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LOCOMOTOR PATTERN GENERATION IN THE SPINAL CORD: STUDIES IN ADULT LAMPREY AND ZEBRAFISH

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Dedicated to my mother (Hasina Aktar Begum) and my father (Suruj Alam Chowdhury): Without them I wouldn’t be here

ABSTRACT

The overall objective of this thesis is to characterize the mechanisms involved in the generation of locomotor activity in the spinal cord. To this end, we initially used the lamprey spinal cord to determine the transmitter phenotype of commissural interneurons (CINs). In addition, we developed a novel preparation of the brainstem-spinal cord of the adult zebrafish and used it to determine the mechanisms of locomotor pattern generation. The results obtained show:

(1) That the left–right alternation is maintained because commissural glycinergic interneurons outnumber the glutamatergic ones. It was also shown that CINs display a graded rostrocaudal distribution and are immunoreactive to both glycine and glutamate. The difference in the proportion of inhibitory and excitatory CINs represents an anatomical substrate that ensures the predominance of alternating activity during locomotion.

(2) That adult zebrafish spinal cord can produce locomotor activity and be used to study the organization of the locomotor circuitry. In this study we developed both a semi-intact and an *in vitro* preparation of the juvenile/adult zebrafish spinal cord that are able to generate a rhythmic motor pattern with characteristics similar to swimming in intact animals. In the *in vitro* preparation, spinal cord neurons were accessible for patch-clamp recordings to study their pattern of activation during fictive locomotion.

(3) That 5-HT is released within the locomotor circuitry and acts as an intrinsic modulator to set the baseline locomotor activity. 5-HT decreases the frequency of the locomotor rhythm by increasing the mid-cycle inhibition and delaying the onset of the following on-phase excitation. Thus endogenous 5-HT sets the balance between excitation and inhibition and set the baseline locomotor frequency.

(4) That a brief stimulation of descending inputs at a defined region located at the first segments of the spinal cord induces long-lasting coordinated swimming activity. The burst amplitude, frequency and duration of the episode can increase by changing the frequency and strength of the stimulus pulses. The descending inputs seems to act as a switch to turn on the activity of the spinal locomotor network in the caudal spinal cord that relies mostly on iontropic glutamate receptors.

In summary, our results provide anatomical evidence underlying the dominance of reciprocal inhibition over excitation during locomotion. We also showed that our newly developed *in vitro* adult zebrafish spinal cord preparation can be used to study spinal circuitry underlying locomotion.

Key words: lamprey, spinal cord, CPG, glycine, glutamate, GABA, zebrafish, NMDA, strychnine

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III.	Gabriel JP, Ausborn J, **Mahmood R**, Ampatzis K, Eklöf-Ljunggren E, and El Manira A. (2009) Principles governing recruitment of motoneurons during swimming in zebrafish. Nat. Neurosci. 14(1): 93-9.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AHP	afterhyperpolarization
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
beo	*bandoneon*
CNS	central nervous system
CPG	central pattern generator
CCINs	contralateral and caudally projecting interneurons
CiD	circumferential descending
CINs	commissural interneurons
CoBL	commissural bifurcating longitudinal
dINs	descending interneurons
DCs	dorsal cells
DLR	diencephalic locomotor region
ECs	edge cells
EINs	excitatory interneurons
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
EMG	electromyogram
GABA	gamma-amino butyric acid
GIs	giant interneurons
GPCRs	G-protein coupled receptors
ir	immunoreactivity
INs	interneurons
INs	inhibitory interneurons

IPSC	Inhibitory post synaptic current
IR-DIC	infrared differential interference contrast
LINs	lateral interneurons
LTP	long term potentiation
mGluR	metabotropic glutamate receptor
MLR	mesopontine locomotor region
MNs	motoneurons
MCoD	multipolar commissural descending
NMDA	N-methyl-D-aspartate
PB	phosphate buffer
PBS	phosphate buffered saline
pMNs	primary motoneurons
sAHP	slow afterhyperpolarization
SK channel	small conductance Ca^{2+}-activated K^{+} channel
TTX	tetrodotoxin
VeMe	ventromedial
X-PBS	triton-X in PBS

1 INTRODUCTION

1.1 Central pattern generator: a historical perspective

Motor tasks are the key element of the behavioral repertoire of all animals (Grillner, 1975; Orlovsky et al., 1999). Although animals exhibit complex motor activity, some of the simplest forms of motor behavior require the integrated activity of a diverse set of neural networks. These behaviors include breathing, mastication, scratching and locomotion that are well studied in both 'vertebrate' and 'invertebrate' model systems (Roberts et al., 1981; Keifer and Stein, 1983; Ramirez and Richter, 1996; Roberts et al., 1998; Marder and Bucher, 2001; Grillner, 2003; Marder et al., 2005; Grillner, 2006; Kiehn, 2006; Lund and Kolta, 2006; McDearmid and Drapeau, 2006; Fetcho et al., 2008; Roberts et al., 2008).

There have been long standing efforts to understand how the nervous system is organized and functions to perform various motor behaviors. At the beginning of the last century, Sherrington (Sherrington, 1906) published the monograph 'The Integrative Action of Nervous System' where he outlined the idea that complex motor behaviors, including locomotion, were generated by chained reflexes. Later on, Brown (Brown, 1911) selectively eliminated virtually all sensory inputs and was able to show that animals so treated could still produce alternation between a flexor and an extensor muscle around one joint. These experiments led to the first explicit proposal for a centrally generated rhythmic activity (Brown, 1914). In contrast to the chained reflex model proposed by Sherrington, Brown proposed that the circuits within the spinal cord could produce the motor patterns for walking in the complete absence of sensory feedback. His 'half-center' model is based on reciprocal inhibition between two flexor and extensor networks. Subsequently, Brown's initial concept has been generalized to give the definition of a CPG; a network of neurons that is capable of generating an organized pattern of motor activity without any inputs from sensory afferents (Grillner and Zangger, 1979). For many years research was aimed at either supporting whether either chains of reflexes or central oscillators produced rhythmic movements. The central oscillators theory stood out when Wilson (Wilson, 1961) showed the most compelling evidence that the isolated CNS of the locust was capable of producing rhythmical motor pattern. Using deafferented cats, it was shown for the first time that the CPG responsible for hindlimb locomotion is localized in lumber spinal cord that maintains both rhythm and coordinated activity in multiple joints required for locomotion (Grillner and Zangger, 1979). They also demonstrated that despite the absence of sensory feedback neither locomotion nor coordination between different muscle groups is disrupted.

The concept of central pattern generation for locomotion for vertebrates has been further corroborated in spinal dogfish (Grillner et al., 1976) and *in vitro* lamprey spinal cord preparations (Cohen and Wallen, 1980; Poon, 1980), where it has been shown that locomotor activity can be generated in the absence of any higher or afferent inputs. Indeed, by isolating the lamprey spinal cord *in vitro* and applying pharmacological or electrical stimulation of the CPG it has been possible to generate rhythmical output in the ventral roots. The recorded motor pattern is known as fictive locomotion, which is the neural correlate of locomotion (Poon, 1980; Wallen and Williams, 1984). Today, many preparations have been shown to generate 'fictive motor patterns' that would normally drive muscle movements. Some of these include: the cat (Deliagina et al., 1981) the Xenopus tadpole (Roberts et al., 1981), the neonatal mouse and rat (Cazalets et al., 1995; Kiehn, 2006), the salamander (Ryczko et al.) and larval zebrafish (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006).

In my thesis I will describe a new model system to study fictive locomotion, which is developed exclusively in our laboratory.

1.2 The swimming CPG

Studies in lamprey, Xenopus and zebrafish have provided key insights into the organization and synaptic connections that produce swimming movements (Dale and Kuenzi, 1997; Roberts et al., 1998; Grillner, 2003; McDearmid and Drapeau, 2006; Fetcho and McLean, 2010; Gabriel et al., 2010). While the swimming movement is much simpler and differs markedly from complex limb-dependent motor behavior seen in terrestrial vertebrates, the fundamental underlying organization and neuronal makeup of the locomotor system is remarkably conserved (Grillner, 2003; Kiehn, 2006). Because of this, better understanding of the functional organization of the swimming CPG will give us important clues about the network structure of the locomotor CPG in higher vertebrates.

Here I will give a brief overview regarding the motor pattern and synaptic interactions of some of the vertebrate model systems that are being used to study the locomotor CPG. I will describe briefly first the lamprey whose central nervous system is regarded as a vertebrate prototype (Grillner, 2003) and as I go along I will draw comparisons of motor patterns between different vertebrates with special emphasis on two other swimming CPGs, namely the Xenopus tadpole and the zebrafish. The idea is to show how phylogenetically different models show remarkable similarity in terms of cellular and motor pattern and also to provide the foundation for future studies on the cellular architecture of the spinal locomotor network in adult zebrafish.

1.2.1 The lamprey

Lampreys are jawless vertebrates known as cyclostomes that diverged from the main evolutionary vertebrate line around 450 million years before the appearance of ordinary fish. They have since then changed comparatively little during evolution. They share numerous anatomical features with higher vertebrates like basal ganglia, brainstem, spinal cord, sensory organs, and motor apparatus in many respects (Rovainen, 1979; Nieuwenhuys et al., 1998; Jones et al., 2009). The functional and cellular organization of the locomotor control mechanisms are also similar (Orlovsky et al., 1999). Lamprey provides good opportunities for studying neural networks controlling different motor functions. They have fewer nerve cells compared to higher vertebrates (Rovainen, 1979); and the spinal cord can be maintained *in vitro* for several days and generate the rhythmic pattern underlying locomotion (Cohen and Wallen, 1980; Wallen and Williams, 1984; Grillner and Wallen, 2002), In addition the fairly good transparency allows for identification of individual neurons for morphological (Ohta et al., 1991; Buchanan, 2001), electrophysiological (Buchanan, 1982, 2001; Cangiano and Grillner, 2003, 2005; Biro et al., 2008; Kyriakatos et al., 2009) and histochemical (Shupliakov et al., 1996; Pombal et al., 2003; Viloria et al., 2008) analysis.

The lamprey swims by producing a mechanical wave that is transmitted along the body with a frequency range from 0.1 to 10 Hz (Brodin et al., 1985; Grillner, 2003). Locomotion can be initiated by stimulation of a diencephalic (DLR) and a mesopontine locomotor regions (MLR) (El Manira et al., 1997; Sirota et al., 2000; Dubuc et al., 2008; Menard and Grillner, 2008). These two areas project independently and monosynaptically to reticulospinal neurons which in turn activate the spinal CPG to produce locomotor activity (Grillner, 2003).

1.2.2 Classes of neurons in the lamprey spinal cord

The lamprey spinal cord possess uniquely identifiable (Buchanan, 2001) classes of spinal neurons defined on the basis of morphological and physiological characteristics such as axonal projection, placement and size of the soma, and synaptic properties. These are motoneurons (MNs), lateral interneurons (LINs), contralateral and caudally projecting interneurons (CCINs), excitatory interneurons (EINs), commissural interneurons (CINs), giant interneurons (GIs), sensory dorsal cells (DCs) and stretch or mechanosensitive edge cells (ECs). The number of MNs vary from species to species but on an average there is around 100/hemisegment (Rovainen and Dill, 1984). While MNs may form collateral connections within the spinal cord (Buchanan, 1999b), it is not clear if they have a

functional role in the CPG (Rovainen, 1983; Wallen and Lansner, 1984; Quinlan et al., 2004). LINs are inhibitory (50-100 per animal) and located in the rostral part of the spinal cord (Rovainen, 1974; Selzer, 1979). They inhibit the ipsilateral CCINs with appropriate delay which would then release the contralateral side from inhibition (Buchanan, 2001). The CCINs have been estimated to be as few as 10 (Ohta et al., 1991) or between 10-45 per hemisegment (Buchanan, 1982). They can be both excitatory and inhibitory. Their main axonal branch projects contralaterally and caudally from their rostrally positioned cell body. The membrane potentials of CCINs show strong modulation during swimming and these cells are thought to play a key role in motor coordination (Buchanan, 2001).

EINs have been identified electrophysiologically using paired recordings of the presynaptic interneurons and postsynaptic motoneurons and interneurons (Buchanan and Grillner, 1987; Buchanan et al., 1989). Intracellular dye injection showed that the soma diameter of these cells is around 10 μm in their short axis. The axons of EINs may project up to 9 segments caudally (Dale, 1986), but most appear to be much shorter (Buchanan et al., 1989). They are probably quite numerous because, despite their small soma size, they are encountered more frequently with random microelectrode impalements in the grey area of the spinal cord. The membrane potentials of these interneurons are strongly modulated during swimming (Buchanan et al., 1989) and because of that they are thought to impart much of the phasic excitatory drive to motoneurons and interneurons during swimming. Their postsynaptic targets include motoneurons, LINs, CCINs and other EINs (Buchanan et al., 1989). The CINs project both rostrally and caudally in the contralateral side and display a substantial morphological diversity with regard to their soma size and extent of their axonal projection (Buchanan, 2001). They are both inhibitory and excitatory (Ohta et al., 1991; Buchanan, 2001). The excitatory glutamatergic CINs mediate the synchronus locomotor pattern which is unmasked after blocking of predominant glycinergic coupling between two sides of the spinal cord (Cohen and Harris-Warrick, 1984). The glycinergic CCINs and CINs are thought to be major providers of inhibition that ensures left-right alternation of the motor pattern (Cohen and Harris-Warrick, 1984; Ohta et al., 1991; Buchanan, 2001). The giant INs, edge cells and dorsal cells have sensory function (Grillner et al., 1982; Grillner et al., 1983; Rovainen, 1983; Grillner et al., 1984) and thus do not play a role during fictive locomotion (isolated preparation), but only in the freely behaving animal.

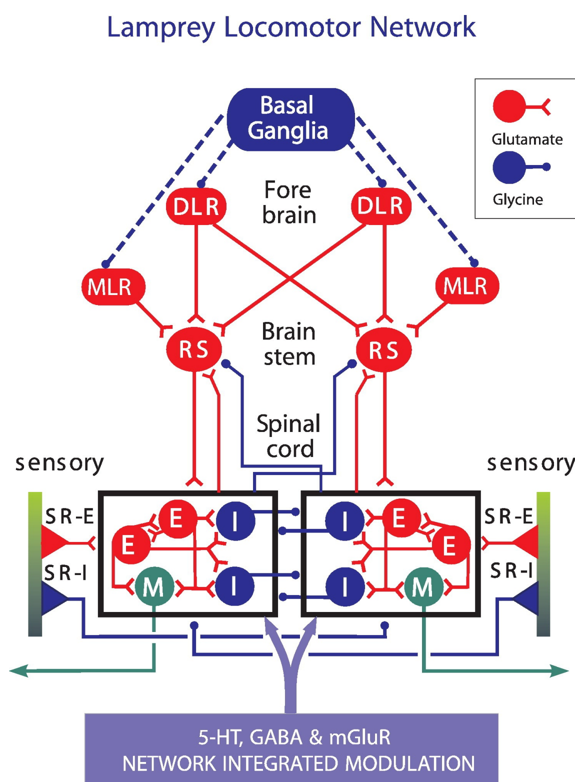

Figure 1: Schematic diagram of the lamprey locomotor network. Selection of locomotor activity takes place in the basal ganglia. Initiation of locomotion is done by disinhibition of the mesencephalic locomotor region (MLR) and the diencephalic locomotor region (DLR) which in turn excite reticulospinal neurons (RS) that provide descending excitation to the spinal CPG neurons and motoneurons. Excitatory stretch receptor neurons (SR-E excite ipsilateral neurons and inhibitory stretch receptor neurons (SR-I) inhibit the contralateral neurons after crossing the midline. In addition neuromodulators also make up an integral part of CPG. (adapted from Grillner et al., 2008)

1.3 Swimming motor pattern

The lamprey and other bony fishes including zebrafish as well as swimming salamanders and frog tadpoles share some common features in their basic motor pattern (Grillner, 1974; Cohen and Wallen, 1980; Fetcho and Svoboda, 1993; McDearmid and Drapeau, 2006; Ryczko et al., 2010). They show left-right alternation which produces lateral undulation of the body, The burst duration occupies nearly one half of the cycle period which means alternating activity with a 50% contralateral phase difference. They display a rostrocaudal wave of activity originating from head that propagates along the body; towards the tail during swimming. With faster swimming, the cycle time decreases proportionately with the burst duration in each segment, hence remaining roughly a constant fraction of the cycle period. There is an exception of this feature seen in larval zebrafish which might result from undersampling of the overall activity during swimming (Masino and Fetcho, 2005). The rostrocaudal phase lag remains largely constant with relation to cycle period. In

lampreys the phase lag is 1% of the cycle period which results in a complete traveling wave over the approximately 100 body segments (Grillner and Matsushima, 1991).

1.3.1 NMDA induced motor pattern

The excitatory amino acid agonist *N*-methyl-D-aspartate (NMDA) produces rhythmic activity in the isolated spinal cord in a range of vertebrates such as lamprey (Cohen and Wallen, 1980; Wallen and Williams, 1984), cat (Douglas et al., 1993), rat (Kudo and Yamada, 1987; Cazalets et al., 1990; Raastad et al., 1997; Kiehn, 2006), mouse (Hernandez et al., 1991), Xenopus (Dale and Roberts, 1984) and zebrafish (McDearmid and Drapeau, 2006). This rhythmic activity is intrinsic to the spinal cord because it can be generated without the descending input from higher centers. The motor pattern induced by NMDA displays left-right alternation and a rostrocaudal delay similar to that seen in freely swimming fish (Cohen and Wallen, 1980; Dale and Roberts, 1984; McDearmid and Drapeau, 2006).

The swimming frequency can vary between preparations from intact animal to spinal preparation. In zebrafish larva, the swimming frequency is similar to that seen in intact animal, that is about 20 Hz (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006), but in lamprey the frequency in isolated preparation is lower, around 0.5-1.4 Hz compared to frequencies of 1.5-7.6 Hz in freely swimming animals (Wallen and Williams, 1984).

The activity pattern of all motoneurons during fictive locomotion is characterized by periodic membrane potential oscillations. The peak depolarization occurs in phase with ipsilateral ventral root discharge or burst while the hyperpolarizing trough phase is synchronous with contralateral burst. This shows that there is an on-cycle excitatory drive that alternates with an active mid-cycle inhibition (Fig 2). This may be described as a push-pull arrangement, which takes place at the premotor interneuronal level in all CPG neurons modulating the motoneuron potential (Roberts et al., 1981; Russell and Wallen, 1983; Endo and Kiehn, 2008; Gabriel et al., 2009).

1.3.2 On-cycle excitation

In lamprey phasic excitation is clearly provided by the population of glutamatergic premotor EINs (Buchanan and Grillner, 1987). They diverge onto many MNs and also all sets of interneurons including other EINs (Buchanan, 2001). During fictive locomotion these EINs mutually excite each other to mediate the excitatory drive (Buchanan et al., 1989; Cangiano and Grillner, 2005). This excitation is the summation of many converging excitatory postsynaptic potentials (EPSPs) onto MNs.

In hatching Xenopus tadpoles the descending interneurons (dINs) mediate the excitatory drive. These neurons are the members of a population of excitatory neurons with ipsilaterally descending axons extending from mid-brain to the spinal cord (Soffe et al., 2009). The dINs directly excite other members of the swimming network via their descending axons. During fictive swimming they are rhythmically active and reliably fire contributing to their strong mutual excitation via electrical and chemical connections resulting in recurrent excitation (Li et al., 2009). Like in other vertebrates the excitatory drive depends on both fast α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and slow NMDA receptors (Li et al., 2004).

In larval zebrafish the excitatory multipolar commissural descending (MCoDs) are active during slow swimming and another group of excitatory interneurons which is ipsilateral, the circumferential descending interneurons (CiDs) are active during fast swimming (Kimura et al., 2006; McLean et al., 2008). One interesting thing came out from these studies is that different classes of neurons are activated depending on the functional dynamics of the fish.

In mammals V2a interneurons expressing the transcription factor Chx10 make putative connections with MNs and CINs (Al-Mosawie et al., 2007; Crone et al., 2008). They are functional homologs to CiDs in zebrafish that express transcriptional factor Alx (Kimura et al., 2006). By selectively ablating the V2a interneurons genetically in mice, it has been shown that they are not essential for rhythm generation, but help in stabilizing the rhythm and activate commissural pathways to maintain left-right alternation (Crone et al., 2008). Recent evidence shows that these interneurons has a speed dependency as they are recruited preferentially at progressively higher speeds (Crone et al., 2009). This is consistent with the role of CiDs in the zebrafish that also show similar frequency dependent recruitment (McLean et al., 2008).

1.3.3 Phasic mid-cycle inhibition

The mid-cycle reciprocal inhibition ensures the left-right alternation of activity in the vertebrate spinal cord during locomotion. This is done by inhibitory CINs via which each of the hemisegmental oscillators are activated with a phase difference (Grillner, 2006).

In the lamprey spinal locomotor network, the phasic inhibition is mediated by CCINs (Buchanan and Cohen, 1982) and inhibitory CINs (Ohta et al., 1991). Intracellular studies of CCINs showed phase-locked activity with respect to the onset of the ipsilateral ventral root burst (Buchanan and Cohen, 1982). The CINs play an active role in mediating mid-

cycle inhibition during a locomotor cycle (Biro et al., 2008). This mid-cycle inhibition is mediated by chloride conductances (Russell and Wallen, 1983).

During locomotion, reciprocal inhibition sets the pattern and frequency of the locomotor rhythm since progressive sectioning in the midline of lamprey spinal cord during locomotion enhances the frequency of the locomotor rhythm (Cangiano and Grillner, 2003). Similarly, low concentration of strychnine increased the locomotor frequency, while at higher concentrations the frequency decreased with concomitant bursting on the both sides of the cord (Grillner and Wallen, 1980). These results show that glycinergic transmission is not only necessary for left-right alternation but also for the generation of rhythm.

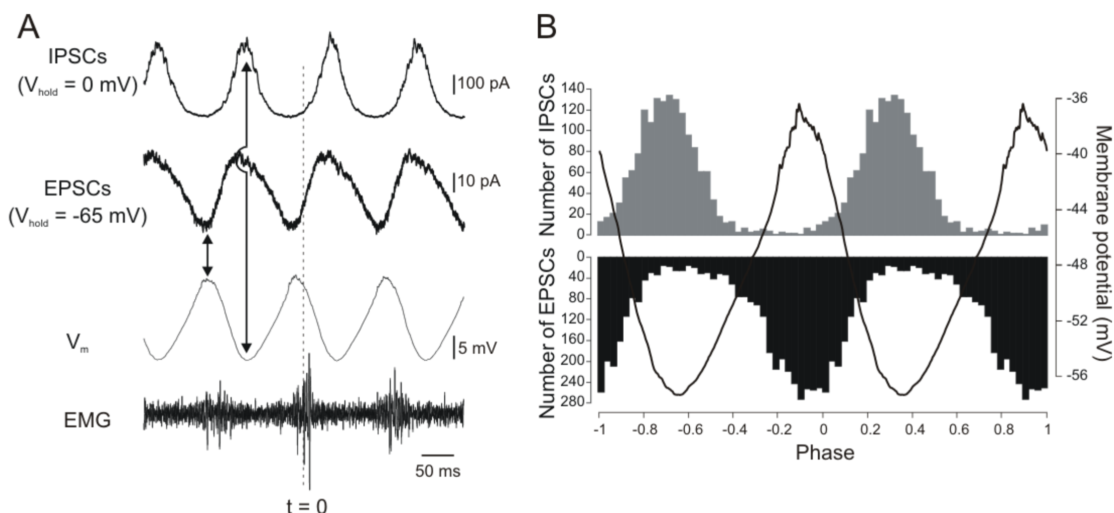

Figure 2: Alternating excitatory and inhibitory currents underlying the phasic oscillations during locomotion. A. Waveform average of intracellular recordings from a spinal neuron during NMDA-induced fictive locomotion in current-clamp and voltage-clamp. The peak of the integrated ipsilateral EMG recording is taken as a reference. B. Histogram showing the distribution of the average number of unitary EPSCs (black) and IPSCs (gray) received by the neuron during different phases of the locomotor cycle. The values correspond to the average number of PSCs per bin. The peak of the integrated ipsilateral EMG recording is used as a reference and the membrane potential oscillation is shown for comparison. (Adapted and modified from paper III)

In the zebrafish locomotor network in the absence of functional glycine receptors, *bandoneon* (*beo*) mutants display simultaneous activation of axial muscles on both sides of the trunk (Hirata et al., 2005). Also targeted knockdown of embryonic glycine receptor α2-subunit disrupted rhythm generating networks and reduced the frequency of spontaneous glycinergic and glutamatergic events in larval zebrafish (McDearmid et al., 2006). In zebrafish two anatomically distinct inhibitory interneurons have been identified and their role during swimming has been evaluated (Liao and Fetcho, 2008). One of them is the commissural bifurcating longitudinal (CoBLs) which has shorter axon length. They are more numerous compared to other cell types and thought to be the good candidate forming

the core interneuron class mediating left-right alternation (Liao and Fetcho, 2008). One of the interesting aspects of the zebrafish locomotor network is the recruitment of different neurons at different locomotor speeds. This gives an additional facet to the role of inhibition within the locomotor network, which is its specificity in silencing different premotor interneurons, depending on the locomotor frequency (McLean et al., 2008). The role of inhibition was also tested during NMDA induced locomotion in the larval zebrafish. When strychnine was applied in this preparation, it severely disrupted the left-right alternation through mid-cycle inhibition (McDearmid and Drapeau, 2006).

In Xenopus tadpoles, like in other vertebrates, glycine is endogenously released during locomotion and set the amplitude of mid-cycle inhibition in motoneurons and CPG interneurons (Soffe et al., 1984; Roberts et al., 1985). Brief application of strychnine depressed the mid-cycle inhibition and increased the locomotor frequency (Soffe, 1987). A local microperfusion of strychnine, which essentially does not affect the overall operation of swimming circuitry also has been shown to reduce the mid-cycle inhibition in different amphibian species including the Xenopus (Perrins and Soffe, 1996). Intracellular recordings in Xenopus tadpole showed that cINs were active during swimming and produced glycinergic mid-cycle inhibition of antagonistic neurons on the opposite side of the spinal cord (Dale, 1985). These interneurons have long ascending and descending axonal projections which implies that they also control the phase of swimming and play a part in coordinating the longitudinal spread of motor activity during swimming (Yoshida et al., 1998; Soffe et al., 2001). In addition to producing alternation, the cINs are also proposed to be important for rhythm generation, due to their ability to trigger post-inhibitory rebound depolarizations in dINs (Roberts et al., 2008).

Overall, mid-cycle inhibition mediated by glycine in locomotor networks helps in the appropriate recruitment of neurons in the different phases of the locomotor cycle. Control of mid-cycle inhibition is thought to plays key role in setting the locomotor frequency. When mid-cycle inhbition is reduced, such as in the case of strychnine, the locomotor frequency is enhanced (Hellgren et al., 1992; Dale, 1995).

1.3.4 Modulation of spinal locomotor pattern

All neuronal networks are modulated by intrinsic and extrinsic modulatory systems. These systems can sculpt the output of a hard-wired network, in which activity is generated by fast acting excitatory and inhibitory synaptic transmission that acts on ligand-gated ion channels. Modulation can adapt network activity to changing internal or external environments, or influence long-term changes that contribute to developmental or learning

induced changes (Harris-Warrick and Marder, 1991; Grillner et al., 1995; Katz, 1995; Parker, 2000). Neuromodulators play important instructive roles in determining the moment-to-moment output of a CPG by altering the synaptic strengths and intrinsic cellular properties of the circuit neurons (Katz and Harris-Warrick, 1990; El Manira et al., 2002). By targeting the excitatory drive and mid-cycle inhibitions, the CPG network can impart profound effects in the motor output (El Manira et al., 2002; Grillner, 2003; El Manira et al., 2008; Sillar et al., 2008). The modultatory system acts through activation of G-protein coupled receptors (GPCRs).

Serotonin (5-HT) is a powerful modulator of the locomotor network in all vertebrates studied so far (Sillar et al., 1998; Schmidt and Jordan, 2000; McLean and Sillar, 2004). In the lamprey, 5-HT slows down the locomotor frequency, increases burst intensity and duration and regularizes the locomotor rhythm (Harris-Warrick and Cohen, 1985; Wallen et al., 1989; Zhang and Grillner, 2000). Spinal 5-HT is supplied by descending fibers, dorsal root ganglia and cells below the central canal throughout the length of the spinal cord, forming a plexus, into which INs and MNs extend their dendrites (Christenson et al., 1989; Zhang et al., 1996). 5-HT is endogenously released during locomotion (Zhang and Grillner, 2000) and decreases the slow postspike afterhyperpolarization (sAHP) in motoneurons, crossed caudal (CCINs), and lateral interneurons (Wallen et al., 1989). The sAHP is mainly due to apamin-sensitive calcium-activated potassium channels of the SK type (El Manira et al., 1994).

In Xenopus tadpole, application of 5-HT increases locomotor burst duration, depresses mid-cycle inhibition, and modulates NMDA receptors during fictive locomotion induced by skin stimulation (Sillar et al., 1992; Sillar et al., 1998). In the newborn mammalian spinal cord, 5-HT is important for generation of the stable locomotor rhythm induced pharmacologically (Cazalets et al., 1992; Kiehn and Kjaerulff, 1996; Liu and Jordan, 2005; Liu et al., 2009).

In both adult and larval zebrafish spinal cord, 5-HT innervations comes from descending raphe projections and intraspinal neurons (Van Raamsdonk et al., 1996; Kaslin and Panula, 2001; McLean and Fetcho, 2004). At the zebrafish larval stage, endogenous 5-HT primarily acts to modulate the duration of the quiescent period between the consecutive active swim periods without changing the frequency of spontaneous swimming (Brustein and Drapeau, 2005).

With my introduction above, I tried to show how different model systems with different levels of complexity display remarkable similarity in their core pattern generation network. The lamprey's CNS, apart from similarities with other vertebrates, is simpler than other

vertebrates. Each spinal segment has around 1000 neurons per segment with a rough total of around 100,000 in the entire cord (Rovainen, 1979). If we compare this to another animal, we can see that in rat with roughly similar body size to lamprey has almost 300,000 neurons per spinal segment and around 8 million neurons within the spinal cord (Bjugn and Gundersen, 1993). The explanation for this difference lies in the much more complex behavioral pattern of the rat involving the use of multi-joined limbs. The comparative simplicity of the lamprey body, transparency, swimming pattern, and of the underlying nervous system make it a much less difficult model to study and understand compared to other complex animals. Another added advantage of the lamprey is that the motor pattern underlying locomotion can be maintained *in vitro* for several days (Grillner and Wallen, 2002). In my first project I took the advantage of both the transparency and few cells of the lamprey spinal cord to label the CINs and anatomically corroborate the evidence of dominance of reciprocal inhibition over excitation.

The zebrafish nervous system has a basic motor pattern generating network similar to lamprey (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006) and Xenopus tadpole (Roberts et al., 1998; Roberts, 2000). The spinal cord of larval and juvenile zebrafish is also transparent which allows for optical approaches to the study of structure and function of the intact animal. Apart from these similarities, zebrafish has the added strength of genetic accessibility that allows for the production of both mutant animals and transgenic fish. Although the zebrafish is comparatively new in motor control studies, rapid progress has been made in identifying cells and circuits in zebrafish. Some key factors contributed to this. The use of calcium imaging *in vivo* allowed for the rapid identification of neurons in a particular behavioral context (Fetcho and O'Malley, 1995; Brustein et al., 2003). Patch clamping allows for studies of cellular properties and synaptic connectivity (McDearmid et al., 1997; McDearmid and Drapeau, 2006; McLean et al., 2007; McLean and Fetcho, 2009; Gabriel et al., 2010).

The newly developed juvenile and adult zebrafish preparation that was developed during the course of my thesis project can add a new level of insight into the investigation of the spinal locomotor network because it also offers the same combination of transparency and genetic tools like larva but at a more mature stage. This will help to bridge the gap between immature and mature systems which has thus far been lacking in other preparations.

2 QUESTIONS ADDRESSED IN THIS THESIS

The overall organization and neuronal makeup of the locomotor system shows remarkable similarities and conservation in different vertebrates. The aim of my thesis work has been to examine the organization of the locomotor network in accessible model systems.

The specific questions addressed are:

- Can the dominance of reciprocal inhibition over excitation in controlling left-right alternation during locomotion be explained anatomically? (Paper I)
- Can locomotor pattern be studied in the adult zebrafish preparation *in vitro*? (Paper II)
- What is the role of serotonin (5-HT) in modulating the adult zebrafish spinal cord locomotor network? (Paper III)
- How are spinal locomotor circuits turned on by descending excitatory inputs and how does the produced motor pattern *in vitro* compare to swimming in intact zebrafish? (Paper IV)

3 METHODS

Two different preparations were used in my thesis. For the anatomical study in paper I, adult river lamprey (*Lampetra fluviatilis*) were used. For the study of the spinal motor pattern in paper II, III & IV different stages of zebrafish were used: juvenile (early juvenile stage: age 30-44 days; late juvenile stage: age 45-89 days) and adult zebrafish: age ≥90 days.

3.1 Lamprey

3.1.1 Retrograde tracing

To retrogradely label CINs in the lamprey spinal cord, a vertical cut was made with the tip of a thin bored needle from near to the midline upto the lateral aspect of the ipsilateral spinal cord in the middle of a 20 segment long spinal cord. Small crystals of Neurobiotin were applied at the injection site after cutting. The tracer was then allowed to travel a considerable distance. After the tracer transport, the tissue was processed for whole-mount and immunohistochemistry.

3.1.2 Whole mount histochemistry

Tracer transported tissue was fixed in formalin and picric acid in phosphate buffer (Gunn et al.) and subsequently washed in PB. The spinal cord was then counter stained with streptavidin-Alexa 488 overnight to detect the Neurobiotin. The tissue was then dehydrated with ascending ethanol series and cleared and embedded in methyl salicylate.

3.1.3 Immunohistochemistry

To detect glycine, glutamate and GABA-immunoreactivity (ir) in the CINs, tissue was fixed in different concetrations of formalin, picric acid and glutaraldehyde depending on the antibody used. After fixation, tissue was cryoprotected with sucrose and 14 μm thick horizontal sections were cut with a cryostat. For co-localization of retrograde tracer with antibody, the sections were incubated with appropriate primary and secondary antibodies.

3.1.4 Confocal analysis

For whole-mount and horizontal tissue sections a Zeiss laser scanning confocal imaging system (LSM 510 Meta) with appropriate laser line was used. To maintain accuracy cells were counted online and after the experiment when data were imported to Adobe Photoshop. Cell counts were corrected using Abercrombie's factor (Abercrombie, 1946).

3.2 Zebrafish

3.2.1 Preparation

All dissection steps were performed in slush frozen saline. For the semi-intact preparation, animals were glued dorsal side up to the recording chamber. The skull was opened and brain was cut caudal to tectum. The muscles were cut dorsally and laterally over 50-75% of the body length. The vertebra overlying the spinal cord were pulled out to expose the spinal cord. The skin was peeled from the muscle and the tail was freed from glue to allow visual observation of motor behavior.

For *in vitro* preparation, the steps were as before but here all of the entire vertebra overlying the spinal cord were removed. The ventral roots were cut close to the spinal cord. For microelectrode penetration during patch-clamp recordings, small gashes were made in meninges overlying the spinal cord with a sharp tungsten pin. The entire spinal cord including the hindbrain was then lifted out of the vertebral column with a fine hook. The preparation was placed lateral side up for intracellular recording or ventral side up for extracellular recording. In paper 3, the preparation was done as described before with slight modifications. Here the entire spinal cord including the hindbrain and a small part of caudal musculature was cut together with the vertebral column and transferred to recording chamber.

3.2.2 Split-bath experiments

For split bath experiments, low melting agar (0.1%) was used. Care was taken to prevent solution leaking from rostral to caudal pool and vice-versa.

3.2.3 Electrophysiology

Extracellular suction electrodes were pulled by a microelectrode puller and fire-polished for ventral root and EMG (electromyogram) recordings. Signals were amplified with a

differential AC amplifier (AM systems) and filtered with appropriate filter settings. Neurobiotin was added for subsequent morphological analysis. Cells were visualized with differential interference contrast (IR-DIC) optics and a CCD camera with a frame grabber (Hamamatsu). Whole cell voltage was amplified with a MultiClamp 700B intracellular amplifier (Axon Instruments) and low-pass filtered. Unitary EPSCs and IPSCs were detected with MiniAnalysis software (Synaptosoft).

3.2.4 Neurobiotin stainings

The spinal cord was fixed with appropriate fixatives and washed in Triton-X in PBS (X-PBS). The cord was then incubated in streptavidin-Cy3, washed and dehydrated in graded ethanol and cleared in methyl salicylate. After clearing, tissue was mounted lateral side up and visualized on a confocal microscope.

3.2.5 Backfills of motoneurons

Small crystals of different dextrans were picked on the tip of sharp tungsten pins and moved over the muscle on a cold anaesthesized fish to severe the motor axons. After the dye uptake, the animals were dissected as described earlier for the patch recordings.

3.2.6 Data acquisition and analysis

Data were digitized with a Digidata A/D converter (Molecular Devices) and acquired on a personal computer using pClamp software. Data analysis was performed in Spike2.

4 RESULTS AND DISCUSSION

4.1 Anatomical basis for the dominance of left-right alternation during locomotion (Paper I)

In lamprey several classes of morphologically identifiable neurons with crossing axons have been described, such as crossed caudally projecting interneurons (CCINs), commissural interneurons (CINs), giant interneurons and edge cells (Rovainen, 1967; Buchanan, 1982; Grillner et al., 1984; Ohta et al., 1991; Fagerstedt and Wallen, 1992, 1993; Buchanan, 1999a, 2001). Of those the edge cells are intra-spinal stretch receptors (Grillner et al., 1984) and giant interneurons are sensory relay neurons (Rovainen, 1974). They are not active during fictive locomotion (Buchanan, 2001). In this paper, I looked at transmitter phenotypes of CINs. I will first give a brief description of my results and later on I will explain the reason for choosing these interneurons for my study and give an explanation of my findings.

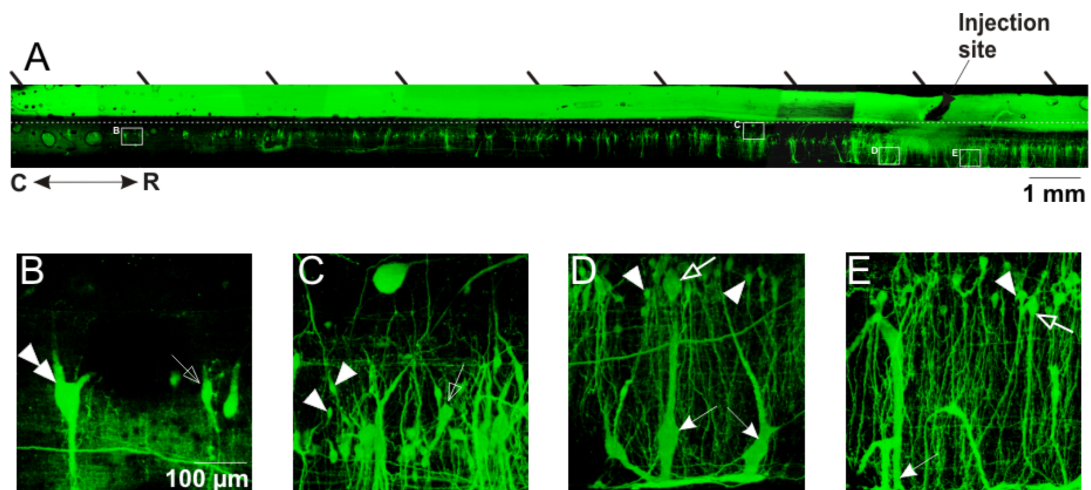

Fig 3: Neurons with contralateral axonal projections in the lamprey spinal cord. A. Projection of contralaterally projecting neurons (CINs) in the whole mount spinal cord. Neurobiotin is injected on one side of the spinal cord (injection site) to label neurons projecting to the contralateral side both rostrally and caudally. Black oblique lines indicate segmental boundaries. Dashed line indicates the midline; R, rostral; C, caudal direction. B. Projection from segment seven caudal to the injection site showing very few caudally projecting CINs of large size (double arrowhead) and a few medium sized CINs (open arrow). C. Projection from segment 2 of caudally projecting CINs showing small (arrowhead) and medium (open arrow) CINs. D. Volume stacks from segment 1 showing small (arrowhead) and medium (open arrow) CINs and large edge cells (filled arrows). E. Projection of rostrally projecting cells in segment 1 showing small (arrowhead) and medium (open arrow) CINs and large edge cells (filled arrow). (Adapted and modified from paper I)

Commissural rostrally and caudally projecting CINs were retrogradely labeled with neurobiotin in the spinal cord *in vitro* (Fig 3). Most of these neurons are located in the

intermediate region of spinal gray matter but some are located in the middle. The tracer faithfully labeled all of the neurons with crossing axons over five segments but very few projected over six segments and beyond (Fig 3). We therefore restricted our study to CINs over six segments rostral and caudal to the site of injection of the dye. The number of CINs gradually decreased from the site of injection. The neurons with rostrally projecting axons are always in greater number than the caudally projecting ones.

To examine the transmitter phenotypes of CINs, we performed experiments with retrograde labeling combined with immunohistochemistry. The immunohistochemistry directed against three different transmitters of the lamprey spinal cord revealed that the distribution pattern of CINs with glycinergic phenotype is predominant with half of the CINs being glycinergic. Approximately one-third of the CINs show immunoreactivity to glutamate, while none of them are GABAergic.

Why look at the projection pattern of CINs instead of the CCINs? In the more recent schematic model of lamprey locomotor network (Fig 1) the crossing inhibitory interneurons are indicated as INs (inhibitory interneurons) which include both the larger CCINs and the smaller CINs that have relatively short axonal projections (Grillner, 2003). The CCINs were original proposed as the inhibitory crossing interneurons that were mediating left-right alternation (Buchanan, 1982; Buchanan and Grillner, 1987). There are a number of features suggesting that they are unlikely to be the candidate for mediating segmental reciprocal inhibition (see Rovainen, 1983). They have long axons that are not consistent with the segmental role, but could suggest a role in intersegmental coordination (Buchanan, 1999a). Their patterns of phasic potential changes are less uniform than motoneurons. The repetitive intracellular stimulation of CCINs has little or no effect on the timing of fictive swimming (Rovainen, 1983; Grillner et al., 1986). In addition there is no experimental evidence from paired recordings that they inhibit the EINs in the opposite hemisegment (Buchanan, 1999b; Parker and Grillner, 1999).

In contrast, retrograde labeling of the CINs showed that they have short axonal projections (Ohta et al., 1991; Fagerstedt and Wallen, 1992, 1993). Electrophysiological analysis suggested that these cells might contribute in segmental reciprocal inhibition (Buchanan, 1999a). Although morphological evidence showed that these neurons constitute 50% of the neurons, direct electrophysiological evidence for the segmental role of these CINs is still only preliminary (Ohta et al., 1991; Biro et al., 2008). The reason for this is the small size of these CINs. The diameter of most of the neurons is around 12 μm in their short axis (Buchanan, 2001).

In this context the anatomical data could prove valuable, because it can complement electrophysiological data by adding or filling up the missing links.

Why look at projection pattern up to 5 segments? As I mentioned before the most logical answer to this question is that these CINs have been suggested to contribute to segmental reciprocal inhibition, which fits with the model of the locomotor network (Fig 1). Besides this it has been shown elcectrophysiologically that coupling signals act over approximately 5 segments (Buchanan, 1999a). In addition previous morphological evidence also points to the fact that CINs do not project beyond 5 segments (Ohta et al., 1991). In our experiments, we further corroborated the previous morphological and electrophysiological data and showed that there are very few CINs projecting beyond 5 segments. By showing the dominance of glycine-ir over glutamate-ir CINs, we anatomically substantiate the previous electrophysiological evidence of the predominant role of reciprocal inhibition over excitation that helps in maintaining alternation between two sides during locomotion (Grillner and Wallen, 1980; Cohen and Harris-Warrick, 1984; Buchanan, 2001; Cangiano and Grillner, 2005).

How does the quantitative data on glutamatergic CINs fit with the locomotor network scheme? It was shown that individual hemisegment unit burst generators are coupled to each other by a prominent commissural reciprocal inhibition and a weaker commissural excitation (Cohen and Harris-Warrick, 1984; Hagevik and McClellan, 1994). Bath application of strychnine during fictive locomotion converts alternating rhythmic bursting activity to synchronous ventral root activity both in lamprey and in other vertebrates (Cohen and Harris-Warrick, 1984; Roberts et al., 1985; Hagevik and McClellan, 1994; Cowley and Schmidt, 1995; McDearmid and Drapeau, 2006; Gabriel et al., 2008). This finding elucidates the role of reciprocal inhibition while at the same time unmasking a commissural excitatory influence. It has been shown previously in lamprey that crossed excitatory connections exists between the CINs and MNs, that produce larger EPSPs in the MNs (Parker and Grillner, 2000). Our findings imply that the smaller glutamatergic CIN population might be responsible for this weaker excitatory coupling between two sides.

In the rat in addition to glycine, there is evidence that left-right alternation also dependent on GABAergic transmission (Cowley and Schmidt, 1995; Bracci et al., 1996; Kremer and Lev-Tov, 1997). In these studies $GABA_A$ antagonist disrupted the left-right alternation. As strychnine can block $GABA_A$ a contribution of these receptors to the alternation activity in these studies could not be excluded. In lamprey during fictive swimming pharmacological blockade of $GABA_A$ by bicuculline or gabazine increased the locomotor frequency without affecting the left-right alternation (Tegner et al., 1993; Schmitt et al., 2004). Our anatomical data also indirectly shows that reciprocal inhibition

does not involve direct GABA transmission. There are only occasional commissural GABA-ir fibers observed in developing and adult lampreys (Melendez-Ferro et al., 2003; Villar-Cervino et al., 2008). However the origin and distribution of the neurons giving rise to these fibers are not yet clear.

In the embryonic or larval zebrafish, commissural interneurons bearing glycinergic or glutamatergic transmitter phenotype have been characterized (Higashijima et al., 2004). Interestingly none of the commissural interneuons was found to be positive for GABA even at this early age. The Xenopus tadpole at stage 37/38 also showed no evidence of presence of GABAergic CINs (Roberts et al., 1988). Different pictures also exist, for example, the neonatal rodents aged 5-8 days and neonatal mouse aged P0-P1 where a proportion of CINs is GABA-ir (Weber et al., 2007; Restrepo et al., 2009) along with glycinergic and glutamatergic CINs. The possible explanation for this is that it is possible in rat and mouse the transmission composition may change, while this is not the case in adult lamprey (Villar-Cervino et al., 2008; Restrepo et al., 2009).

Another important observation from our study is that although we managed to characterize 79% of CINs with our method, we failed to identify the remaining 21% of the CINs. Some of these could still correspond to glycinergic or glutamatergic CINs which we could not label immunohistochemically or some of them might display immunoreactivity for calbindin and calretinin (Megias et al., 2003).

Overall this anatomical study shows that dominant reciprocal inhibition is probably glycinergic while the cross mutual excitation is glutamatergic.

4.2 Adult zebrafish spinal cord *in vitro*: A neuronal correlate of swimming *in vivo* (Paper II)

In this study, we developed an *in vitro* preparation of the isolated spinal cord from juvenile/adult zebrafish. The aim was to gain access to a preparation that can exhibit the same rhythmic pattern that bears all the hallmarks of swimming in the intact freely swimming animal.

First, we used semi-intact preparation and showed that the average frequency of swimming elicited by NMDA was 5 Hz (100 μM), whereas in isolated spinal cord it was 6 Hz (50 μM). These are within the frequency range measured in freely swimming adult zebrafish (Muller et al., 2000). In our experiments locomotor burst frequency also varied with age, it was higher in early juvenile compared with late juvenile and adult zebrafish. In zebrafish larva the frequency is about 30 Hz when locomotion is induced spontaneously or

by light (Buss and Drapeau, 2001; Masino and Fetcho, 2005). When NMDA is applied to spinalized zebrafish larva, the frequency was 18 Hz (McDearmid and Drapeau, 2006). Larval zebrafish required a higher concentration of NMDA than that used to elicit locomotion in the adult *in vitro* preparation. A kinematic study in freely swimming larval zebrafish also revealed that the frequency decreases with increasing age (Muller and van Leeuwen, 2004). This suggests that with development the locomotor network changes resulting in a decrease in motor output as the animal matures. Another interesting suggestion is there is a slow increase of red (slow) muscle fibers at later stages of development (van Raamsdonk et al., 1982; Buss and Drapeau, 2000; Gabriel et al., 2010).

The cross-correlation and autocorrelation studies from both semi-intact and *in vitro* preparations of the zebrafish displayed left-right alternation and rostrocaudal delay of motor bursts. The phase delay related to 60% for a full wave of activity along the body at any given point of time. In larval zebrafish it was shown that a full wave of activity is generated along the body during fictive swimming (Masino and Fetcho, 2005). This is in contrast to what has been shown in our preparation and compared to adult goldfish (Fetcho and Svoboda, 1993). In goldfish they concluded that the mature fish has less flexibility than larval zebrafish. In our case we hypothesized that during maturation the spinal network adapts with the constraints imposed by the stiffening of the body.

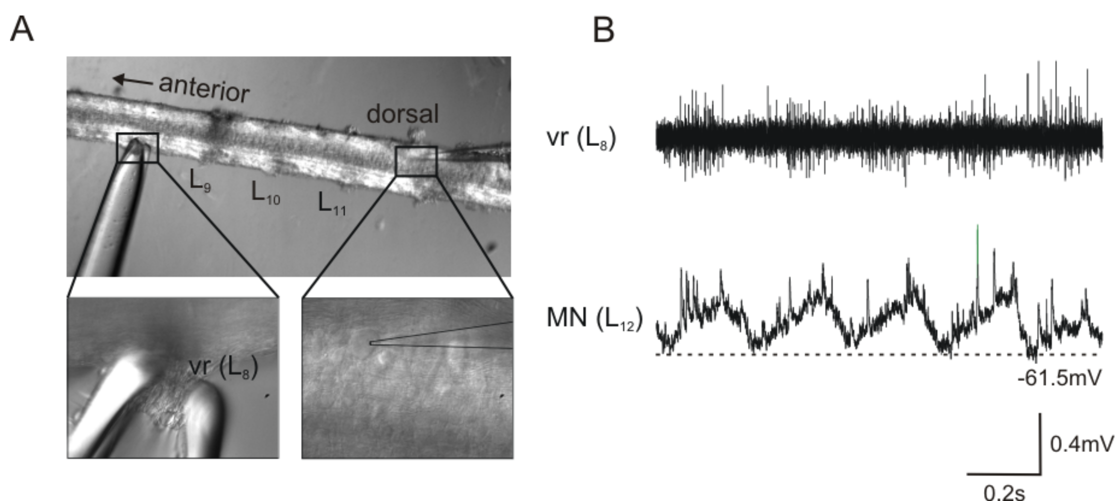

Fig 4: *In vitro* intracellular recording of a primary MN (pMN) during fictive locomotion. A. Top: Lateral view of the zebrafish spinal cord with the extracellular electrode to the left and the intracellular electrode to the right, bottom: magnified images from top. Labels L_9–L_{11} align with the stumps of the left ventral roots in segments 9–11. B. Recording of a pMN showing membrane potential oscillation in-phase with the ipsilateral ventral root in 30 µM NMDA. (Adapted and modified from paper II)

When we recorded the activity of primary motoneurons (pMN), we saw a membrane potential oscillation that is in phase with ipsilateral ventral root activity (Fig 4). The amplitude and frequency increased with increased NMDA concentrations. Unlike pMNs in

larva where they readily fire action potentials during fictive locomotion (McDearmid and Drapeau, 2006), we only observed subthreshold synaptic inputs. This is not surprising because they are only recruited during high frequency swimming or escape (Liu and Westerfield, 1988; Gabriel et al., 2010).

In the *in vitro* preparation, when reciprocal glycinergic inhibition with strychnine was blocked, synchronous motor bursts on both sides and along the rostrocaudal axis was observed, indicating inhibitory synaptic transmission is mediating the alternation and delayed propagation. This has already been described in other preparations (see introduction). It is important to mention that when reciprocal connection is weakened by low doses of strychnine in lamprey, the left-right alternation is preserved with a concomitant increase in the frequency (Grillner and Wallen, 1980; McPherson et al., 1994). However, when glycinergic inhibition is completely blocked with high doses of strychnine, the synchronous motor activity develops (Cohen and Harris-Warrick, 1984; Cowley and Schmidt, 1995) with a dramatic decrease in the frequency (Cohen and Harris-Warrick, 1984; McPherson et al., 1994). In our semi-intact experiments during the wash-in of strychnine (probably representing a situation when the concentration of strychnine inside the cord is still low) there was a transitional period when continuous motor activity was patterned into discrete episodes. Within these episodes we saw an almost 5 fold increase of burst frequency. This is also observed in fictive locomotion of zebrafish larva (Masino and Fetcho, 2005; McDearmid and Drapeau, 2006). Thus two rhythms with different cycle periods and sometimes different phase relationships can be observed at the same time The presence of this two discrete rhythm was also shown in lamprey hemicord preparation (Cangiano and Grillner, 2003). This shows that strychnine is acting on alternation and cycle period through two different mechanisms. In our experiment, strychnine at 0.5 μM decreased the frequency in both semi-intact and *in vitro* preparation. In the zebrafish larva, left-right alternation is lost without changing the frequency which may be due to difference in network architecture(McDearmid and Drapeau, 2006).

Overall our studies in this paper strongly suggest that the locomotor pattern induced by NMDA in the spinal cord of juvenile/adult zebrafish is a neuronal correlate of fictive locomotion in the freely swimming animal that can provide a foundation for future cellular studies on the network architecture in an adult system that can complement studies in zebrafish larva and other species.

4.3 Examining the role of 5-HT on the modulation of fictive swimming frequency of the adult zebrafish brainstem/spinal cord preparation (Paper III)

Serotonin (5-HT) has pronounced modulatory effects on spinal locomotor networks. 5-HT serves as a pivotal modulator of the motor networks, stabilizing the locomotor rhythm and promoting a decrease in burst frequency. In the spinal cord of larval zebrafish 5-HT only decreases the rest or glide period between two consecutive beat or swim periods without changing the frequency of spontaneous swimming (Brustein and Drapeau, 2005). This is in contrast to what has been shown to other model systems and prompted us to examine whether the absence of its effect is only present in larval stages or if it persists in the adulthood. For this we used the *in vitro* brainstem/spinal cord preparation.

We showed that during NMDA induced fictive locomotion, 5-HT decreased the frequency in a concentration dependent manner. The decrease in frequency is not associated with changes in burst proportion. The difference of action of 5-HT in adult and larval zebrafish is probably due to the fact 5-HT modulates the locomotor network differently in larval zebrafish where swimming frequency is much higher than the adult zebrafish.

Having proved that 5-HT modulates the locomotor activity we next tested whether this modulation is endogenous. For this we used citalopram, a reuptake inhibitor of 5-HT, which increases the concentration of 5-HT in the synaptic cleft (Fuller and Wong, 1990). Citalopram mimicked the effect of exogenous application of 5-HT, indicating that there is an endogenous release of 5-HT during locomotion in juvenile and adult zebrafish spinal cord *in vitro*. The released of 5-HT may arise from innervation from intraspinal neurons as well as supraspinal raphe projections which persists from larva till adulthood (Kaslin and Panula, 2001; McLean and Fetcho, 2004).

In other major vertebrate preparations, 5-HT acts differently to modulate the locomotor network. In Xenopus tadpoles, 5-HT increases the intensity and duration of motor bursts mainly by depressing the synaptically driven mid-cycle inhibition (McDearmid et al., 1997; McLean et al., 2000) without having major effects on frequency (Sillar et al., 1992). In the lamprey spinal cord, 5-HT decreases the locomotor frequency and increases burst intensity and burst duration through a depression of the slow afterhyperpolarization (sAHP) (Wallen et al., 1989) and modulation of synaptic transmission (Biro et al., 2006). These reports prompted us to look at the synaptic targets of 5-HT during modulation of swimming in our preparation. We showed that 5-HT depressed mid-cycle inhibition. It also affected the excitatory drive because when strychnine was used to block the glycinergic

synaptic trasmission, an application of 5-HT decreased the frequency (Fig 5). The decrease of frequency also associated with a slowing down of the onset of excitatory drive received

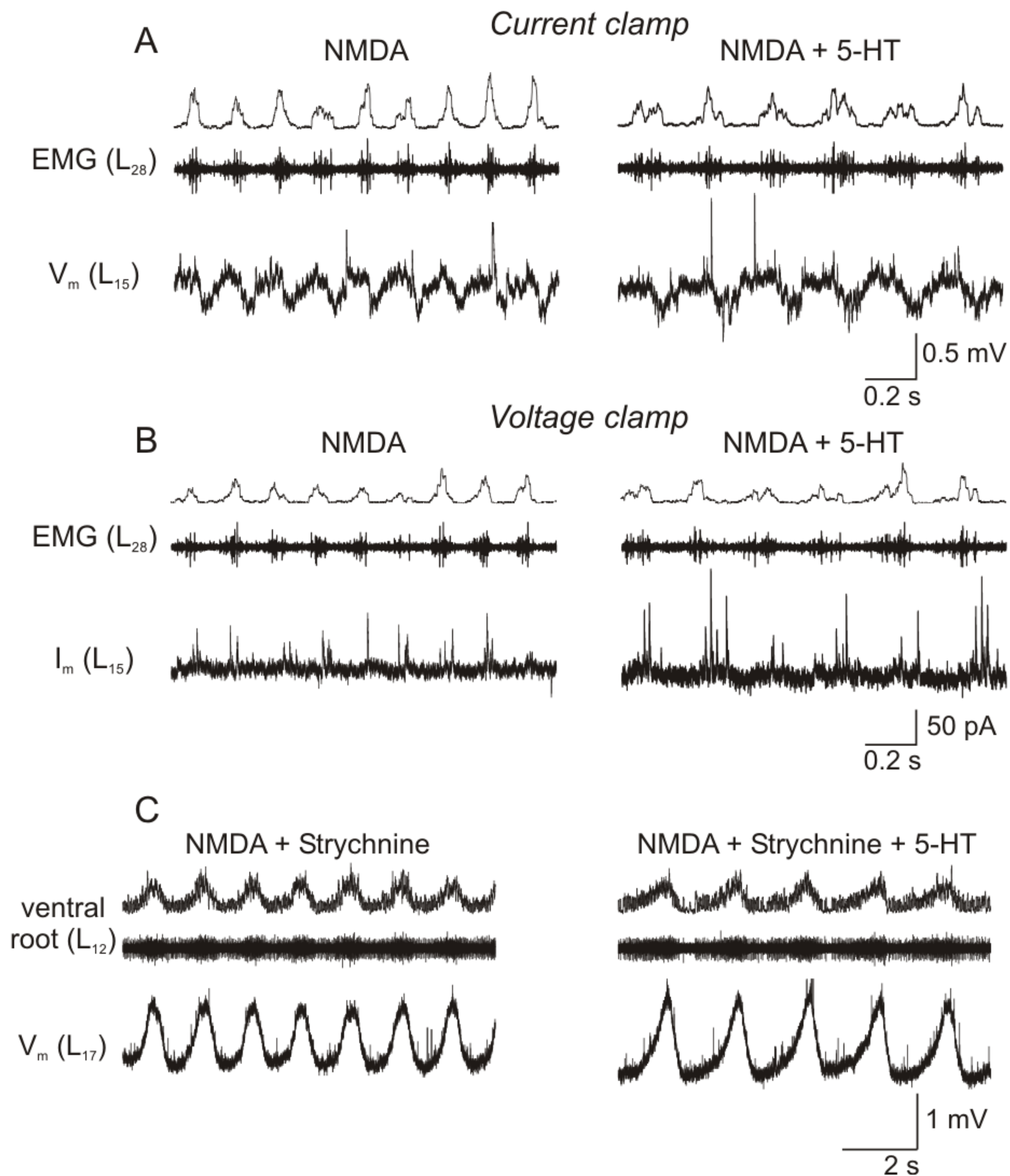

Fig 5: A, B. 5-HT increases the amplitude of mid-cycle inhibition during locomotion. A. Intracellular recording of a spinal neuron showing IPSPs during hyperpolarized phase of the oscillations. In 5-HT the locomotor frequency decreases and the IPSPs increase in amplitude and number. B. Rhythmic IPSCs recorded in the same spinal neuron voltage-clamped at a holding potential of 0 mV also increases in amplitude and number in 5-HT. C. 5-HT delays the onset of the excitatory synaptic drive. When glycinergic inhibition is blocked by strychnine, NMDA induces slow membrane potential oscillations in spinal neurons that occur in phase with the ipsilateral ventral root burst. Application of 5-HT decreases the burst frequency and slows down the depolarizing phase of the excitatory drive (Adapted and modified from paper III)

by motoneurons. This indicates that 5-HT acts on both inhibitory and excitatory synaptic transmission. Computer simulations also proved that an increase in mid-cycle inhibition can effectively decrease the locomotor frequency by delaying activation of the contralateral side (Hellgren et al., 1992). In larval zebrafish, the decrease in rest period by 5-HT was proposed to be mediated by an effect on the chloride homeostasis and as a result decrease in glycinergic synaptic transmission.

Overall in this paper we showed how endogenously released 5-HT affects the activity of the locomotor network in the adult zebrafish and characterized the underlying synaptic mechanisms.

4.4 Initiation of locomotion by stimulating the excitatory descending drive: A new model in adult zebrafish (Paper IV)

Here we examined at how a brief stimulation at a specific region in the *in vitro* brainstem-spinal cord preparation can induce long lasting swimming activity. We identified the optimal region to induce sustained swimming and examined how the pattern and frequency of the swimming activity compares with that of the freely swimming animal. In addition we also investigated the interaction of two distinct behavioral patterns of the zebrafish, namely swimming and escape, in this *in vitro* preparation.

Our results show that a brief stimulation with appropriate frequency and intensity induces swimming that outlasts the stimulation with alternation between left and right side (Fig 6). The burst amplitude and frequency as well as duration of the episode increased by increasing the frequency and strength of the stimulus pulses. The swimming activity induced by electrical stimulation displays a whole range of frequencies (1-12 Hz) unlike the locomotor rhythm induced by NMDA which becomes locked to one frequency (Fig 6). The fictive swimming induced by electrical stimulation of descending inputs bears similarities with that of freely swimming animals, so this is more physiological than the pharmacological approach.

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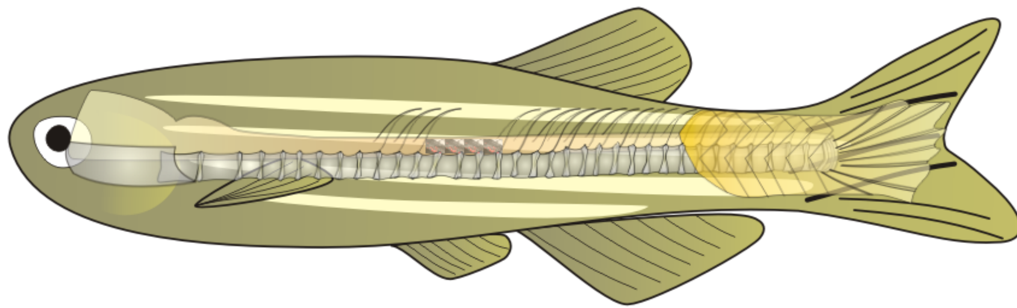

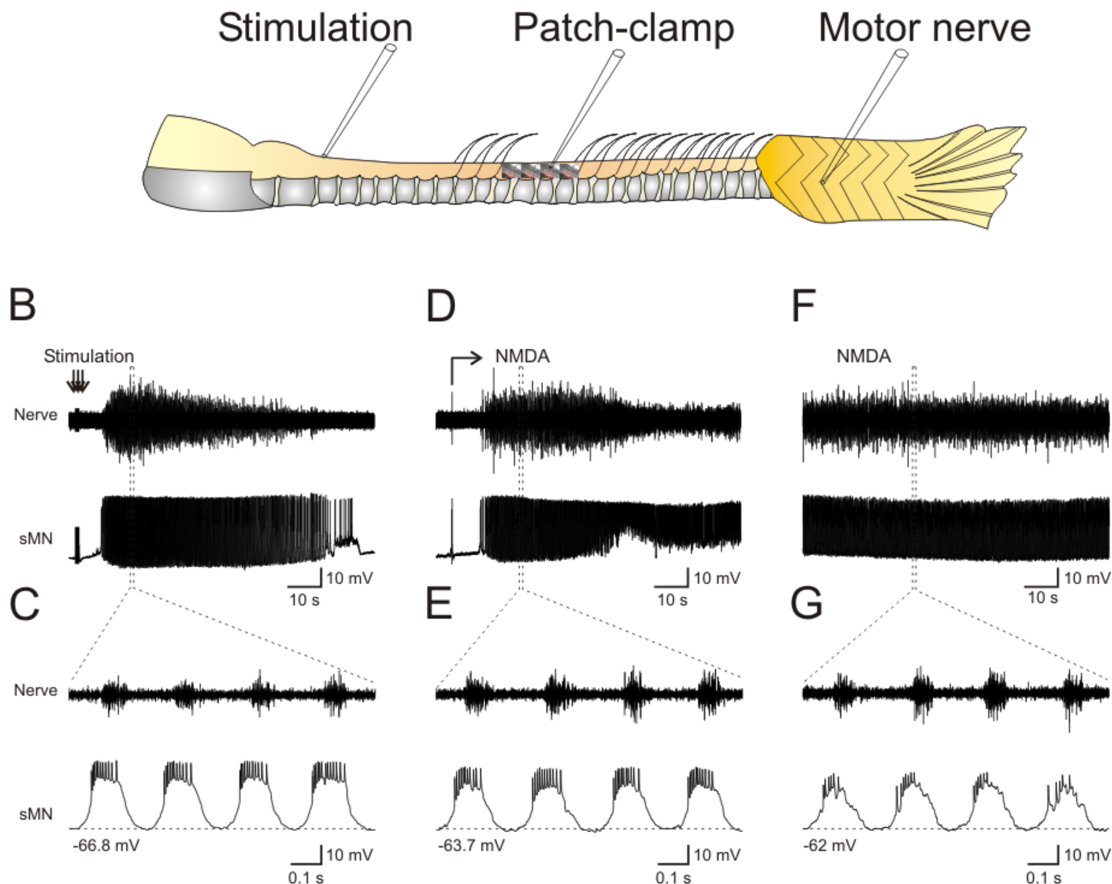

Fig 6: A. Experimental setup of the stimulation induced fictive swimming. B. Stimulation of descending inputs induced a long episode of swimming burst in the motor nerve associated with membrane potential oscillation in the secondary motoneuron (sMN). C. Action potentials occurs during the plateau depolarization of the sMN and has constant amplitude. D, E. At the onset of NMDA application membrane potential oscillations and firing of the sMN resembles those seen during stimulation-induced swimming. F, G. When the swimming activity reaches a steady-state frequency, the membrane potential of the sMN becomes slightly depolarized and the shape of the membrane oscillations changed in comparison to those occurring at the onset of NMDA application. The action potentials already occurring during the rising phase of the oscillation and their amplitude is shunted by the depolarization of the membrane potential. (Adapted and modified from manuscript)

It has been shown that hindbrain excitatory neurons are responsible for sustained swimming in *Xenopus* tadpole and larval zebrafish (Chong and Drapeau, 2007; Soffe et al., 2009). In our preparation we showed that this is not the case because blocking chemical synaptic transmission and in the brainstem and rostral spinal cord did not prevent the induction of swimming by stimulation.

The results presented in this study provide a characterization of how spinal locomotor circuits are turned on by descending excitatory inputs and some initial insights into the interactions between swimming and escape circuits.

5 CONCLUDING REMARKS

Many important insights into the organization and function of spinal locomotor networks have emerged from accessible animals. Recently, it has become increasingly important to combine different techniques and have access to genetically amenable preparations. This thesis aimed at defining the anatomical organization of interneurons controlling left-right coordination during locomotion and developing novel experimental model systems for studying the locomotor networks.

Reciprocal inhibition is a key component of the pattern generation because it ensures the characteristic left-right alternation during locomotion. Although it has been known that CINs are composed of both inhibitory and excitatory interneurons, the reason for the dominance of inhibition has been unclear. By examining the projection pattern and transmitter phenotype of CINs, we have shown that glycinergic interneurons outnumber the glutamatergic ones. Thus these data provide support for the dominance of reciprocal inhibition over excitation. It is also possible that there is in addition a difference between the dynamics of inhibitory and excitatory synaptic transmission that ensure the predominance of inhibition. In this regard the accessibility of the adult zebrafish *in vitro* preparation would be valuable for examining the dynamics of synaptic transmission from identified locomotor network interneurons.

The lamprey as a model system has provided many insights into the operation of the locomotor network. However, its inaccessibility to genetic and molecular tools has led us to develop an additional model system. Our goal is to understand the function of the locomotor network beyond interference of developmental changes. We therefore developed an *in vitro* preparation of the adult zebrafish. We show that a completely isolated brainstem-spinal cord can generate swimming activity with frequencies similar to those seen in freely moving animals. We have identified a region that when stimulated transiently produces long-lasting swimming episodes. This preparation is also accessible for patch-clamp recordings from interneurons and motoneurons. In a recent study, we showed that motoneurons can be divided into four groups that are recruited at a given frequency threshold (Gabriel et al., 2010). This preparation allows for the analysis of the locomotor network from larvae to juvenile and adult zebrafish to see what mechanisms are conserved and those that are changing to adapt to the developmental demands.

One of the features of the adult zebrafish is the ability to recover locomotor function after spinal cord injury. Many of the studies have focused on determining the capacity of

the injured axons to regenerate. However, it could be that intrinsic plasticity takes place within the spinal locomotor network that contributes to the recovery of motor activity. The *in vitro* preparation of the adult zebrafish will help in determining the changes that occur in the properties of identified neurons after injury and how they change with time.

In conclusion, the work of this thesis has provided insights into the organization of the interneurons responsible for left-right alternation during locomotion. The newly developed preparation of the zebrafish has started yielding results about the mechanisms of recruitment of neurons at different speeds of locomotion and therefore has a strong potential to help gaining more knowledge about the spinal networks controlling locomotion.

6 SOME FUTURE PROJECTS

Recently our labratory has used a photoablation technique to ablate the excitatory V2a interneurons which is zebrafish homolog of Chx10 in mammalian spinal cord (Kimura et al., 2006). After the ablation, the excitability of the network is decreased in the larval zebrafish (Eklöf-Ljunggren et al., 2010). It would be interesting to see what happens if these animals were allowed to grow into juvenile stages. Will they still show a perturbed behavior and if so what is the mechanism behind this? It will be also possible to look at the morphology of the other non ablated V2a interneurons at the juvenile stage to see how they change their dendritic arborization to compensate the behavioral perturbation if they are present at later stage

It was also shown by Liao and Fetcho (Liao and Fetcho, 2008) that commissural bifurcating longitudinal ascending (CoBL) interneurons in the larval zebrafish are glycinergic and are rhythmically active during swimming. They are the more numerous of all the cell classes being encountered electrophysiolgoically and are thought to be the core interneuron class mediating left-right alternation during swimming. It would be interesting to see if photoablating these cells affects the left-right alternation. It would be also interesting to see if there is any perturbed behavior if they will persist until juvenile stage.

Another interesting project would be looking at the interneuronal targets of 5-HT. Two previous observations suggested co-locallization of 5-HT in two different interneurons in the spinal cord. One has a unipolar process that projected ventrolaterally and dorsally into the motor column. The axonal process appears to be descending (McLean and Fetcho, 2004). They are different from VeMe interneurons which are identified by another group as the 5-HT neurons (Brustein and Drapeau, 2005). Since 5-HT modulates the locomotor pattern differently in adult spinal cord, it would be interesting to see which interneuron class contains the 5-HT in adult spinal cord. It would be also interesting to what happens if we drive the network to generate higher swimming frequency with our stimulation paradigm in the brainstem/spinal cord preparation and look at modulation of 5-HT at high swimming speeds.

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