in the spinal segments. The firing patterns of V2a interneurons are heterogeneous and are not always correlated to rhythmicity of the network (Dougherty *et al.*, 2010). Selective ablation of V2a interneurons with diphtheria toxin A disrupts the left-right alternation at intermediate locomotor frequencies, but does not affect the flexor-extensor alternation and locomotor frequency (Crone *et al.*, 2008; Crone *et al.*, 2009). This suggests that V2a interneurons are not rhythm generating neurons. Instead Shox2 interneurons, which partially overlap with V2a interneurons, have been shown to participate in generating the rhythmicity (Dougherty *et al.*, 2013).

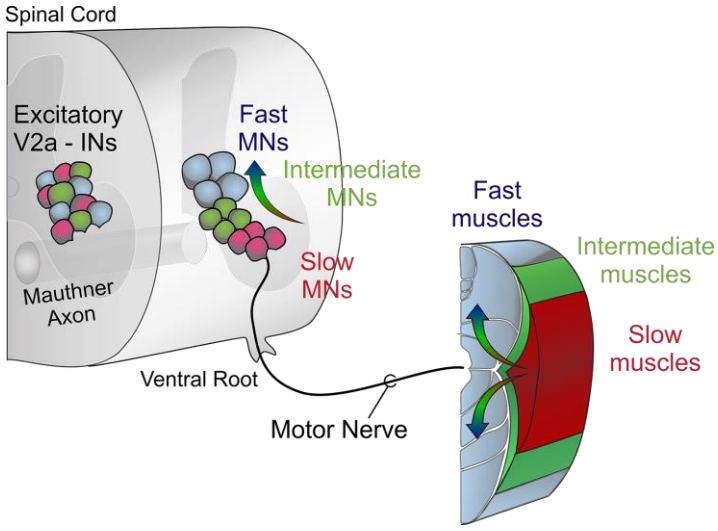

Figure 3. Schematic cross section of adult zebrafish showing the distribution of muscles, motoneurons and V2a interneurons. The axial muscles are very well segregated into slow, intermediate and fast types, which are innervated by topographically organized slow, intermediate and fast motoneurons respectively. Contrast to motoneurons, V2a interneurons are not topographically organized.

1.2.2 Motoneurons – size principle and final common pathway

Spinal motoneurons are derived from the pMN progenitor domain of the spinal ventricular zone. During development, each motoneuron sends its axons to innervate a target group of muscle fibers, hence the concept of motor unit. A motor unit has two components: the motoneuron and its innervated muscle fibers. The axon terminals of the motoneuron communicate to the muscle fibers at the neuromuscular junctions.

Final common pathway: The motoneurons in the spinal cord serve as a final common pathway that receive, integrate and transmit the neural information from the CPG network or the sensory inputs. The final common pathway theory indicates that the motoneurons in the spinal cord receive, integrate and transmit the commands from the

upper network (Liddell *et al.*, 1925; Sherrington, 1925; Heckman *et al.*, 2012). The only established way for motoneurons to communicate with the premotor network is through the recurrent inhibition via the Renshaw cells, which are thought not to be involved in the rhythm generation (McCrea *et al.*, 1980; Zhang *et al.*, 2014).

Size principle: Three types of motor units are identified according to their intrinsic properties: slow (type S), fast fatigue resistant (type FR), and fast fatigable (type FF) (Burke *et al.*, 1974). The recruitment of motor units is believed to follow the size principle (Harris *et al.*, 1977; Powers *et al.*, 1996). Henneman compared the recruitment order of motoneurons by stretching the triceps muscle in decerebrate cat to the amplitude of their impulses. He suggested that the smallest motoneurons had the lowest thresholds for stretch and fired first, while larger motor neurons had higher thresholds and fired last. The size principle can also be interpreted as the input resistance being the main factor determining the recruitment order of motoneurons (Henneman, 1957; Kiehn, 2006; Goulding, 2009). However the size principle is limited to the timing of recruitment of motor units and cannot easily explain the recruitment of the motor units in synergistic muscles (Koehler *et al.*, 1984). Furthermore, there is also an assumption in the size principle that all the motoneurons receive equal synaptic inputs simultaneously, which may not necessarily the case.

In the larval zebrafish, all motoneurons are uniform and participate in the swimming activity by following the size principle (McLean *et al.*, 2007). However, accompanying the development of the muscle types, the motoneurons in the adult zebrafish spinal cord become topographically organized into slow, intermediate and fast groups. During swimming with increasing speed, the order of motoneuron recruitment is from slow to intermediate and fast. Voltage-clamp experiments have shown that slow, intermediate and fast motoneuron pools receive different excitatory and inhibitory synaptic currents during locomotion. The recruitment order of the different motoneuron pools is not correlated with their input resistance and hence does not follow the size principle (Gabriel *et al.*, 2011; Ampatzis *et al.*, 2013). This makes the adult zebrafish an attractive model for reconsidering the size principle and finds out more about how the CPG network is organized to drive the motoneurons during swimming activity.

1.3 NEURAL MECHANISMS OF BEHAVIOR SELECTION

In face of changing situations in the environment, animals usually make the appropriate necessary behavioral selection. Sensory cues coming from auditory, visual and olfactory stimulation are integrated in the related area of nervous system, which gates behavior selection by immediately stopping one or more ongoing behaviors and simultaneously initiating the desired behavior (Romo *et al.*, 2003; Preuss *et al.*, 2006; Palmer *et al.*, 2011). Initiation of behaviors requires the activation of their command neurons (several neurons) or command system (a small neural network) (Kristan, 2008) by sensory inputs, to drive the CPG networks underlying these behaviors. To understand the neural mechanisms responsible for behavioral selection, several invertebrate and vertebrate preparations have been developed to investigate certain

behavioral selections. The neural mechanisms underlying behavioral choice is a sensory-gated process, during which the nervous system is biased to one behavior by activating its command neurons or command system, and meanwhile counteracts the other behaviors by inhibiting their command neurons or command systems. Even though the neural circuits underlying behavioral choice vary, they do share similar mechanisms. Below are some of the important mechanisms responsible for behavioral selection.

Presynaptic inhibition: Presynaptic inhibition refers to an inhibitory control of synaptic efficacy at the axon terminal, arising from descending inputs, or interneurons or sensory afferents that is found in both invertebrate and vertebrate (Eccles, 1964; Fu *et al.*, 1978; El Manira *et al.*, 1994). In the leech, feeding is the top priority behavior that completely overrides the locomotor activities (swimming or crawling) via presynaptic inhibition. Locomotion is controlled by activation of mechanosensory neurons (P cells) which excite anterior pagoda (AP) neurons and start the locomotor central pattern generator. Feeding behavior in leech increases the firing rates of serotonergic neurons in the cephalic ganglion that in turn produces presynaptic inhibition at the synapse between P-AP neurons. This leads to silencing of the locomotor CPG and prevents any locomotor activities (Gaudry *et al.*, 2009).

Competition between circuits: Behavioral selection may involve competition between two local circuits, which share the same elementary neurons. In the sea slug, rhythmic feeding is generated by a CPG network, within which interaction between excitatory I2 neurons and inhibitory I1 neurons generates the rhythmic feeding activity. Once swimming is generated in the sea slug, the CPG neurons excite A-ci1 neurons, which constantly inhibit I1 interneurons in the feeding CPG network and terminate the feeding activity (Jing *et al.*, 1995). The fact that swimming overrides feeding appears to be a behavioral selection mediated by the interaction between two CPG networks which allows the sea slug to escape away from danger during feeding.

Neuromodulator-mediated behavioral selection: Neuromodulators acting on their receptors are known to influence the properties of single neurons, synapses, or local circuits to eventually affect the behavior output (Bargmann *et al.*, 2013). Roaming and dwelling behaviors in *C. elegans* are mediated by distinct neural circuits – PDFR-1 neural circuits and serotonergic neural circuits, respectively. Serotonin released from serotonergic neurons promotes dwelling behavior while the neuropeptide pigment-dispersing factor (PDF) produced in the PDFR1-expressing neurons enhances roaming behavior. Increasing serotonin release changes ongoing roaming behavior to dwelling behavior, while enhancing PDF production switches dwelling behavior to roaming behavior (Flavell *et al.*, 2013).

Behavioral selection gated by sensory neurons: Activating the decision-maker neurons can bias the decisions that animal makes (Brozović *et al.*, 2007; Cohen *et al.*, 2008; Kristan, 2008). In an experiment on monkey, a micro-stimulor was placed in the receptive field of the column of recorded sensory neurons of the temporal visual area.

The monkey's preference as to whether motion of the visual stimulus was in the preferred or null direction was recorded in the column under study. Micro-stimulation of one or a few middle temporal columns of visual cortical area, given while the monkey viewed a random dot stimulus, could strongly bias the monkey's perceptual judgments toward the direction preferred by the stimulated column (Nienborg *et al.*, 2012). These studies demonstrate that sensory neurons can act as decision makers and gate the behavioral choice.

1.4 ENDOCANNABINOID NEUROMODULATION

In the nervous system, endocannabinoids are released from postsynaptic neurons and act on presynaptic cannabinoid receptors 1 (CB1 receptors) to mediate synaptic transmission as a retrograde messenger (Kano *et al.*, 2009; Castillo *et al.*, 2012; Tanimura *et al.*, 2012; Younts *et al.*, 2014). CB1 receptors, G-protein coupled receptors, are the most abundant cannabinoid receptors and are expressed mostly at the presynaptic terminals (Katona *et al.*, 2006; Gregg *et al.*, 2012), which further support their reported retrograde effects on synaptic transmission. Activation of CB1 receptors depresses synaptic transmission, which involve distinct mechanisms either by modulating the activity of voltage-gated Ca^{2+} channels (VGCCs) (Kreitzer *et al.*, 2001; Wilson *et al.*, 2001) and K^{+} channels (Daniel *et al.*, 2001; Nazzaro *et al.*, 2012) or by affecting protein synthesis (Alger, 2009; Heifets *et al.*, 2009; Tsetsenis *et al.*, 2011).

1.4.1 Endocannabinoid-mediated synaptic plasticity in the spinal network

Synaptic plasticity mediated by endocannabinoids has been studied mainly in acute brain slices. While the synaptic connections in slices are still intact, it is not always possible to evaluate their effects on the entire neural circuit. Lamprey spinal cord preparations with an intact CPG network and inducible locomotor activity provide the possibility to study the endocannabinoid modulation on a functional neural network.

Previously, we showed that endocannabinoids embedded in the spinal network set the baseline of the locomotor frequency (Kettunen *et al.*, 2005). Activation of cannabinoid receptors 1 (CB1 receptors) increases excitatory synaptic transmission in the network neurons, but decreases inhibitory synaptic transmission, which overall increases the network excitability and potentiates locomotor activity (Kyriakatos *et al.*, 2007). Similar to endocannabinoids, NO is released in the lamprey spinal locomotor network and participates in setting the frequency of locomotor activity by modulating synaptic transmission (Kyriakatos *et al.*, 2007; Kyriakatos *et al.*, 2009). Inhibition of nitric oxide synthesis or scavenging of NO in the lamprey spinal network impairs endocannabinoid-induced potentiation of locomotor activity. This indicates that there is an intrinsic interaction of endocannabinoids and NO in spinal locomotor network which by an unknown mechanism modulate the synaptic plasticity.

1.4.2 Endocannabinoid 2-AG synthesis and degradation

The two main endocannabinoids in the nervous system are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). The former was discovered first and acts as a partial agonist on CB1 receptors (Sugiura *et al.*, 2002). On the other hand, 2-AG is the more common endocannabinoid in the nervous system and its level in the nervous system is in the order of nanomoles per gram, which is much higher than that of anandamide (Sugiura *et al.*, 2006). 2-AG is a full agonist of both CB1 and CB2 receptors with a higher affinity for both receptors than anandamide (Sugiura *et al.*, 2006).

The main pathway of biosynthesis of 2-AG is via two enzymes: phospholipase Cβ (PLCβ) and diacylglycerol lipase (DGL). Presynaptic glutamate release acts on postsynaptic metabotropic glutamate receptors 1 (mGluR1) (Maejima *et al.*, 2001; El Manira *et al.*, 2010) to activate phospholipase enzyme PLCβ, which hydrolyzes phosphatidylinositol into diacylglycerol (DAG) (Hashimotodani *et al.*, 2007). Meanwhile, depolarization of postsynaptic neurons triggers a Ca^{2+} influx, which activates the enzyme DGLα to catalyze DAG into 2-AG (Tanimura *et al.*, 2010; Yoshino *et al.*, 2011). It is worth noting that mGluR activation by an agonist, DHPG, is sufficient to trigger 2-AG-mediated short- or long-term synaptic plasticity, even if Ca^{2+} is chelated in the postsynaptic neurons (Marsicano *et al.*, 2002; Chevaleyre *et al.*, 2003; Galante *et al.*, 2004; Jung *et al.*, 2005; Heifets *et al.*, 2009). Even though Ca^{2+}-influx or activation of G protein-coupled receptors (GPCRs) can trigger 2-AG synthesis independently, they work in a synergistic way most of the time (Brenowitz *et al.*, 2005; Hashimotodani *et al.*, 2005; Kreitzer *et al.*, 2005; Edwards *et al.*, 2008; Heifets *et al.*, 2009). The degradation of the endocannabinoid 2-AG mainly involves the enzyme, monoacylglycerol lipase (MGL) (Dinh *et al.*, 2002; Gulyas *et al.*, 2004; Blankman *et al.*, 2007).

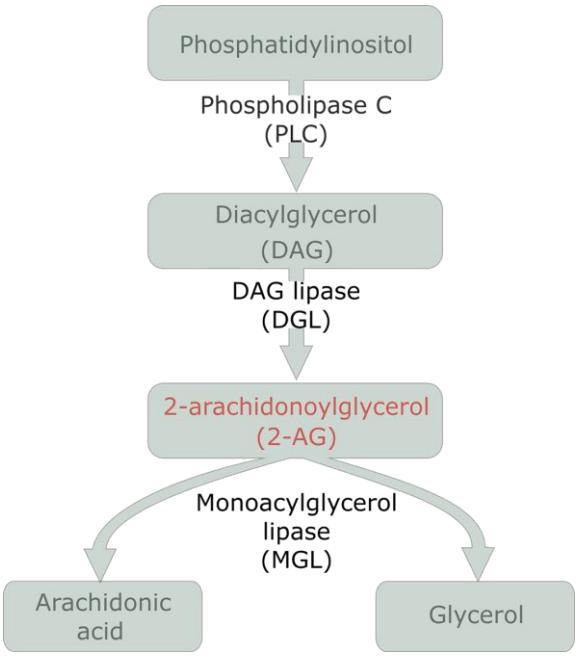

Figure 4. The synthesis and degradation pathway of endocannabinoid 2-AG.

1.5 THE ELECTRICAL SYNAPSE – HISTORY AND FUNCTION

Over a century ago, the British neurophysiologist Charles Sherrington introduced the word ‘synapse’ to describe the connection zone between neurons. For a long time after, all synapses were considered to operate in a manner of electrical transmission. However, in 1921 the German pharmacologist Otto Loewi discovered that the signaling from the vagus nerve to the heart muscle in frog is transduced by the chemical compound acetylcholine (ACh). For this finding he was rewarded the Nobel Prize in Physiology or Medicine in 1936. However, more importantly, it also triggered a long lasting debate on the role of electrical vs chemical synaptic transmission in the nervous system for decades. After improvements of electrophysiology techniques and ultrastructural methods in 1950s and 1960s, the presence of both electrical and chemical synapses was confirmed in the nervous system.

1.5.1 The electrical synapse in the nervous system

The term ‘gap junction’ derives from a structural characteristic view of the electrical synapse and refers to the gap junction channels connecting two neurons at an electrical synapse. The synaptic cleft of an electrical synapse is about 4 nm (chemical synapse: 20 nm) and is bridged by gap junction channels. A single gap junction channel is composed of two hemi-channels, which are termed ‘connexon’ and located separately in the pre- and the postsynaptic cell membranes separately. The pore of the gap-junction channel has a large diameter (~1.2 nm), which allows inorganic ions, small organic molecules and fluorescence makers to flow between the two cells. Each hemi-channel consists of six identical subunits, named connexins. Connexin proteins (Cx) are encoded by a gene family with more than 20 members in mammals (Willecke *et al.*, 2002). Cx36 (homolog to fish Cx35) is considered to be the prevalent connexin in the mammalian nervous system (Condorelli *et al.*, 2000; Pereda, 2014). Although connexins have highly conserved sequences, gap junction channels with different connexin assemblies show different properties with respect to channel conductance, and permeability (Bennett *et al.*, 2004) Generally, most electrical synapses are bidirectional with similar permeability to ions in either direction. The rectifying synapse (for example the crayfish giant motor synapse) is a special case with different combinations of connexins into connexons locating at the pre- and postsynaptic neuronal membrane. Voltage-dependent gap junction channels only allow the depolarizing current to transmit unidirectionally (Bennett, 1997).

1.5.2 The function of gap junctions in the nervous system

Compared to the chemical synapse, the electrical synapse (gap junction) provides the neuronal interconnections with faster speed, simpler transmission and bidirectional reciprocity (Bennett, 1997; Pereda, 2014). One prominent function of gap junctions in the nervous system is to synchronize the activity of a group of neurons. Interneurons in hippocampus, thalamus, neocortex, cerebellum, striatum, brainstem or olfactory bulb are electrically coupled. This synchronizes their activity and output to generate global

effects (Mann-Metzer *et al.*, 1999; Rekling *et al.*, 2000; Galarreta *et al.*, 2001; Connors *et al.*, 2004; Yaksi *et al.*, 2010). In the spinal locomotor network, motoneurons innervating the same muscles are electrically coupled well with each other to synchronize their output (Tresch *et al.*, 2000; Zhang *et al.*, 2009). Blockage of all chemical synaptic transmission with TTX, the rhythmic activity is still produced by NMDA through gap junctions in neonatal rat spinal cord (Tresch *et al.*, 2000). During development of the spinal network, gap junctions have been found to play important roles in regulating the formation of the spinal neural circuits (Saint-Amant *et al.*, 2000; Kiehn *et al.*, 2002). Although gap junction expression declines during development, in adult vertebrate spinal cord, the gap junction protein Cx36 is widely expressed in both interneurons and motoneurons (Rash *et al.*, 1996; Chang *et al.*, 1999; Parenti *et al.*, 2000), however its function is unknown.

2 SPECIFIC AIMS

The overall aim of this thesis was to explore the neural mechanisms controlling the generation and selection of motor behaviors. These mechanisms involve both hard-wired neural circuits with specific neuronal connectivity and endocannabinoid neuromodulation at the cellular level. This thesis is divided into four projects with the following aims:

1. To characterize the identity of the endocannabinoids in the spinal locomotor network and how it interacts with NO to modulate the locomotor activity.

2. To investigate the neural circuits encoding the behavior selection between escape and swimming and the role of the endocannabinoids in promoting this behavioral selection.

3. To map the specific connectivity pattern between V2a interneurons and motoneurons to reveal how the spinal network conveys excitation to motoneurons.

4. To examine how electrical coupling enables motoneurons to control the activity of premotor V2a interneurons and the locomotor rhythm generation in the spinal network.

3 METHODS

3.1 LAMPREY

The experiments were performed on *in vitro* preparations of the isolated spinal cord from adult lampreys (*Lampetra fluviatilis*) of both genders. The details of the preparation are described in paper I.

Fictive swimming activity was induced by application of NMDA. Locomotor burst activity was recorded by glass suction electrodes placed on ventral roots. Intracellular recordings were made from spinal neurons with 3 M potassium acetate-filled thin-walled glass microelectrodes. The concurrent recordings of the locomotor frequency and of the activity of MNs allowed us to correlate changes in the synaptic amplitude to changes in locomotor frequency.

To examine the modulation on ipsilateral excitatory or inhibitory synaptic transmission, the recording chamber was divided into two pools by an agar barrier. Locomotor activity was induced in the rostral pool by application of NMDA while the spinal cord in the caudal pool was perfused with strychnine to block inhibitory synaptic transmission, or with kynurenic acid to block ionotropic glutamate receptors. The peak-to-trough amplitude of the locomotor-related synaptic input was measured and monitored under control conditions and in the presence of different pharmacological agents.

3.2 ZEBRAFISH

3.2.1 Animals

Zebrafish (*Danio rerio*) were raised and kept in a core facility at the Karolinska Institute according to established procedures. To enable selective target of V2a interneurons, we used a zebrafish line (Chx10:GFP) in which the expression of GFP was driven in V2a interneurons by the premotor of transcription factor Alx (homolog of the mammalian Chx10).

3.2.2 Zebrafish *in vitro* preparation

The adult zebrafish were anesthetized in a slush of frozen extracellular solution and eviscerated. Afterwards the brain was exposed and the cerebellum was gently removed to allow the stimulation electrode to have access to the Mauthner system. The epaxial musculature was carefully removed up to the caudal end of the dorsal fin, leaving the musculature at the tail area intact. Then the bones were removed four to five segments rostral to the dorsal fin to allow access with intracellular recording electrodes. For *in vitro* escape and swimming experiments, preparations were fixed dorsal side up with

Vaseline. For whole-cell patch clamp recordings, preparations were transferred to a recording chamber, placed lateral side up and fixed with Vaseline.

3.2.3 Optogenetics

To be able to express halorhodopsin in motoneurons specifically, we employed the Gal4/UAS system. This system consists of two components: the driver line, which has the Gal4 gene under a tissue-specific promotor, and the reporter line with an upstream activation sequence (UAS). When the two lines are crossed, Gal4 binds to UAS, which activates transcription of the reporter gene in a tissue specific manner.

We used a zebrafish line $Gal4^{s1020t}$ (Et(-0.6hsp70l:Gal4-VP16)s1020t), in which Gal4-VP16 was expressed mainly in motoneurons (Wyart *et al.*, 2009). Crossing the $Gal4^{s1020t}$ line with the Tg(UAS:NpHR-mCherry) line yielded zebrafish expressing of NpHR-mCherry in motoneurons. By crossing these zebrafish with another zebrafish line Tg(Chx:GFP), expressing of GFP in the V2a interneurons, we finally obtained zebrafish simultaneously expressing NpHR-mCherry in motoneurons and GFP in V2a interneurons.

Motoneurons were inhibited optogenetically by using a light stimulation (yellow light: 586 nm), which was generated by a custom made LED system.

3.2.4 Retrograde labeling of motoneurons

Retrograde labeling of descending brainstem neurons and motoneurons was performed using the fluorescent tracer Rhodamine-dextran or neurobiotin. The animals were anaesthetized and the retrograde tracer was then injected into either the spinal cord or muscle fibers in order to labeled descending brainstem neurons or motoneurons, respectively. Afterwards the animals were kept overnight to allow for retrograde transport of the tracer. For whole-mount imaging of the brain, a laser scanning confocal microscope was used.

3.2.5 Electrophysiology

Extracellular recordings were performed from the motor nerves. Two stimulation electrodes were used; one was placed on the dorsal part of the rostral spinal cord to elicit swimming and the other over the Mauthner cell region in the hindbrain to elicit escape. In some experiments, the application of drugs that interfere with endocannabinoid signaling, or a high divalent cation solution was restricted only to the spinal cord to avoid affecting escape command neurons in the brainstem. For this we used a split-bath recording chamber that was divided into two pools separated by a tight Vaseline- or low-melting agar (0.1%) barrier. The rostral pool contained the brainstem and rostral spinal cord and was always perfused with normal saline. The caudal pool contained the caudal spinal cord where motoneurons and peripheral nerves were

recorded, and was perfused with blockers of endocannabinoid signaling or with a high divalent cation solution.

Whole-cell recordings were performed from spinal V2a interneurons and motoneurons. Motoneurons were always recorded from the contralateral side of the stimulated Mauthner cell area. Subthreshold monosynaptic excitatory (EPSPs) and polysynaptic inhibitory (IPSPs) potentials as well as excitatory currents (EPSCs) were induced by stimulation of the Mauthner cell area in the brainstem. Different drugs were applied to the physiological solution to examine the effect of endocannabinoids or excitatory synaptic transmission.

3.2.6 Immunohistochemistry

Zebrafish were fixed in 4% PFA, washed in PBS solution with 1% Triton X-100, and blocked in 0.15% normal horse serum with 5% bovine serum albumin (BSA) and 1% Triton X-100 in PBS before incubation with the primary antibody overnight at 4°C. After rinsing in PBS, the preparations were incubated with a secondary antibody overnight at 4°C. Whole-mount imaging of the spinal cords was carried out in a laser scanning confocal microscope.

3.2.7 *In vivo* behavioral analysis.

A final volume of 2 µL was intraperitoneally injected in anaesthetized zebrafish to achieve a concentration of 1mg/kg for AM-251 and 4mg/kg for JZL184. The animals were moved back to the tank to recover from anaesthesia and experiments were performed 2-3 hours after the injection. For sound-induced escape, a sound generator and a high-speed camera were used to induce and record escape behaviors.

4 RESULTS AND DISCUSSION

In this thesis, four projects were performed to study the neural mechanisms underlying the generation and selection of motor behaviors in the spinal locomotor network.

4.1 NITRIC OXIDE GATES THE POLARITY OF ENDOCANNABINOID-MEDIATED SYNAPTIC PLASTICITY IN THE SPINAL LOCOMOTOR NETWORK

4.1.1 The identity of the endocannabinoids in the spinal locomotor network

Endocannabinoids are released within the lamprey spinal cord and participate in setting the baseline of the locomotor frequency (Kettunen *et al.*, 2005). However, the identity of these endocannabinoids has remained unknown. To determine the identity of the endocannabinoids in the spinal cord, the endocannabinoid 2-AG was applied to the lamprey spinal cord preparation. Application of 2-AG induced a long-term potentiation of the locomotor frequency by elevating the excitability of the entire network. This was achieved by increasing excitatory synaptic transmission and decreasing inhibitory synaptic transmission. To further confirm that 2-AG was the endocannabinoid released in the spinal locomotor network, specific inhibitors of 2-AG synthesis and degradation were added to the perfusion solution. Blockage of the 2-AG synthesis by tetrahydrolipstatin (THL) depressed the locomotor frequency by decreasing excitatory synaptic transmission and increasing inhibitory synaptic transmission. By contrast, inhibition of 2-AG degradation with JZL184 induced a long-lasting increase of the locomotor frequency by potentiating excitatory synaptic transmission and depressing inhibitory synaptic transmission. These results suggest that 2-AG is continuously synthesized and released during locomotion to maintain the excitability of the locomotor network.

There are two endocannabinoids in the nervous system, anandamide and 2-AG. We previously showed that the release of endocannabinoids requires activation of mGluR1 (Kyriakatos *et al.*, 2007; El Manira *et al.*, 2010), which activates phospholipase enzyme PLCβ to hydrolyze phosphatidylinositol into DAG. The enzyme, DGL, hydrolyzes DAG into 2-AG. Disrrupting the synthesis and degradation of the endocannabinoid 2-AG significantly affects the locomotor frequency, which is consistent with the modulatory effect of endocannabinoids on locomotion. These results suggest that 2-AG is the endogenous endocannabinoid acting as retrograde messenger to modulate the synaptic transmission in the spinal locomotor network.

4.1.2 The interaction between endocannabinoid and NO mediates synaptic plasticity

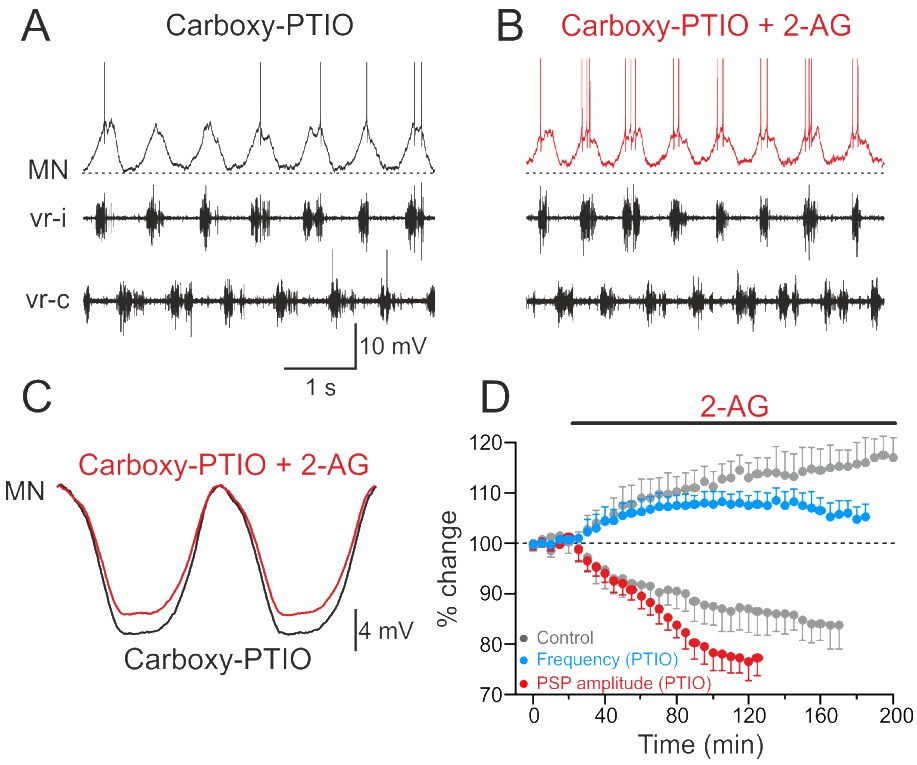

Figure 5. Nitric oxide contributes to the potentiation of the locomotor burst frequency by the endocannabinoid 2-AG. (A) Locomotor rhythm was induced by NMDA in the presence of the NO scavenger carboxy-PTIO and was recorded in opposing ventral roots. Intracellular recording from a MN that received on-cycle excitation in phase with the ipsilateral ventral root (vr-i) activity that alternated with midcycle inhibition occurring in phase with the contralateral ventral root (vr-c) burst. (B) Application of 2-AG in the presence of carboxy-PTIO was still able to increase the burst frequency that was associated with a decrease in the oscillation amplitude. (C) Averaged synaptic inputs received by the MN showing a decrease in the amplitude of the oscillation by 2-AG. (D) Averaged data from all the experiments showing the time course of the effect of 2-AG on synaptic transmission and the locomotor burst frequency in the presence of carboxy-PTIO. The decrease of the oscillation amplitude was more pronounced (red), while the potentiation of the locomotor burst frequency was attenuated (blue) in preparations preincubated with carboxy-PTIO compared with controls (gray). (From Paper I)

Previously, we showed that there is an interaction between endocannabinoids and NO, which mediates the synaptic plasticity induced by the mGluR1 activation (Kyriakatos *et al.*, 2007). Later we examined how NO interacts with the endocannabinoid 2-AG to modulate synaptic transmission in the spinal network. We found that the depression of inhibitory synaptic transmission induced by 2-AG does not require NO. However, the potentiation of excitatory synaptic transmission was NO dependent. These results suggest that NO is necessary for the potentiation of excitatory synaptic transmission by 2-AG. There is an interaction between 2-AG and NO that results in a more pronounced shift in the balance between excitation and inhibition within the locomotor circuit. This is further supported by the effect of scavenging NO on the potentiation of the locomotor frequency by 2-AG. Indeed, the NO scavenger carboxy-PTIO attenuated the 2-AG-induced potentiation of locomotor burst frequency. This suggests that an interplay between 2-AG and NO produces a more pronounced shift in the excitability of the spinal locomotor network and potentiates the locomotor frequency.

It was shown previously that inhibition of Nitric oxide synthase (NOS) decreases the locomotor frequency in the lamprey spinal cord (Kyriakatos *et al.*, 2009) and blocks the endocannabinoid-dependent potentiation of the locomotor frequency induced by mGluR1 activation. Now we showed that NO interacts with endocannabinoids to potentiate excitatory synaptic transmission. NO seems to act as a metamodulator to determine the polarity of the endocannabinoid-mediated excitatory synaptic transmission. The balance between excitatory and inhibitory synaptic transmission in the spinal locomotor CPG determines the speed and force of the locomotor output. The gating of the polarity of endocannabinoid-mediated synaptic plasticity by NO represents a novel mechanism by which the excitability balance is precisely defined within the locomotor CPG network and allows a more flexible execution of motor behavior.

4.2 NEURAL MECHANISMS OF BEHAVIORAL SELECTION BETWEEN ESCAPE AND SWIMMING

4.2.1 A hard-wired circuit mediates the switch between swimming and escape

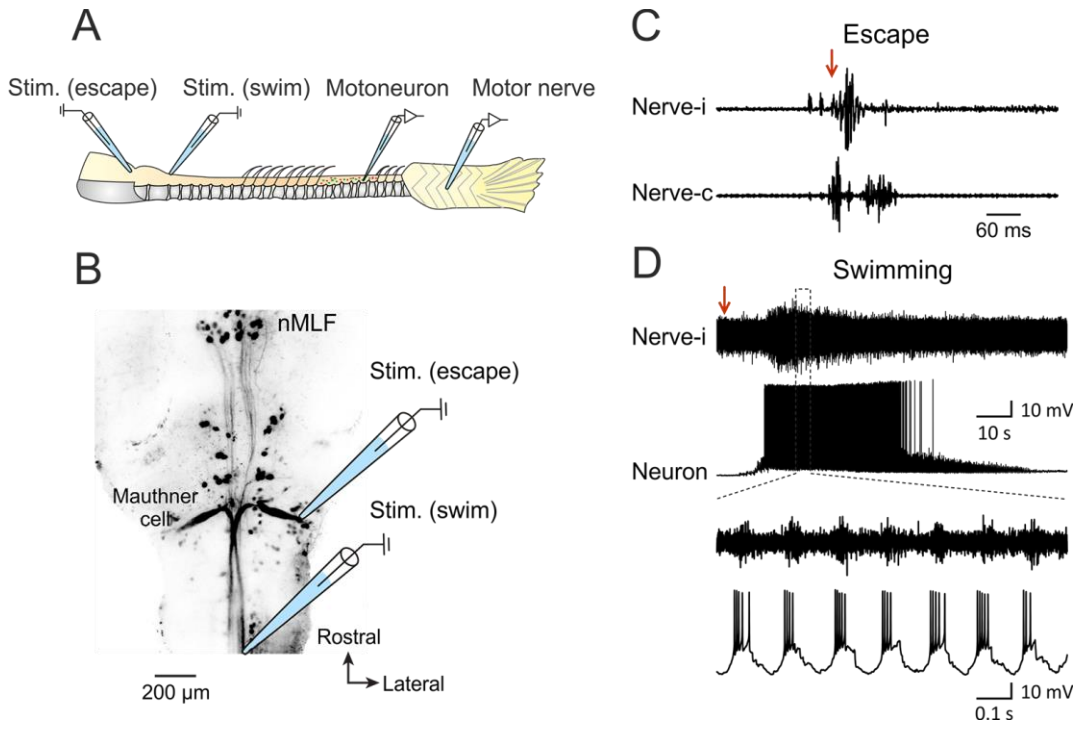

Figure 6. Experimental setup for electrophysiological studies. (A-B) Schematic drawing (A) and retrograde staining of mauther cells. (B) in adult zebrafish brain stem and spinal cord showing the stimulating (escape and swimming) and recording sites. (C) Characterization of the escape behavior. (D) Swimming activity extracellularly recorded from the motor nerve and a single whole-cell recorded neuron rhythmically firing at most cycles of swimming bouts. (Modified from Paper II)

A behavioral choice hierarchy favors vital motor actions and overrides competing ones through a context-dependent integration of sensory inputs. In freely swimming

zebrafish, escape overrides on-going swimming activity (Kyriakatos *et al.*, 2011). To investigate the neural underpinnings of this interaction, we developed an *in vitro* zebrafish brain stem-spinal cord preparation in which induced escape suppresses on-going swimming for a few cycles. We found that the behavioral selection between escape and swimming is mediated by switching between fast and slow motoneuron pools. The fast motoneuron pool underlying escape is engaged via direct excitation from the Mauthner system, while the slow motoneuron pool underlying swimming is disengaged via indirect inhibition that decouples these motoneurons from the premotor swim circuit. After escape, the swimming bursts always resumed without any resetting of the phase. This is further supported by the analysis of the synaptic currents received by slow motoneurons. The excitatory currents underlying the rhythmic depolarization of slow motoneurons were not affected by escape, which suggests the premotor CPG circuits are not inhibited by escape commands. These data suggest that the selection of escape over swimming is initiated by descending commands and executed by a local hardwired circuit in the spinal cord that disengages slow motoneurons while engaging fast motoneurons.

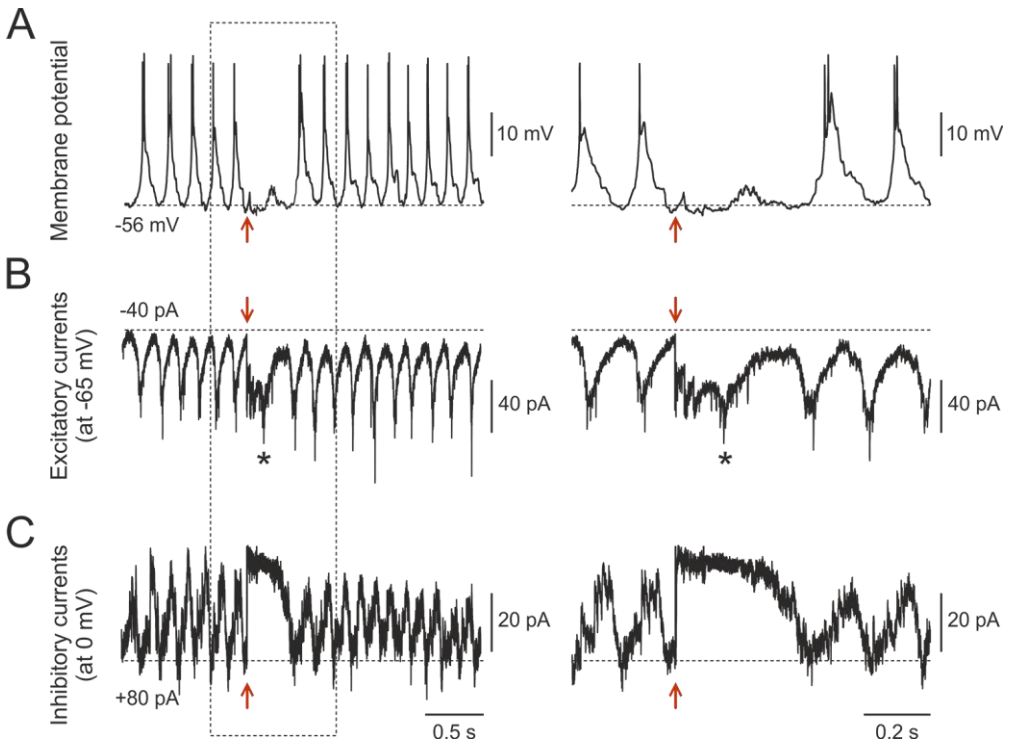

Figure 7. Escape suppresses swimming by inhibiting slow motoneurons. (A) Swimming activity recorded in a slow motoneuron in current-clamp. Escape (red arrow) produced a transient suppression of swimming-related membrane potential depolarization in the slow motoneurons. (B) The swimming-related rhythmic excitatory currents were not suppressed by escape and continue to occur (asterisk). The voltage-clamp recording is from the same neuron as in (A) with holding the membrane potential at -65 mV. (C) Escape induced a strong and long-lasting inhibitory current in the same motoneurons as in (A-B). The holding potential of the motoneuron was at 0 mV. (Modified from Paper II)

Although escape has the primacy over swimming in zebrafish, the transition from escape to swimming needs to occur in a smooth manner to continue the motion of the animal. Escape transiently stops the swimming motor output, which always resumes with the original frequency without disruption of the phase. The transient interruption

of swimming and its immediate recovery to the original frequency are achieved by directly disengaging slow motoneurons without influencing the activity of the premotor swim circuit, which continues to generate the swimming rhythm. In this manner, the basic rhythm is still generated by premotor swimming CPG circuits, but is no longer conveyed to the muscles, and hence no swimming activity is recorded. Our results reveal a novel mechanism for behavioral selection acting as a clutch-like to switch between slow and fast motoneuron pools without interfering with the premotor swim circuit.

4.2.2 Tonic endocannabinoid release promotes behavioral selection between escape and swimming

To examine if endocannabinoids modulate the behavioral selection between escape and swimming, we disrupted with endocannabinoid signaling. Blocking CB_1 receptors with AM-251 decreased the duration of the inhibition in slow motoneurons and also shortened the associated pause of swimming activity. Furthermore, polysynaptic IPSP amplitude in slow motoneurons was depressed with AM-251. Inhibiting 2-AG synthesis with THL also decreased the duration of swimming inhibition in slow motoneurons by inhibiting polysynaptic IPSPs in slow motoneurons. Conversely, blocking the 2-AG degradation with JZL184 increased the suppression of swimming by potentiating the inhibitory synaptic transmission in slow motoneurons. Furthermore, endocannabinoids regulated the threshold of firing of fast motoneurons by enhancing both electrical and chemical excitatory synaptic transmission induced by escape command. These results show that the hardwired circuit is supplemented by an embedded endocannabinoid modulation that defines the extent of swimming suppression and modulates the threshold of initiation in escape. The change in the duration of the swimming pause was similar whether AM-251, THL or JZL184 were applied to the whole brainstem-spinal cord preparation or only to the caudal spinal cord, suggesting that the endocannabinoid modulation occurs in the spinal cord, not in the brain stem.

The release of endocannabinoids in the CNS requires increased intracellular Ca^{2+} levels (Alger, 2002; El Manira *et al.*, 2008; Alger, 2009; Kano *et al.*, 2009; El Manira *et al.*, 2010; Cachope *et al.*, 2012; Castillo *et al.*, 2012; Ohno-Shosaku *et al.*, 2014; Younts *et al.*, 2014). Dialyzing a given fast motoneuron with either THL or BAPTA prevented the tonic endocannabinoid modulation of the direct excitation, indicating that the released 2-AG originates entirely within the same motoneuron. This also shows that there is no spread of the endocannabinoid 2-AG from neighboring motoneurons. However, applying the same treatment to slow motoneurons did not alter the effect of endocannabinoids on indirect inhibitory synaptic transmission in these motoneurons. These results suggest that there is a tonic synthesis and release of the endocannabinoid 2-AG from fast motoneurons which acts as a positive feedback mechanism to potentiate the monosynaptic EPSPs and hence lower the threshold for escape initiation. The modulation site for IPSPs in slow motoneurons could reflect increased excitatory synaptic transmission to the intercalated inhibitory interneurons, which could be the

source of the tonic release of 2-AG. Our results also suggest that there is a limited spread of endocannabinoids between different neurons within the spinal cord, which makes it unlikely that slow motoneurons are affected by endocannabinoids from fast motoneurons. This is in agreement with results in the hippocampus where the spread of endocannabinoids has been estimated to be limited to the width of the extracellular space (<15 µm; (Alger, 2012)).

4.3 MODULAR ORGANIZATION OF THE SPINAL LOCOMOTOR CPG

4.3.1 Selective connectivity pattern between V2a interneurons and motoneurons

An important source of excitation within the locomotor network is V2a interneurons (Eklöf-Ljunggren *et al.*, 2012). These interneurons in the adult zebrafish spinal cord are recruited in an incremental manner following an increase in swimming frequency (Ausborn *et al.*, 2012). Similar to the motoneurons, a Gaussian mixture model analysis of their minimum recruitment frequencies indicates that there are three main clusters of V2a interneurons. Both V2a interneurons and motoneurons are organized in three distinct sub-classes that are sequentially engaged during swimming activity as speed increases from slow to intermediate and fast. To test if there is a selective connectivity pattern between V2a interneurons and motoneurons, we mapped the connectivity by using dual whole-cell patch-clamp recordings. The strength and distribution matrix of monosynaptic EPSPs clearly showed strong and reliable connections between V2a interneurons and motoneurons of functionally related modules but only weak and sparse connections between interneurons and motoneurons of modules with adjacent recruitment frequencies. There is a clear segregation of the cumulative distribution of the EPSP amplitude from connections within the same module compared to connections between different modules.

The ‘‘size principle’’ assumes that all motoneurons receive uniformly distributed excitatory inputs from a common premotor source and that the order of recruitment is dictated by their input resistance (Heckman *et al.*, 2012). In contrast to the ‘‘size principle’’ assumption, we show that the locomotor network does not consist of a uniform unit, but can be deconstructed into three microcircuit modules. Each module includes a distinct sub-class of excitatory V2a interneurons that make selective monosynaptic connections with slow, intermediate, or fast motoneurons. These data suggest that the spinal locomotor network is composed of three discrete microcircuit modules with selective and strong synaptic connections between, and within, the three modules.

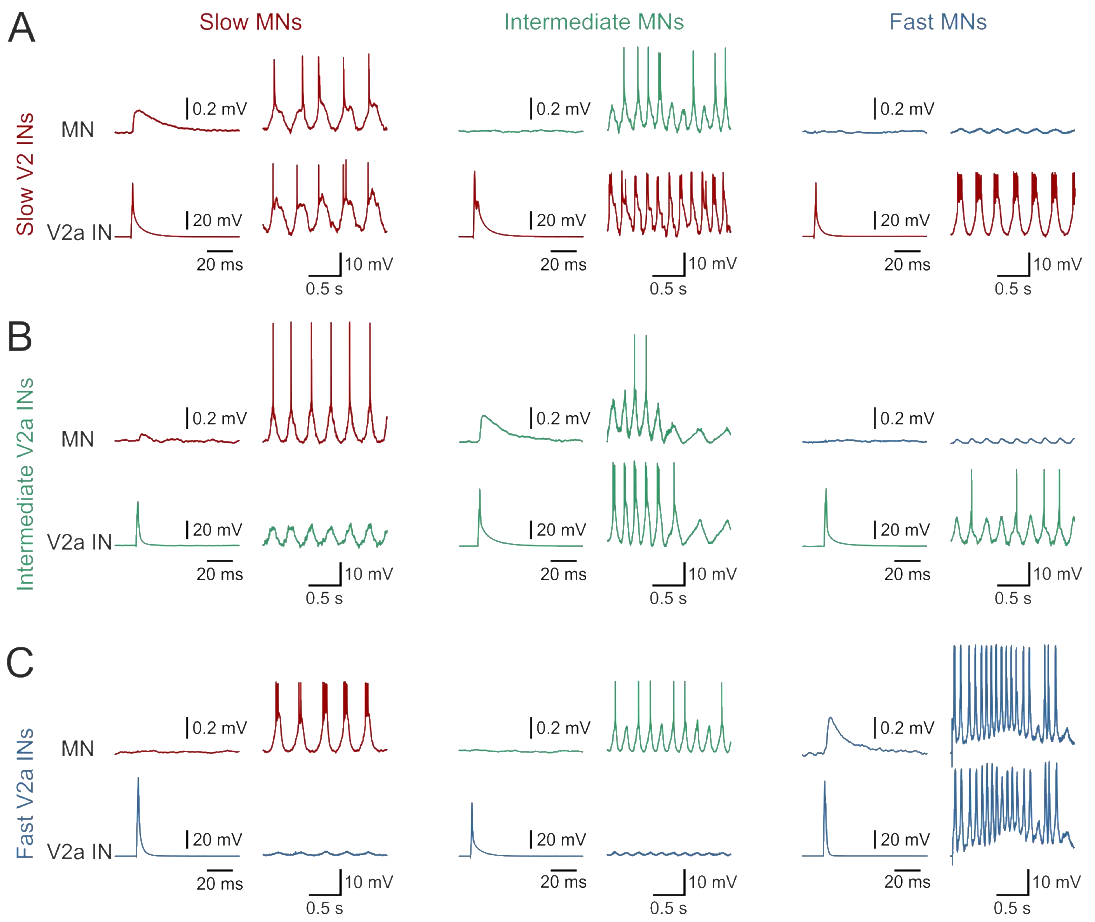

Figure 8. Modular microcircuit organization of the locomotor network. (A) Left panel: paired recordings showing the existence of a strong monosynaptic connection between a slow V2a interneuron (red) and a slow motoneuron (red) that were simultaneously recruited during swimming at slow speeds. Middle panel: paired recordings from a slow V2a interneuron (red) and an intermediate motoneuron (green) without any detectable monosynaptic connections. Right panel: paired recordings from a different slow V2a interneuron (red) and a fast motoneuron (blue) that were not monosynaptically connected. (B) Left panel: paired recordings showing the existence of a weak monosynaptic connection between an intermediate V2a interneuron (green) and a slow motoneuron (red) that were not simultaneously recruited during swimming at slow speeds. Middle panel: paired recordings a intermediate V2a interneuron (green) and an intermediate motoneuron (green) showing a strong monosynaptic EPSP. These two neurons were recruited at intermediate swimming frequencies. Right panel: paired recordings from a different intermediate V2a interneuron (green) and a fast motoneuron (blue) that were not monosynaptically connected. (C) Left panel: paired recordings showing the lack of monosynaptic connections between a fast V2a interneuron (blue) and a slow motoneuron (red) that were not simultaneously recruited during swimming at slow speeds. Middle panel: paired recordings showing the lack of monosynaptic connections between a fast V2a interneuron (blue) and an intermediate motoneuron(green). Right panel: paired recording from a different fast V2a interneuron (blue) and a fast motoneuron (blue) that were strongly connected. These two neurons were recruited simultaneously at fast swimming frequencies. (Modified from Paper III)

4.3.2 Speed control is mediated by the modular organization

To understand how the modular organization governs the speed control, we analyzed the firing onset of V2a interneurons and motoneurons during swimming activity. The onset of action potential firing in slow, intermediate, and fast V2a interneurons always occurred prior to that of the corresponding motoneurons during locomotion. There is a sequential recruitment of V2a interneurons and motoneurons, in which V2a interneurons are recruited first and then recruit the motoneurons to produce the swimming activity. Analysis of excitatory drives in both V2a interneurons and

motoneurons showed a linear relationship, which suggests that the modular organization of V2a interneurons and motoneurons, combined with their overlapping activation during swimming ensures a smooth transition between locomotor speeds during acceleration or deceleration by sequentially engaging or disengaging successive microcircuits.

4.4 ELECTRICAL COUPLING EXTEND THE INFLUENCE OF MOTONEURONS ONTO THE PREMOTOR CIRCUIT GENERATING THE LOCOMOTOR RHYTHM

4.4.1 Gap junctions between motoneurons and premotor V2a interneurons

To examine if electrical coupling exists between motoneurons and the premotor V2a excitatory interneurons, three complementary sets of experiments were performed. First, the occurrence of dye coupling from motoneurons to V2a interneurons was revealed by injection of neurobiotin into muscles. This led to the labeling of V2a interneurons via dye coupling from the retrogradely labeled of motoneurons. Second, using immunohistochemistry, connexin 35/36 was localized on the dendrites of motoneurons in close apposition to the presynaptic axons of V2a interneurons. Moreover, we also showed that in the dendrites connexin 35/36 was present in close proximity to synaptophysin-labeled presynaptic terminals. Thirdly, the occurrence of electrical coupling was confirmed using whole-cell recordings from pairs of V2a interneurons and motoneurons. There was a bidirectional electrical communication between V2a interneurons and motor neurons. Further we showed that the electrical coupling occurs only in pairs of V2a interneurons and motoneurons connected via chemical synaptic transmission. These results show that gap junctions occur between the presynaptic terminals of V2a interneurons and the dendrites of motor neurons. This enables propagation of electrical signals backwards from motoneurons to the upstream excitatory V2a interneurons.

Electrical synapses occur in the adult vertebrate spinal cord (Sotelo *et al.*, 1972; Rash *et al.*, 1996; Chang *et al.*, 1999; Parenti *et al.*, 2000), however their function has remained unclear. To address this, we tested if the backward propagation of electrical signals from motoneurons influences synaptic transmission from V2a interneurons. A steady depolarization of the membrane potential in the recorded motoneurons enhanced the EPSPs, while a hyperpolarization depressed the EPSPs. Importantly, in the pairs that were only connected via chemical synapses, the amplitude of the EPSPs was not affected by steady changes in the membrane potential of the motoneurons. There was a linear relationship between the coefficient of electrical coupling between the motoneurons and the V2a interneurons and the changes in the EPSP amplitude. Moreover, in pairs connected by gap junctions, a change of the motoneurons membrane potential altered the firing threshold of the presynaptic V2a interneurons. A steady hyperpolarization of the motoneurons significantly increased the firing threshold of the V2a interneurons and inhibited repetitive firing. Conversely, a steady depolarization of

the motoneurons decreased the firing threshold of the V2a interneurons and increased their repetitive firing. This suggests that motoneurons have the capacity to modulate the firing of premotor V2a interneurons which are the source of excitation driving swimming activity in zebrafish.

These results show that gap junctions enable backward propagation of analog electrical signals from motoneurons to premotor V2a interneurons, and that this contributes to defining their synaptic strength and firing threshold.

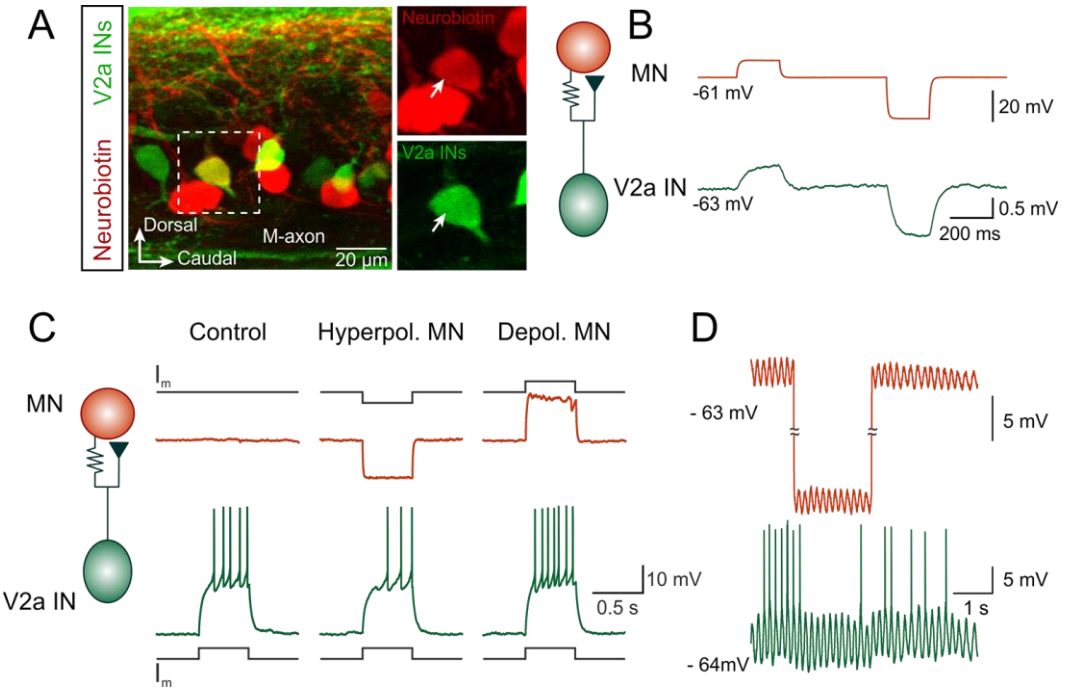

Figure 9. Motoneurons influence the firing threshold and the recruitment of V2a interneurons. (A) Dye coupling between motoneurons and V2a interneurons in the adult zebrafish spinal cord. (B) Dual patch-recordings showing the fluctuation of membrane potential in a motoneuron affecting that in a V2a interneuron. (C) The firing of a V2a interneuron was strongly influenced by changes in the membrane potential of the electrically coupled motoneuron. (D) In the electrically coupled pair of a V2a interneuron and a motoneuron, a steady hyperpolarization of the membrane potential in the MN reversibly prevented the firing in the V2a IN, which displayed only subthreshold membrane potential oscillations. (Modified from Paper IV).

4.4.2 Motoneurons influence premotor rhythm generation through gap junctions

Motoneurons are traditionally considered to be the final common pathway for the execution of the command from the premotor locomotor network (Kiehn, 1991; Brownstone, 2006; Heckman *et al.*, 2012). However, their strong influence on V2a interneurons prompted us to reassess their role in the locomotor rhythm generation. Whole-cell recordings were made from pairs of V2a interneurons and motoneurons connected with gap junctions. Both the V2a interneurons and the motoneurons were rhythmically active during locomotion. Remarkably, hyperpolarization of the membrane potential in the motoneurons reversibly inhibited the V2a interneurons, which were de-recruited and displayed only subthreshold rhythmic synaptic activity.

These results revealed a prominent role for motoneurons in defining the recruitment threshold of the V2a interneurons, which are a pivotal component of the rhythm generator circuit. Next we examined if acute inhibition of motoneurons during ongoing locomotor activity alters features of the locomotor rhythm generation. During ongoing locomotor activity, optogenetic inhibition of motoneurons reversibly de-recruited some V2a interneurons. At the circuit level, optogenetic inhibition of motoneurons significantly decreased the burst frequency and the duration of the locomotor activity.

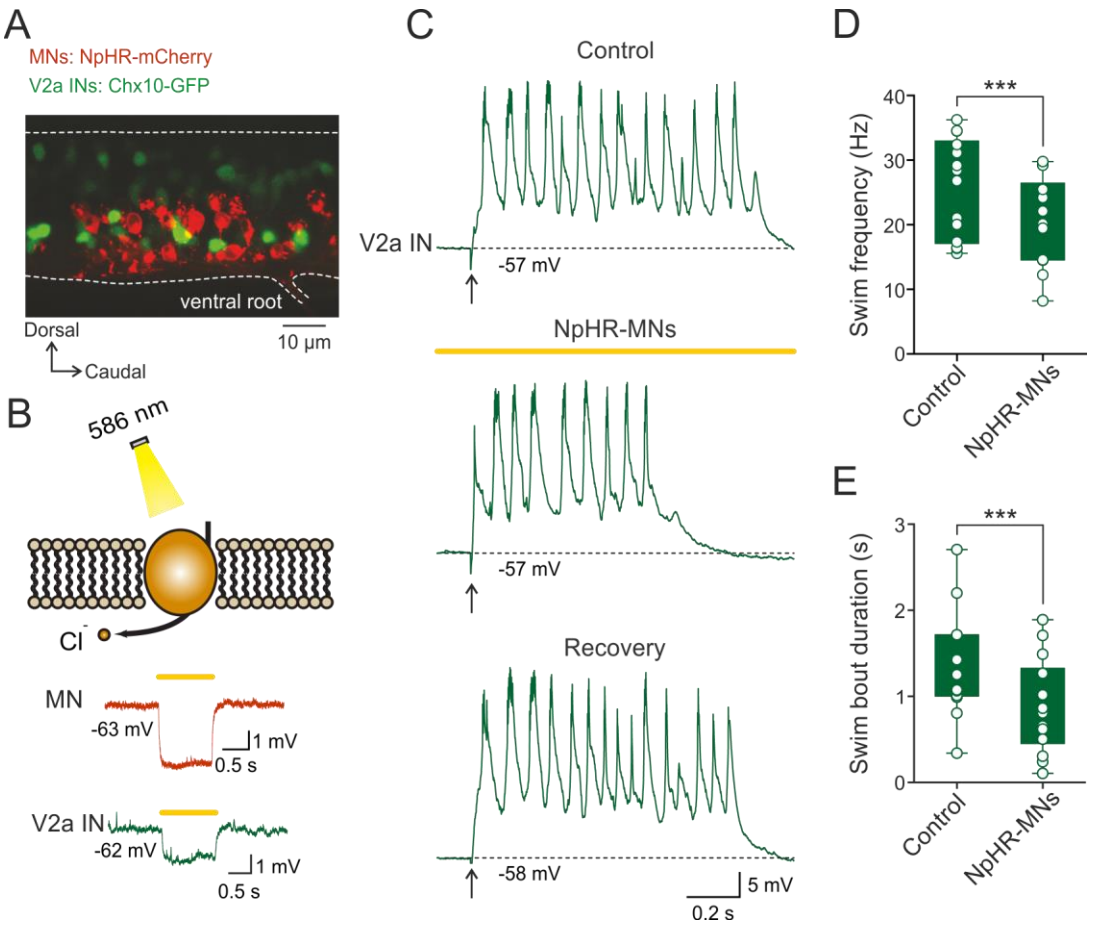

Figure 10. Optogenetic inhibition of motor neurons decreases the frequency of locomotion. (A) A transgenic zebrafish line was used in which halorhodopsin (NpHR-mCherry) was expressed in motoneurons while GFP was expressed in V2a interneurons. (B) Yellow light stimulation induced hyperpolarization in the membrane potential of the motoneurons and their electrically coupled V2a interneurons. (C) In most of the preparations, long yellow light stimulation decreased the burst frequency and duration of the swimming bout. (D) Graph showing the decrease in the swimming burst frequency by optogenetic inhibition of motor neurons. (E) Graph showing the decrease in the swimming bout duration by optogenetic inhibition of motoneurons. (Modified from Paper IV)

Until now, the only reported path for motoneurons to influence the spinal central pattern generator has been to activate Renshaw cells, which produce recurrent inhibition (McCrea *et al.*, 1980; Noga *et al.*, 1987; Kiehn, 2006). However, this pathway is not considered to contribute to the generation of the locomotor rhythmicity (Noga *et al.*, 1987; Zhang *et al.*, 2014). An important function of electrical coupling via gap junctions is to synchronize the activity of neuronal ensembles. In the spinal cord, gap junctions have been thought to play a role during the early development of spinal

circuits (Walton *et al.*, 1991; Frank, 1993; Saint-Amant *et al.*, 2000), in the specification of neuron types and in circuit formation (Chang *et al.*, 2000; Tresch *et al.*, 2000; Zhong *et al.*, 2010). In addition, they can synchronize the activity of motoneurons to produce rhythmic activity (Sillar *et al.*, 1994; Tresch *et al.*, 2000). We now demonstrate a novel and direct path that mediates a simultaneous and continuous integration of the ongoing activity of the motoneurons with the upstream excitatory interneurons. The existence of electrical coupling between motoneurons and excitatory V2a interneurons suggests that a major revision of the traditional view of the construction of locomotor circuits is required. Rather than acting as feed-forward units, the backward transfer of electrical signals via electrical coupling unites motoneurons and interneurons into functional ensembles endowed with an analog processing essential for the elaboration of the locomotor rhythm.

5 CONCLUDING REMARKS

This thesis focuses on studying the fundamental neural circuits of swimming and escape as well as the endocannabinoid modulation in these circuits. Detailed neural mechanisms that underlie neural control of swimming speed and the choice of escape overriding swimming are suggested. These mechanisms involve not only the basic neuronal circuits of the spinal swimming and escape networks, but also the modulatory effect of endocannabinoids on these networks.

Revealing the neural circuit substrates and mapping the connectivity pattern among network neurons is fundamental to understand the basic network structure underlying the neural control of motor behavior and the behavioral selection. This would inform us exactly how the neural networks operate with respect to the behavior – movement pattern, speed control or behavioral selection. Although the neural circuits and the connectivity patterns differ for different behaviors, the connections among the network neurons are mediated through chemical synapses, electrical synapses or both. Neuromodulators in the neural circuits can dynamically modulate the connectivity of network neurons via tuning the strength of synaptic transmission by acting on presynaptic or postsynaptic components. The modulatory effects occur at the level from single cells to neural circuits, which can have a profound influence on the physiological state and the behavioral output. In general, the fundamental structure of neuronal networks together with adjustable modulatory systems generates flexible behaviors and promotes appropriate behavioral selection in response to the environmental requirements.

How can our results from the zebrafish be applied to other nervous systems? Firstly, the modular organization of premotor V2a interneurons and motoneurons explains how the spinal premotor network channels excitation to motoneurons, a finding that might apply also to interneurons mediating reciprocal inhibition. Furthermore, in mammals, each limb contains a few muscles, which work in a coordinated way to perform the movement. As the UBG concept suggests, muscles in the given joint share the same central pattern generator. These muscles contain a mixture of slow, intermediate and fast muscle fibers, which might also be driven by a modular organized network. Secondly, a clutch-like mechanism in the spinal locomotor circuit of zebrafish has been revealed to control the behavioral selection between escape and swimming. In vertebrates, different spinal descending inputs govern distinct behaviors. Behavioral selection might also involve a competition of the related descending pathways. The overall organization of the vertebrate locomotor system is highly conserved and a clutch-like mechanism might help to explain the neural mechanisms underlying behavioral selection also in other vertebrates. Thirdly, gap junctions enable the motoneurons to mediate the activity of premotor interneurons and adjust the locomotor frequency. This finding may also apply to other vertebrates.

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