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EPHA4 AND V2 INTERNEURONS IN THE MAMMALIAN LOCOMOTOR NETWORK

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ABSTRACT

Central pattern generators (CPGs) are neural networks that can execute half-automated movements without supraspinal or sensory input. Hindlimb locomotion in mammals is dependent upon such a CPG which is located ventrally in the spinal cord lumbar enlargement. The key features of mammalian locomotion are ipsilateral excitatory interneurons which execute rhythm generation, as well as commissural inhibitory interneurons which execute left-right coordination and flexor-extensor coordination. Little is known about the ipsilateral excitatory interneurons in the mammalian CPG, and our goal was to identify and describe the role of such excitatory, ipsilateral interneurons.

First we identified the EphA4-positive neurons as a key component of the mammalian CPG, a component which normally is restricted to one side by the signaling induced by the interaction of the axon guidance molecules ephrinB3 and EphA4. We found that in EphA4 and ephrinB3 null mice, CPG neurons aberrantly cross the midline. This results in an abnormal synchronized bilateral coordination ("hopping") due to an increased crossover of excitatory, normally EphA4-expressing neurons, which make connections to the CPG and thus override the normal alternating cross-coordination in the locomotion system.

Next, we directly demonstrated that EphA4-positive neurons are rhythmically active and that a subset of these are ipsilaterally projecting, excitatory interneurons with projections onto motor neurons. This positions them as an identified excitatory interneuron group in the mammalian CPG and suggests that the group may be defined by the combined expression of EphA4 and glutamate release.

We then went on to look for a possible overlap between EphA4-positive interneurons and the developmentally defined group of ventral V2 interneurons in the spinal cord. We found that the vast majority of V2 interneurons express EphA4 and further characterized the V2 interneurons as segmental ipsilaterally projecting, excitatory (V2a) or inhibitory (V2b) interneurons located in a position which suggests that they are members of the mammalian CPG. Furthermore, we show that there are more EphA4-expressing interneurons than what can be accounted for by V2 interneurons and motor neurons, and the aberrant crossing of processes from EphA4-positive neurons in *ephA4*$^{LacZ-/-}$ mice does not originate from the V2a population.

Finally, we used a mouse model where excitatory ipsilaterally projecting, Chx10-positive (V2a), interneurons are specifically ablated. In the absence of Chx10 neurons, the locomotor burst activity displayed increased variation, but flexor-extensor coordination was unaffected while left-right alternation was disrupted. Evidence for a direct excitatory input of V2a interneurons onto commissural interneurons was provided by anatomical tracing studies. Among the commissural interneurons contacted were the V0 (Evx1 positive) interneurons which are involved in left-right alternation. These observations point to an essential role for V2a interneurons in the control of left-right alternation.

Together, work presented in this thesis has identified essential components of the mammalian CPG for walking, leading us towards a better understanding of the fundamental principles for the organization of the mammalian locomotor network. Our hope is that our contribution will help to improve clinical neuro-rehabilitation of spinal cord injured patients.

LIST OF PUBLICATIONS

I. Kullander K, Butt SJ, Lebret JM, **Lundfald L**, Restrepo CE, Rydström A, Klein R, Kiehn O. *Role of EphA4 and ephrinB3 in local neuronal circuits that control walking.* Science. 2003. 299(5614):1889-92.

II. Butt SJ*, **Lundfald L***, Kiehn O. *EphA4 defines a class of excitatory locomotor-related interneurons.* Proc Natl Acad Sci U S A. 2005. 102(39):14098-103.
* These authors contributed equally.

III. **Lundfald L**, Restrepo CE, Butt SJ, Peng CY, Droho S, Endo T, Zeilhofer HU, Sharma K, Kiehn O. *Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord.* Eur J Neurosci. 2007. (11):2989-3002.

IV. Crone SA, Quinlan KA, Zagoraiou L, Droho S, Restrepo CE, **Lundfald L**, Endo T, Setlak J, Jessell TM, Kiehn O, Sharma K. *Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord.* Neuron. In press.

Paper not part of this thesis

Kiehn O, Quinlan KA, Restrepo CE, **Lundfald L**, Borgius L, Talpalar AE, Endo T. *Excitatory components of the mammalian locomotor CPG.* Brain Res Rev. 2008. (1):56-63. Review.

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryotamine (=serotonin)
β-gal	β-galactosidase (the protein made from the *LacZ* gene)
CNS	Central nervous system
CIN	Commissural interneuron
CPG	Central pattern generator
CST	Corticospinal tract
dCIN	Descending commissural interneuron
DTA	Diphtheria toxin A-chain
E	Embryonic day (the morning of the vaginal plug is E0.5)
EphA4	Ephrin receptor A4
FP	Fluorescent protein
GABA	Gamma-aminoburytic acid
GFP	Green fluorescent protein
GlyT1	Glycine transporter 1
GlyT2	Glycine transporter 2
iL	Ipsilateral lumbar
KO	Knock out
L	Lumbar
MLR	Mesencephalic locomotor region
NMDA	N-methyl-D-aspartic acid
P	Postnatal day
sCIN	Segmental commissural interneuron
T	Thoracic
vGluT2	Vesicular glutamate transporter 2

1 INTRODUCTION

1.1 ORGANIZATION OF MOTOR SYSTEMS

The central nervous system (CNS) in vertebrates is a fascinating and complex system which performs a wide range of tasks including control of physiological regulation, processing of sensory information, formation of memory and learning as well as the control of movements. When it comes to initiating movements, the CNS can be thought of as organized into three levels. The strategy is taken care of by the highest level which is the premotor cortices and the basal ganglia. The tactics are then calculated at the middle level by the motor cortex and the cerebellum. Finally, at the lowest level, the brain stem and the spinal cord execute the orders and make the muscles contract (Bear *et al.*, 1996). However, while goal directed movements (e.g. reaching) require a high degree of cortical input, the timing and coordination of muscle activation during vertebrate rhythmic locomotion is controlled by local neural networks in the spinal cord. These networks are capable of generating rhythmic motor activity in the absence of sensory input (Brown, 1911) and are called central pattern generators (CPGs) (Grillner, 1981; 2003; Gordon & Whelan, 2006a). CPG networks are however influenced by sensory inputs. Thus sensory input into the CPG will modify the output, but is not needed in order to generate locomotion patterning (Iizuka *et al.*, 1997; Gordon & Whelan, 2006a).

The interest in understanding neural networks probably arises from our understanding that so many vital body functions are controlled by the brain and spinal cord. One such fundamental function controlled by the CNS is the control of gait. The importance of the CNS in this kind of locomotion is obvious when observing patients suffering from spinal cord injury or a stroke, rendering them unable to walk. While our goal is to understand the fundamental principles for the organization of the mammalian CPG for walking these studies are also a crucial step in improving clinical neuro-rehabilitation of spinal cord injured patients (Rossignol *et al.*, 1998; Arenkiel *et al.*, 2007; Edgerton *et al.*, 2008).

1.2 CENTRAL PATTERN GENERATORS

CPGs have been found in species throughout the animal kingdom (Delcomyn, 1980). They exist for a number of essential partially automated body functions depending on the animal in question, including movement of the gut muscles, blinking of an eye, chewing, swimming, breathing and walking. A CPG is defined as a unit of neural network that can produce rhythmic repetitive output without the influence of any sensory or supraspinal input (Grillner, 1981). Locomotor CPGs have been described extensively in several species, among others lamprey (Buchanan, 2001; Grillner, 2003), zebrafish (Fetcho *et al.*, 2008), tadpoles (McLean *et al.*, 2000), mice (Gordon & Whelan, 2006a; Kiehn, 2006), rats (Butt *et al.*, 2002b; Kiehn, 2006) and cats (Jankowska, 2008) and evidence of human CPGs has also emerged (Dimitrijevic *et al.*, 1998).

Normally CPG activation is initiated from higher brain centers. Locomotion is induced by activity in the mesencephalic (MLR) or diencephalic locomotor regions,

which in turn activate reticulospinal neurons in the medial reticular formation (reviewed by Grillner *et al.*, 2008). The activity then descends through the fibers of the reticulospinal tracts which terminate in the spinal gray matter (Kandel *et al.*, 2000). In lamina VII and VIII they activate interneurons and subsequently motor neurons driving locomotion. Thus, stepping movements can be induced by electrical stimulation of neurons in the MLR (Edgerton *et al.*, 2008). Similarly in isolated spinal cords (see below) with the brainstem attached, locomotion patterns can be recorded from the ventral roots when reticular spinal neurons are stimulated electrically (Zaporozhets *et al.*, 2006). Locomotion can also be initiated by sensory input to the CPG such as sensory afferent information from the limb muscles or tail pinching in living animals (Lev-Tov & Delvolve, 2000). The speed of locomotion is regulated by the degree of activity in the descending fibers (Grillner *et al.*, 2008).

1.3 THE ISOLATED RODENT SPINAL CORD PREPARATION

Until the late 1980's most information on mammalian locomotion came from *in vivo* experiments in cats. Then, inspired by the success in using *in vitro* preparations of tadpoles and lamprey, the isolated neonatal rat spinal cord preparation was developed (Kudo & Yamada, 1987; Smith & Feldman, 1987). This system has proved valuable for a number of reasons. First of all, like the other systems used, it provides a measurable electrical output making it possible to directly relate the network activity with an actual behavior. Second, it produces a locomotion pattern which is similar to that in adult animals (Kiehn & Kjaerulff, 1996). Furthermore the preparation can be kept alive for a long time *in vitro* and it is readily available for intracellular recordings and manipulations of the network activity by pharmacological agents. A decade later, the similar neonatal mouse preparation became available (Nishimaru *et al.*, 2000). In addition to the above mentioned qualities it has the advantage that the molecular code for the embryonic determination of several classes of spinal cord neurons has been laid out (Jessell, 2000), providing the possibility to genetically manipulate the system.

As mentioned, locomotor activity can be elicited *in vitro* by electrical stimulation of either the brainstem or the sacral roots including the dorsal afferents (Lev-Tov & Delvolve, 2000; Whelan *et al.*, 2000; Zaporozhets *et al.*, 2006). These pathways operate via a glutamatergic mechanism which provides the extrinsic drive to the CPG (Douglas *et al.*, 1993). However, it is also possible to chemically induce locomotion by application of drugs such as N-methyl-D-aspartic acid (NMDA), 5-hydroxytryotamine (5-HT) and dopamine (Kudo & Yamada, 1987; Smith & Feldman, 1987; Douglas *et al.*, 1993; Cowley & Schmidt, 1994a; b; Kiehn & Kjaerulff, 1996; Kiehn *et al.*, 1999; Nishimaru *et al.*, 2000) thus replacing the descending drive. Both electrical as well as pharmacological induction of *in vitro* locomotion has been shown to be relevant by *in vivo* experiments in animals as well as man: It is possible to induce stepping movements in paraplegic mice with NMDA (Guertin, 2004; 2005), and in humans with long term spinal cord injuries it is possible to elicit EMG patterns of stepping movements by stimulating the ventral funiculus where fibers from the MLR are running (Dimitrijevic *et al.*, 1998; Guertin, 2004; 2005, see also review by Edgerton *et al.*, 2008).

In normal neonatal rodent *in vitro* spinal cord locomotion the ventral root L2 is predominantly flexor related while L5 ventral root activity is extensor related and the right and left L2 roots are alternating as are the ipsilateral L2 and L5 ventral roots (Kiehn & Kjaerulff, 1996; Whelan *et al.*, 2000). This allows the analysis of extracellular ventral root recordings in order to determine the phase of the flexor and extensor bursts during the locomotor cycle as well as the relationship between the right and left sides of the spinal cord.

Evidence from activity labeling and lesion studies as well as electrophysiology experiments have suggested that the location of the CPG for hindlimb locomotion in rodents is in the lower thoracic and lumbar enlargement of the ventral part of the spinal cord from T13-L6 (Kjaerulff & Kiehn, 1996; Kremer & Lev-Tov, 1997; Whelan *et al.*, 2000 and references in Kiehn, 2006). Microlesion studies confirmed that locomotor-related neurons are located in laminae VII, VIII and IX (Kjaerulff & Kiehn, 1996 and references in Kiehn, 2006). The rhythm generating ability is stronger in the rostral part of the CPG and declines toward the caudal spinal cord (Kjaerulff & Kiehn, 1996; Kremer & Lev-Tov, 1997). These findings help us to focus on the right regions in the jungle of nerve cells present in the spinal cord when studying the CPG.

1.4 THE LOCOMOTOR NETWORK

While the mechanisms underlying locomotion are relatively well understood in lamprey, less is known about mammalian locomotion. This is in part due to the more complex situation of not only coordinating between left and right, but also between muscles that flex and extend the limbs during walking. A further complication is the significantly larger amount of cells in the mammalian spinal cord. However, accumulating evidence from lamprey and tadpole has revealed the basic core features of vertebrate locomotor CPGs to be ipsilateral excitatory interneurons as well as commissural inhibitory interneurons (reviewed in Grillner, 2003) (see **figure 1**). The excitatory ipsilateral interneurons are glutamatergic and activate NMDA and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors on ipsilateral motor neurons, other excitatory interneurons and on the inhibitory commissural interneurons (CINs). The inhibitory CINs are glycinergic and provide inhibition onto the contralateral side. These essential features ensure that while locomotor neurons on one side of the spinal cord are activated, the corresponding neurons on the contralateral side are inhibited, hence coordinating bilateral alternation.

Owing to the added complexity of walking in higher vertebrates, a detailed outline for the locomotor network is missing for mammals. It is clear though, that the key features are i) excitatory rhythm generation, ii) left-right coordination and iii) flexor-extensor coordination (Kiehn, 2006). Different groups of CINs coordinating between left-right and flexor-extensor have been characterized (see below) as well as some groups of ipsilateral inhibitory interneurons which include Renshaw cell and Ia interneurons (McCrea *et al.*, 1980; Pratt & Jordan, 1987; Gosgnach *et al.*, 2006). However, detailed information on ipsilateral excitatory interneurons in the mammalian CPG has been missing.

Ventral, commissural, locomotor related interneurons have been characterized both anatomically (Eide *et al.*, 1999; Stokke *et al.*, 2002; Nissen *et al.*, 2005) and

electrophysiologically (Butt *et al.*, 2002b; Butt & Kiehn, 2003; Lanuza *et al.*, 2004; Quinlan & Kiehn, 2007; Jankowska, 2008). They can anatomically be divided into four groups: ascending CINs, descending CINs (dCINs), bifurcating CINs and segmental CINs (sCINs) projecting less than 1.5 segments. The latter group has recently been described by Quinlan and Kiehn (Quinlan & Kiehn, 2007) who showed that sCINs provide mixed excitatory and inhibitory input onto contralateral motor neurons. The majority of the pre-motor sCINs and dCINs recorded from had excitatory connections to the contralateral side where they either excited motor neurons directly or inhibited them indirectly by projecting to local inhibitory interneurons. Furthermore, their connectivity and firing pattern suggested an active role in segregating left-right activity (Quinlan & Kiehn, 2007).

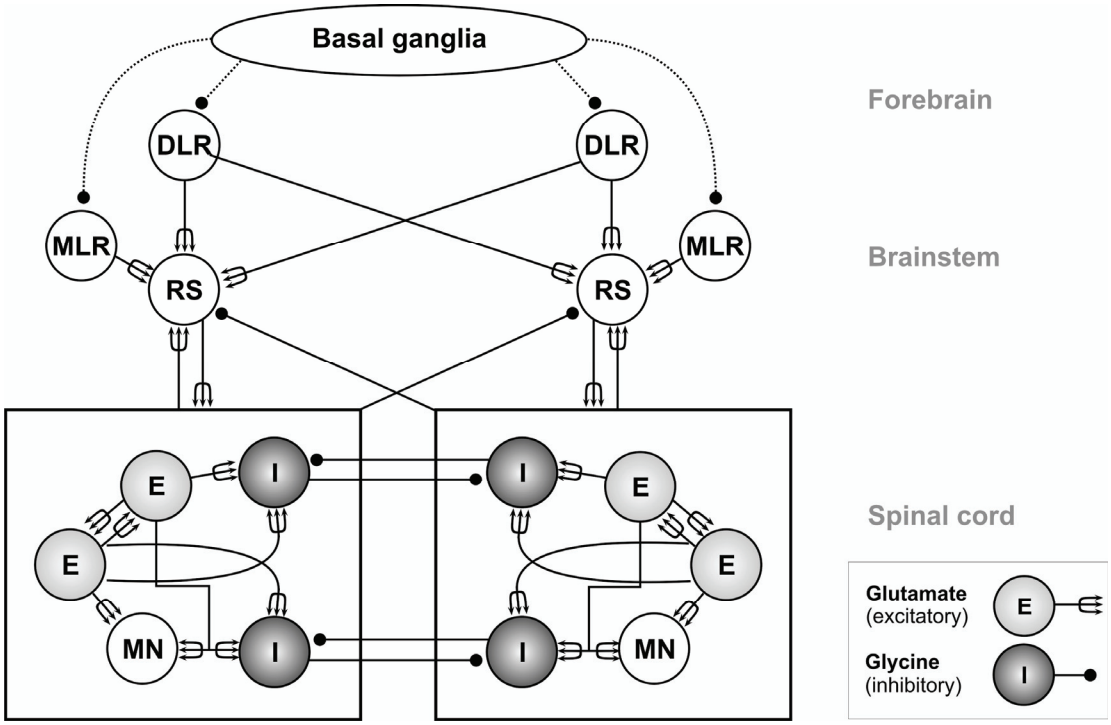

Figure 1. The lamprey locomotor network model. The forebrain, brainstem and spinal cord constitute the core of the neural circuitry which generates locomotion. The basal ganglia control the activity of the DLR and the MLR by inhibiting their activity. Once disinhibited, the DLR and MLR excite the reticulospinal (RS) neurons which in turn trigger locomotion via descending excitatory pathways. Excitatory interneurons in the spinal cord excite all types of neurons as indicated by their projections. The inhibitory glycinergic interneurons make crossed connections to inhibit all types of neurons on the contralateral side. Adapted from Grillner, 2003. For references, see Grillner *et al.*, 2008.

dCINs have been described by Butt and Kiehn (Butt & Kiehn, 2003). Similar to the sCINs, they have contralateral input to motor neurons in one of three ways: glycinergic monosynaptic, excitatory monosynaptic or inhibitory disynaptic by means of glutamatergic dCINs connecting onto local GABAergic interneurons. A fourth group was polysynaptic at rest, but switched to direct excitation during locomotion (Butt & Kiehn, 2003). The dCINs were active during all phases of the cycle period, meaning that they are probably involved in more than just left-right alternation. This notion was

confirmed when the authors used DC recording and found that in the L2 segment there are both flexor- and extensor-related neurons and the flexor dCINs are on average located more ventro-lateral than the extensor-related dCINs which are located deeper in the ventral spinal cord (Butt & Kiehn, 2003). This indicated a role for CINs in coordinating across the spinal cord so that the right flexor motor neurons in the upper lumbar spinal cord are activated simultaneously with the left extensor motor neurons in the lower lumbar spinal cord.

As opposed to the extrinsic drive needed to initiate locomotion, the rhythm that determines the locomotor pattern is derived from within the spinal cord. Experiments in the neonatal rodent spinal cord have shown that the rhythm generating capabilities are also dependent upon glutamatergic transmission (see Kiehn, 2006 for review), indicating that the rhythm generator is to be found among the many excitatory neurons in the ventral spinal cord.

Not much is known about the identity of the excitatory interneurons and their involvement in generating mammalian locomotion. Therefore further anatomical as well as electrophysiological data is much needed. Aside from the ipsilateral EphA4-positive, excitatory interneurons (**Paper I-II**) and the ipsilateral V2a interneurons (**Paper III-IV**) described in this thesis, the only other group of mammalian ipsilateral excitatory interneurons functionally described in rodents is the Hb9 interneurons. They are glutamatergic and receive input from excitatory interneurons in phase with the ventral root burst and they also fire in phase with the ventral root burst. Overall, they fulfill many of the criteria for being rhythm generating (Hinckley *et al.*, 2005b; Wilson *et al.*, 2005; Hinckley & Ziskind-Conhaim, 2006 and reviewed by Brownstone & Wilson, 2008) but there is still some controversy as to what their role is in locomotion. It is the aim of this thesis to define excitatory components of the mouse hindlimb locomotor CPG, and to describe their role during locomotion.

1.5 UNRAVELING NETWORK STRUCTURE

Complicated structures are easiest understood by dissecting them into smaller and simpler elements. Therefore, vertebrates such as tadpoles, lamprey and zebrafish with less overwhelming numbers of cells in their spinal cords can teach us a lot about the basic principles of locomotion. By studying how the locomotor networks are assembled, we can learn something about the structure of the network. The large knowledge on developmental neurobiology together with an array of genetic manipulation methods is useful for this purpose. Some methods are already being used, such as labeling of neuronal populations with fluorescent proteins (Tamamaki *et al.*, 2003; Wilson *et al.*, 2005; Zeilhofer *et al.*, 2005) and knocking out gene function (Dottori *et al.*, 1998; Kullander *et al.*, 2001a; Kullander *et al.*, 2001b). Other methods include removal of cell groups, either by knocking them out completely or by killing them with diphtheria toxins at a certain stage of development when a particular gene of interest is expressed (Ivanova *et al.*, 2005, **Paper IV)**. However, when a neural network is perturbed it may cause activity-dependent reorganization of the CPG (Myers *et al.*, 2005), and therefore yet other methods aim for the specific electrical activation or inactivation of cells by hyperpolarizing shunts (Callaway, 2005; Gosgnach *et al.*, 2006) or by photo-stimulation or inactivation (Arenkiel *et al.*, 2007; Zhang *et al.*, 2007a).

Traditionally, mammalian locomotion has been investigated by means of electrophysiology and anatomy in order to describe projection and connectivity patterns as well as the location of the participating cells. With **Paper I** of this thesis, combining these methods with those of developmental and molecular biology has brought to attention the potent synergistic effects of combining these fields in the study of the CPG in mice. As correct axon guidance and cell migration during embryogenesis is crucial for assembling the neural networks involved in locomotion, and since we have used mice with assembly defects in the CPG, the next sections are dedicated to describing essential features of axon guidance and developmental biology important for this thesis.

1.6 FINDING THE WAY: AXON GUIDANCE

The correct path finding and target specificity of outgrowing axons is dependent upon a great number of axon guidance molecules expressed both on the axon and in the surrounding environment. Many different types of molecules possess axon guidance properties including cell adhesion molecules, tyrosine kinase receptors, extracellular matrix proteins, morphogens and growth factors (Tessier-Lavigne & Goodman, 1996; Plachez & Richards, 2005) all of which can be combined in a number of ways through clustering into membrane bound complexes (Murai & Pasquale, 2004). Furthermore electric activity in developing neurons is also an important factor for correct path finding (Hanson & Landmesser, 2004).

Long and short range attraction as well as long and short range repulsion influences the path taken by the growth cone at the tip of the developing axon (Tessier-Lavigne & Goodman, 1996). It can react differently to cues in the environment depending on the molecules which it expresses itself. It may be either attracted, repelled or induced to collapse. A fourth possibility is that a cue in the environment may allow the growth cone to continue to grow in spite of repellents in the surrounding (Tessier-Lavigne & Goodman, 1996; Plachez & Richards, 2005). This is the consequence not just of the axon weighing the different cues but also of different signals being combined in a hierarchical fashion, with one signal silencing the response to another (Stein & Tessier-Lavigne, 2001). These principles for axonal outgrowth are of paramount importance for establishing the correct network architecture.

Developing axons in the spinal cord face a number of challenges upon growing. They need to decide whether they are to grow rostrally or caudally, ventrally or dorsally and whether they should cross the midline or not. These principles have been studied extensively in vertebrates with respect to CINs (Tessier-Lavigne & Goodman, 1996; Plachez & Richards, 2005). As a general principle the axons of these neurons first grow towards the midline. This is mediated by both repulsion away from the site of origin as well as attraction from cues in the surrounding and the midline. The midline acts as a checkpoint for decision making. Upon arrival to the midline, some guidance molecules need to be downregulated in order to cross the midline. After crossing, other sets of molecules are expressed in order to both repel the axon from the midline as well as to attract it to new cues in the contralateral half. In this way the newly crossed axon is prevented from re-crossing, while at the same time allowing it to orient towards new cues in rostral and caudal directions.

Ipsilateral axons likewise have to respond to cues in the surroundings, but in contrast to CINs they are not to cross the midline. Instead upon reaching the midline, growth cone collapse is induced, ensuring that the axon will not cross the midline. If molecules involved in this midline repulsion are defective, axons may cross aberrantly (Tessier-Lavigne & Goodman, 1996; Dottori *et al.*, 1998; Coonan *et al.*, 2001; Kullander *et al.*, 2001a; Kullander *et al.*, 2001b; Yokoyama *et al.*, 2001; Plachez & Richards, 2005). This can result in animals with locomotor defects. In mice, this is the case when the axon guidance molecules involved in midline repulsion, EphA4 or ephrinB3, are knocked out.

1.7 EPHA4 AND EPHRINB3

The Eph receptors comprise a large family of tyrosine kinase receptors divided into the EphA and the EphB groups. Their ligands, the ephrins, are also divided into two groups: ephrinA which is attached to the membrane by a glycophosphatidylinositol anchor, and ephrinB which traverses the membrane. EphAs primarily bind ephrinAs while EphBs primarily bind ephrinBs. However, both groups of receptors may bind both groups of ligands, the best described example of which is ephrinB3 binding to EphA4. Both classes of ligands may act as signaling molecules although this is much better described for the ephrinB group. Upon ligand binding large signaling complexes are assembled, hence allowing intricate signaling to induce repulsion or (sometimes, depending on context) cell adhesion (reviewed in Kullander & Klein, 2002; Murai & Pasquale, 2004; Lackmann & Boyd, 2008).

EphrinB3 is expressed in the midline of the spinal cord (Kullander *et al.*, 2001a; Yokoyama *et al.*, 2001; Kadison *et al.*, 2006) and EphA4 is expressed in the ventral gray matter on the growth cones of developing axons (Greferath *et al.*, 2002). When EphA4 binds ephrinB3, the axon growth cone collapses due to activation of signaling pathways associated with cytoskeletal motility (Wahl *et al.*, 2000; Kullander *et al.*, 2001a). In this way ephrinB3 acts as a midline barrier confining EphA4-expressing axons to stay on the ipsilateral side of the spinal cord as the cell body from which it originates (Kullander *et al.*, 2001a; Yokoyama *et al.*, 2001).

Many anatomical defects have been described in the EphA4 null mouse. Those involving the spinal cord are aberrant re-crossing of corticospinal axons (Dottori *et al.*, 1998; Coonan *et al.*, 2001; Kullander *et al.*, 2001b) and shallowing of the dorsal funiculus (Kullander *et al.*, 2001b; Yokoyama *et al.*, 2001). Re-crossing of corticospinal tracts (CST) has also been described for ephrinB3 defective mice (Yokoyama *et al.*, 2001). In mice with a knock out (KO) of ephrinB3 there are many misguided axons in the vicinity of the ventral midline (Kadison *et al.*, 2006). They exhibit an increased number of forked transverse commissurals, indicating that the axons are “confused” while crossing the midline and therefore seem to stay within the same segment upon aberrantly crossing the midline. In EphA4 KO animals, axons crossing the midline also terminate at the level at which they cross (Coonan, *et al.*, 2001).

The most prominent behavioral observation of the EphA4 and ephrinB3 KO mice is their hopping phenotype (Dottori *et al.*, 1998; Kullander *et al.*, 2001a; Yokoyama *et al.*, 2001; Kullander *et al.*, 2003; Akay *et al.*, 2006). Instead of walking

with normal alternation between the right and left legs, they instead display synchronous movement of the hindlimbs to produce a hopping gait. As EphA4 KO mice display defects in CST projections (see references above) and since EphA4 is of significant importance for motor neuron axon guidance (Eberhart *et al.*, 2000; Eberhart *et al.*, 2002; Coonan *et al.*, 2003; Hanson & Landmesser, 2004) (both CST tracts and motor neuron axons are involved directly in motor behavior), we found it a thrilling challenge to investigate the locomotion of the *in vitro* preparation of EphA4 KO animals. We did this in order to see if the behavior was due to aberrantly connected CSTs or motor neuron axons, or whether there was a change in the spinal locomotor network (**Paper I**).

1.8 GENETIC DETERMINATION OF SPINAL INTERNEURONS

The development of the spinal cord starts with neurulation when the ectoderm of the gastrula forms the neural plate and starts to invaginate. The neural tube is made when the neural folds are brought together in the dorsal midline (Kandel *et al.*, 2000). Neural tube closure begins in the mouse at E8.5, initiating at the level that later becomes the rostral spinal cord. Hereafter the tube "zips up" to eventually close at early E10.5 and develop into the brain and spinal cord (Copp *et al.*, 2000).

The genes expressed by individual cells determine the placement, transmitter phenotype and projection patterns of the neurons born and causes cells to be born with different identities. These identities are set up already during neural tube formation when the floorplate and, later, the notochord start to secrete Sonic hedgehog (Shh) while the neural folds and, later, the roof plate secrete bone morphogenetic protein (BMP) (Tanabe & Jessell, 1996). This sets up a ventral-dorsal gradient of Shh and BMP and is of crucial importance in setting up the patterning of the spinal cord (Ericson *et al.*, 1997a, reviewed in Tanabe & Jessell, 1996; Jessell, 2000). Thus in the spinal cord, this gradient leads the cells in the ventricular zone to express different patterning genes, dividing the ventricular zone into different progenitor domains. In the dorsal part of the spinal cord these domains are pd0-6 which give rise to 8 classes of dorsal interneurons (Zhuang & Sockanathan, 2006) while the ventral part houses p0-p3 as well as pMN, which give rise to the ventral classes of V0-V3 interneurons as well as to the motor neurons (Briscoe *et al.*, 2000, reviewed by Poh *et al.*, 2002) (see **Figure 2**). These ventral interneurons and the motor neurons are born from around E9.5 to

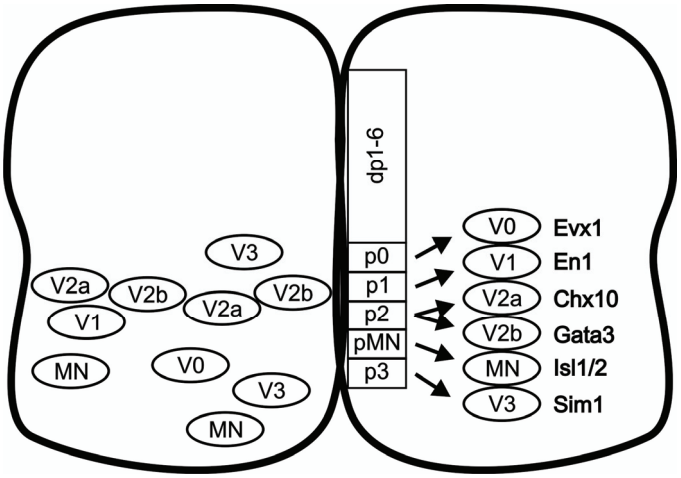

Figure 2. Ventral interneurons. During embryonic life, the progenitor domains are located in the ventricular zone along the midline (right side). Once newborn ventral cells become postmitotic and start to migrate laterally they differentiate into the ventral interneuron groups V0-V3 and motor neurons (MN). The genes which specify the differentiated ventral interneuron groups are indicated to the right. The approximate positions of the ventral interneuron groups in late embryonic mice are indicated on the left side of the spinal cord. See text for references.

around E13.5 (Nornes & Carry, 1978; Briscoe *et al.*, 1999; Pierani *et al.*, 1999; Sander *et al.*, 2000; Moran-Rivard *et al.*, 2001; Vallstedt *et al.*, 2001; Smith *et al.*, 2002; Peng *et al.*, 2007) and probably continue to increase in numbers until E16.5 (Nissen *et al.*, 2005). Once the post mitotic interneurons are born, they immediately start to migrate laterally to their final position in the cord and meanwhile they start to send out their axonal projections in order to make connections to other neurons (Leber & Sanes, 1995; Saueressig *et al.*, 1999).

The main anatomical features of the ventral interneurons are as follows: V0 interneurons are identified by the post mitotic transcription factor Evx1 (and before that the progenitor specific factor Dbx1) (Pierani *et al.*, 1999; Moran-Rivard *et al.*, 2001; Pierani *et al.*, 2001). They are mixed inhibitory and excitatory (Lanuza *et al.*, 2004) and project contralaterally where after they become ascending for one to four segments with the majority projecting up to one and a half segments in the ventral funiculus (Moran-Rivard *et al.*, 2001). V1 interneurons express En1 postmitotically and are ipsilaterally projecting (Burrill *et al.*, 1997; Saueressig *et al.*, 1999; Alvarez *et al.*, 2005) and ascending for maximally two segments (Saueressig *et al.*, 1999) (however, Renshaw cells are both ascending and descending for maximally one segment (Sapir *et al.*, 2004)). They are glycinergic (Alvarez *et al.*, 2005) and GABAergic (Saueressig *et al.*, 1999; Wenner *et al.*, 2000; Sapir *et al.*, 2004; Alvarez *et al.*, 2005), some connect directly to motor neurons (Saueressig *et al.*, 1999; Wenner *et al.*, 2000) and include the physiologically defined Ia interneurons as well as the Renshaw cells (Wenner *et al.*, 2000; Sapir *et al.*, 2004; Alvarez *et al.*, 2005). V2 interneurons derive from a common progenitor population expressing Lhx3 (Peng *et al.*, 2007) and are divided into V2a, expressing Chx10 (and to a large degree also Sox14, L. Zagoraiau and T. M. Jessell, personal communication, see also Hargrave *et al.*, 2000) and V2b expressing Gata2/3 (Ericson *et al.*, 1997b; Zhou *et al.*, 2000; Karunaratne *et al.*, 2002; Poh *et al.*, 2002; Smith *et al.*, 2002), which are generated in equal numbers (Peng *et al.*, 2007). Their projection pattern has been hypothesized to be ipsilateral and one to five segments (Saueressig *et al.*, 1999; Lee & Pfaff, 2001), but data describing this has never been published. The transmitter phenotype (V2a interneurons use glutamate and V2b interneurons use GABA and glycine) of this group was unknown until **Paper III** of this thesis. The V3 group of interneurons is identified on the basis of Sim1 expression (Briscoe *et al.*, 1999). No anatomical data has been published on the V3 interneurons but they are described as a group of primarily excitatory CINs (Goulding & Pfaff, 2005; Nissen *et al.*, 2005; Geiman *et al.*, 2006) with both ascending as well as descending projections (described as unpublished results in Nissen *et al.*, 2005).

Due to the fact that many of the transcription factors defining the interneuron groups are downregulated shortly after their birth, it can be difficult to follow these populations of interneurons. Therefore studies on ventral interneuron groups are often done at the embryonic ages E11.5 and onwards. Features such as projection pattern and cell location are established already at this age allowing us to extrapolate at least some of the data to the neonatal mouse spinal cord. When most of the interneurons are born around E13.5 (Nornes & Carry, 1978), the locomotor network starts to establish itself and to become rhythmically active (Nakayama *et al.*, 2002; Hanson & Landmesser, 2003) further boosting the network development (Hanson & Landmesser, 2004).

As these developmentally defined groups of interneurons are being anatomically characterized in terms of projection pattern and transmitter phenotypes, it allows us to start speculating about their possible overlaps with the interneuron classes identified on the basis of electrophysiology and anatomy (described above). Furthermore, the molecular characterization of early neuronal cell populations has provided a new basis for genetically dissecting the neuronal circuits that control locomotion in the vertebrate spinal cord. Thus in recent years, it has been shown that the V0 interneurons are involved in left-right coordination (Lanuza *et al.*, 2004), while the V1 interneurons have a role in the regulation of locomotor speed (Gosgnach *et al.*, 2006) and V3 interneurons play an important role for maintaining rhythmicity and stability of the locomotor network (Zhang *et al.*, 2007b). With **Paper IV** of this thesis we add to the growing picture by revealing a role for the V2a interneurons in rhythm stabilization and left-right coordination probably through ipsilateral projections to V0 interneurons. Considering the strong tool of genetic manipulation, mouse models with defects or markers in the developmentally defined interneuron classes create a large playground for determining the individual contributions of the many constituents of the mammalian locomotor network.

2 AIMS OF THE THESIS

The general aim in this thesis is to unravel the role of a subset of excitatory interneurons during hindlimb locomotion in the murine spinal cord. The specific aims were to:

Paper I

- Investigate the role of the axon guidance molecule EphA4 in the organization of the mouse hindlimb locomotor CPG.

Paper II

- Directly demonstrate the physiological relevance of EphA4-positive neurons during mouse hindlimb locomotion.

Paper III

- Thoroughly describe the location, projection pattern and transmitter phenotype of the V2 group of interneurons.
- Investigate whether V2 interneurons express EphA4 and whether their axons cross the midline aberrantly in the absence of EphA4 signaling.

Paper IV

- Study the physiological role of excitatory, ipsilaterally projecting Chx10-positive (V2a) interneurons in locomotion.

3 METHODOLOGICAL CONSIDERATIONS

The work presented in this thesis involved the use of a number of techniques, all of which have been described in detail elsewhere. As the experimental procedures regarding materials and methods are mentioned in the papers, I will only briefly discuss advantages and disadvantages of some of the methodologies used.

All animal experiments performed in the Mammalian Locomotor Laboratory have been carried out in accordance with the rules and guidelines required for animal experiments at the Karolinska Institutet which are in accordance with recommendations from the Federation of European Laboratory Animal Science Associations and with the European legislation. Furthermore all experiments on animals have been approved by the relevant animal experiment committees, either in Stockholm or in the city and country where the experiment was carried out.

3.1 GENETICALLY MODIFIED MICE

A number of different transgenic mice were used in these studies. In the introduction I have briefly touched upon some of the advantages of using genetically modified mice. There are also disadvantages and the significance of these is highly dependent upon the gene missing or manipulated and on whether the animal is heterozygous or homozygous for the gene in question. Generally, knocking out genes has much less drastic effects (if any) in heterozygous animals than in homozygous animals. Also, marker genes (such as fluorescent protein (FP) or Cre) usually have little or no effect on the phenotype, they basically just make the cells visible. With markers, the biggest problems are typically that the marker may be expressed unspecifically (as is the case with the mouse where green fluorescent protein (GFP) is expressed under the Hb9 promoter (Wilson *et al.*, 2005)) or alternatively is not expressed in as many cells as could be expected (as is the case with the Lhx3-Cre mice (Sharma *et al.*, 1998)). Therefore it is of great importance to carefully evaluate the expression pattern of the transgene before using it for functional studies. In other cases, the marker disrupts gene function because it replaces parts or all of a gene as is the case with the $ephA4^{LacZ}$ mice. Finally, the time in development when the gene is normally expressed may influence the effect of the (missing or) trans-gene. Thus a missing transcription factor gene important for cell type determination may lead to that cell becoming another type of cell, while a cell surface molecule may instead have effects on axon guidance.

The expression of the attenuated diphtheria toxin A-chain (DTA) can be driven by a gene specific promoter so that when the gene of interest is expressed, DTA is induced, and the cell expressing the particular gene is killed. The advantage of using DTA ablation of Chx10 cells over completely abolishing Chx10 expression in any neuron as in a Chx10 KO is that the Chx10 cells will initially develop but then die at the age when Chx10 becomes expressed. This reduces the risk of changing the composition of the cell population in the ventral spinal cord in favor of another ventral interneuron group, a change which in itself causes reorganization of the connectivity (Lanuza *et al.*, 2004). With the Chx10-DTA there is still the risk that neurons which should have projected to Chx10 cells will instead project aberrantly. However, there is

also the possibility that the axons are simply collapsing and withdrawn as their targets (the Chx10 cells) are missing. It will be interesting to shed more light on this issue and therefore we eagerly await genetically manipulated mice where it is possible to just excite or silence the neurons of interest by shining a light or adding a chemical to the locomotor drugs in order to see a possible change in the normal network configuration.

When using hypomorphic alleles as is the case in the *ephA4*^{LacZ} mouse where the EphA4 is gene trapped (Leighton *et al.*, 2001) care must also be taken in evaluating the transgene before making conclusions on the effect of the mutation. Gene trapping relies on insertion of a gene-trap vector into an intron of a coding gene. Subsequent transcription of the endogenous (in our case) promoter and enhancer elements simultaneously mutates the trapped gene and reports its expression pattern. This way the N-terminal signal sequence is captured and an active β-galactosidase (β-gal) fusion protein is generated (Skarnes *et al.*, 1995; Stanford *et al.*, 2001). There is a risk that an endogenous splice site may be used more efficiently than that of the gene trap vector resulting in normal transcripts which are spliced around the vector (McClive *et al.*, 1998). This will result in the lack of a null allele and, therefore, lack of an obvious phenotype. However, as the *ephA4*^{LacZ} mouse does have the obvious hopping phenotype and also morphologically resembles the EphA4 KO, it seems not to be a problem in our case.

3.2 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) has been used extensively throughout this thesis work. The advantage of IHC is that, with appropriate resolution, it is possible to determine if the antigen in question is expressed by a cell and whether it co-localizes with another marker of interest. However, care must be taken to be sure that the co-localization is true. This is relatively easy when staining for nuclear transcription factors or for GFP located in the entire cell as is the case in **Paper III** and **IV**. It is a bit more difficult when looking for nerve terminals as in **Paper IV** or when trying to identify the EphA4 status (by the means of β-gal expression) of the *ephA4*^{LacZ} mice as in **Paper I-III**. Here, the granular appearance (which is due to the trapping of β-gal in the golgi apparatus and endoplasmatic reticulum, see Skarnes *et al.*, 1995) makes it difficult to decisively determine a cell as EphA4 (β-gal) positive when looking in only one focal plane. However, careful examination by focusing up and down in a normal light microscope as well as taking confocal pictures in 3D has convinced us that if we see enough granules surrounding the cell nucleus, we can determine that the cell is EphA4-positive. This is also the method used by Skarnes and colleagues (Skarnes *et al.*, 1995) who developed the technique of gene trapping. We have described the details of determining the EphA4 status in the materials and methods section in **Paper III**. It should be noted that our selection criterion may result in false negatives, and therefore we should not put too much focus on negative results. With respect to the transcription factor staining we have often applied the use of the magic wand tool in Adobe Photoshop in order to select the transcription factor stained cells with a sufficient intensity level above background. This also may result in a lower cell count, but at the same time ensures that weak cells out of focus are not counted as false negatives in terms of co-localization with β-gal stained granules.

3.3 *IN SITU* HYBRIDIZATION

As the vGluT2 and GlyT2 proteins are located in the synaptic terminals, we had to use ISH in order to determine whether EphA4 and V2 interneurons were glutamatergic or glycinergic. One potential problem with ISH is that there is no guarantee that the protein is expressed. However, from studying spinal cord literature extensively and comparing mRNA expression patterns to those of protein expression, there seems to be a very good correlation between mRNA and protein expression. Thus, in our hands, we obtain very similar results when quantifying the number of glycinergic V2b interneurons with ISH and IHC (**Paper III**). Furthermore our own ISH studies in a mouse where vGluT2 expressing cells express protein markers show excellent correspondence between the ISH and the IHC (L. Borgius, personal communication).

With the development of non-radioactive *in situ* probes, the spatial resolution can be quite good although the sensitivity may be a problem. As we are looking for mRNA in the cell body and this mRNA is often squeezed into the cytoplasm and is visible as a clear black band around the cell nucleus, it makes it relatively easy to determine whether the mRNA co-localizes with other proteins found in the cell body.

3.4 RETROGRADE TRACING

We have used retrograde tracing for two purposes: 1) to identify CIN elements in the genetically manipulated mice used and to 2) reveal the projection patterns of V2 interneurons. In both cases we have used fluorescent or biotin labeled dextran amines applied by either holding a crystal at the application site (where a small cut was made) or by injection of saturated dextran into the cut site. The method was developed and beautifully described by Glover and colleagues (Glover *et al.*, 1986) and mostly has advantages. The major disadvantage is that the dye is not only transported in the neural processes but also diffuses into the tissue which results in the application site becoming fluorescent. Therefore the method is stronger for identifying projection patterns at longer distances away from the site of application. This may explain our difficulties in determining the short range projection patterns of the V2 interneurons and why it is so difficult to use this tracing method to show segmental V2 projections to CINs.

3.5 *IN VITRO* LOCOMOTOR EXPERIMENTS

The advantages of the isolated neonatal rodent spinal cord preparation have already been described in the introduction. In short, it is a very good model for mammalian locomotion. In the Mammalian Locomotor Laboratory, stable locomotion of the *in vitro* spinal cord preparation is routinely induced by use of 5-HT and NMDA. In spite of all the advantages mentioned in the introduction, a disadvantage of drug induced locomotion is that the drugs bathes the spinal cord and therefore hits all neurons with 5-HT and NMDA receptors. Not necessarily all neurons with these receptors are normally activated when the CPG is activated from the brain. A more natural way of inducing locomotion is to stimulate the brainstem in the area of the reticulospinal neurons. However, in general, brainstem stimulation gives a locomotor pattern comparable to that of drug-induced locomotor activity.

Three parameters are important in the analysis of locomotor data: the period, the amplitude and the phase of activity. Analysis of both the period and the amplitude is described in **Paper IV**. When it comes to analyzing the phase of the root activity, we use circular statistics. This method requires a detection threshold and it also requires that the locomotor cycles are independent, which is not the case for any two consecutive locomotor periods. To circumvent this problem we instead analyze random locomotor cycles as described in **Paper IV** (Kjaerulff & Kiehn, 1996).

The method of DC recording used in order to reveal synaptic connectivity is sometimes criticized for underestimating the inhibition because the equilibrium potential for chloride is very close to the resting membrane potential of motor neurons. This issue has been commented on earlier (Butt & Kiehn, 2003; Quinlan & Kiehn, 2007). However, as we look at excitatory cells in both **Paper II** and **Paper IV** and furthermore are able to pharmacologically block inhibitory potentials by use of GABA and glycine blockers, this is not a problem.

4 RESULTS AND DISCUSSION

The possibility of generating genetically manipulated mice in combination with the development of the *in vitro* mouse spinal cord preparation has opened a range of possibilities to closer investigate how the locomotor CPG is organized in mammals. Electrophysiological, anatomical and molecular biological methods are increasingly being combined in attempts to unravel the networks of locomotion and are also here in the studies constituting this thesis.

4.1 PAPER I – EXCITATORY EPHA4 INTERNEURONS IN LOCOMOTION

Paper I (Kullander *et al.*, 2003) provided the first attempt to investigate mammalian locomotion by combining the two traditionally separated fields of molecular biology and electrophysiology. Previous studies had shown that the phenotype of both EphA4 and ephrinB3 null mice was a rabbit-like hopping gait (Dottori *et al.*, 1998; Kullander *et al.*, 2001a; Yokoyama *et al.*, 2001). Anatomical studies demonstrated that EphA4 acts as an axon guidance cue to keep axons from crossing the spinal cord midline due to its expression of ephrinB3 (Coonan *et al.*, 2001; Kullander *et al.*, 2001a). Thus, EphA4 and ephrinB3 are important for establishing correct projections of the CST (Dottori *et al.*, 1998; Coonan *et al.*, 2001), and it was proposed that the hopping behavior was due to a bilateral innervation from the CSTs. We therefore set out to investigate this notion directly by looking at locomotion in the *in vitro* spinal cord preparation.

In **Paper I** we demonstrated that mice with knocked out EphA4 receptors (or their ephrinB3 ligands) do not only have a hopping phenotype, their spinal cords also "hop" in the absence of supraspinal input when examined *in vitro*. Thus when recording from the ventral L2 (mainly flexor motor neurons) and L5 (mainly extensor motor neurons) roots (Kiehn & Kjaerulff, 1996; Whelan *et al.*, 2000), the left L2 was in phase with the right L2 and the left L5 was in phase with the right L5 while the L2s and L5s were out of phase, indicating that the mice simultaneously activated flexor muscles on both sides of the spinal cord, and that the flexors were alternating in activity pattern with the extensors (**figure 1, Paper I**). This gave an indication that the hopping phenotype was not due to aberrant CTS projections but instead were due to a CPG defect in the EphA4 KO mice.

In the normal spinal cord, CINs coordinate the activity between the two halves of the cord (Kjaerulff & Kiehn, 1996; 1997; Butt *et al.*, 2002a; Butt & Kiehn, 2003; Quinlan & Kiehn, 2007). We therefore asked whether a change in the CIN system was the underlying cause of bilateral synchrony in EphA4 KO animals. This seemed not to be the case as, in addition to the CINs in the normal location and number, we discovered that EphA4 KO animals display crossing over of processes that in wildtype animals are ipsilateral. These processes were shown to come from cells that were normally EphA4-expressing (in contrast to CINs), indicating that indeed the wiring in the EphA4 KO spinal cord is incorrect as determined from the errors in projection pattern and the increased overcrossing of fibers (**figure 2, Paper I**).

Since excitatory CINs mediate synchronous coordination between the left and right sides when blocking inhibition in the spinal cord (Bracci *et al.*, 1996; Cowley &

Schmidt, 1997; Kremer & Lev-Tov, 1997; Hinckley *et al.*, 2005a), we speculated that the hopping locomotion was due to an increased excitatory drive due to aberrant crossover of excitatory EphA4-positive neurons. Anatomical support for the existence of excitatory EphA4 neurons in the spinal cord came when we, with a combination of *in situ* hybridization and immunohistochemistry, showed that in the ventral half of the spinal cord, 23% of EphA4 cells are glutamatergic while 51% of all glutamatergic cells express EphA4 (**figure 4, Paper I**).

By adding sarcosine (a glycine uptake inhibitor) to the "hopping" *in vitro* preparation, we could reverse the spinal cord to "walk" again, and the same result was also seen using the glutamate blockers CNQX and AP5 (**figure 3, Paper I** and unpublished data). Taken together our experiments suggested that the normal network for left-right alternation was not affected and that the hopping phenotype was not due to the crossing over of the CSTs in the brainstem as previously speculated (Kullander *et al.*, 2001a; Yokoyama *et al.*, 2001). Instead, our results indicate that the hopping is due to an increased excitatory drive due to additional aberrantly crossing local excitatory processes. The increased excitation thus overrides the normal left-right alternating system in the CPG and prevents it from driving the two sides into alternation. *In vivo*, this statement is supported by reports from Akay and colleagues (Akay *et al.*, 2006) noting that when adult EphA4 null animals wake up from isofluorane anesthesia (which potentiates $GABA_A$ receptor signaling, increasing the inhibitory drive (see references in Akay *et al.*, 2006) the mice walk normally for a few minutes before resuming their abnormal hopping gait.

Since **Paper I** was published, we have learned that sarcosine is a more potent blocker of GlyT1, which functions to regulate the glycine concentration at NMDA receptor glutamatergic synapses (Supplisson & Bergman, 1997; Lim *et al.*, 2004; Issberner & Sillar, 2007, see Zafra *et al.*, 1997 for review), than of GlyT2 which is located at glycinergic synapses where it clears glycine from the synaptic cleft (Liu *et al.*, 1993, see Zafra *et al.*, 1997 for review). As the glycine site on the NMDA receptor is not normally saturated, it can be expected that sarcosine addition will potentiate NMDA receptor signaling by increasing glutamatergic signaling, as has been shown in Xenopus larvae and *in vivo* in rats (Kinney *et al.*, 2003; Issberner & Sillar, 2007). Thus, it can be speculated that our results on reverting the "hopping" to "walking" could have come about instead by increasing the excitatory drive through NMDA receptor signaling onto both excitatory and inhibitory CINs, which are both involved in left-right alternation (Quinlan & Kiehn, 2007). However, in Xenopus addition of sarcosine merely blocked locomotion entirely, and only in combination with strychnine (or with a specific GlyT1b antagonist alone) was the potentiating effect seen on NMDA receptors (Issberner & Sillar, 2007). Likewise, in zebrafish lacking GlyT1 (the *shocked* mutation) swimming is impaired and can be induced when applying strychnine. In the same study, wildtype zebrafish were incapable of swimming in the presence of sarcosine (Cui *et al.*, 2005). Thus, sarcosine may still either have some inhibitory effect on GlyT2 or, alternatively, generally elevate extracellular glycine levels. This in combination with an increased drive to CINs, may account for the reversion of the locomotion pattern.

Aberrantly crossing CSTs are not responsible for abnormal locomotion in the newborn EphA4 KO animals and we propose that this is also the case in adult mice.

Our proposal is supported by a number of additional observations. First, the CSTs do not reach the lumbar enlargement until P7-9 (Gianino *et al.*, 1999) and therefore cannot affect the locomotor network at the early neonatal stage when we conducted our investigations (P0-P4). Second, the EphA4 KO mice walk with normal alternation when waking up from isoflurane anesthesia (Akay *et al.*, 2006) although the CSTs are aberrantly crossing in these animals. Third, the CST axons arise in layer V of the motor cortex (Gianino *et al.*, 1999; Beg *et al.*, 2007) which initiates voluntary movements (Kandel *et al.*, 2000) whereas locomotion is initiated by activity in the MLR of the brainstem (Grillner *et al.*, 2008).

Recently four research groups have described mutant animals with defects in the EphA intracellular signaling pathway (Beg *et al.*, 2007; Fawcett *et al.*, 2007; Iwasato *et al.*, 2007; Wegmeyer *et al.*, 2007). All of these mutant animals are described to have defects similar to the EphA4 and ephrinB3 KO animals described in **Paper I** (shallowing of dorsal funiculus, aberrant overcrossing of CST axons as well as additional overcrossing of fibers in the lumbar spinal cord, a hopping phenotype and "hopping" *in vitro* spinal cords), further strengthening our hypothesis of EphA4 mice hopping due to a defect in their CPG.

In conclusion, we identified EphA4-positive neurons as a key component of the mammalian CPG and found that normally this component is restricted to one side by the signaling induced by the interaction of ephrinB3 and EphA4. We found that in the EphA4 and ephrinB3 null mice, CPG neurons aberrantly cross the midline. This results in an abnormal synchronized bilateral coordination (hopping) due to an increased crossover of excitatory, normally EphA4-expressing neurons, which make connections to the CPG and thus override the normal cross-coordination in the locomotor system.

4.2 PAPER II – EXCITATORY INPUT TO MOTOR NEURONS

Paper II (Butt *et al.*, 2005) is the logical extension of **Paper I**. While the first paper strongly indicated an excitatory input from EphA4-expressing glutamatergic neurons onto motor neurons, **Paper II** demonstrated that this is indeed the case. Since the experiments for this paper were done in $ephA4^{LacZ}$ (EphA4 hypomorph) mice instead of the EphA4 KO, we started out by demonstrating that $ephA4^{LacZ-/-}$ mice "hop" as well and that $ephA4^{LacZ+/-}$ behave as functional wildtype mice (**figure 1, Paper II**). We did note a larger variability in the $ephA4^{LacZ}$ animals as compared to the EphA4 KO animals, but as we were interested in the synaptic effect of EphA4 neurons on ipsilateral motor neurons, we could counter the increased bilateral drift by calculating the spike activity recorded in the iL2 relative to the iL5 ventral root, as the L2 and L5 roots were always in strict alternation.

We blindly patched neurons in the ipsilateral, ventral L2 segment and found the average membrane potential to be -49 ± 6mV (SD) and an average input resistance of 427 ± 206 MΩ (SD) at rest. There was no statistical difference between groups of cells according to genotype. 51% of the recorded cells showed spontaneous action potentials in absence of ventral root bursts. All cells recorded from were filled with Lucifer yellow. Post recording, the spinal cords were fixed and sliced, and a number of the Lucifer filled cells were recovered and immunostained to reveal their EphA4 status (**figure 2A, Paper II**). Both the EphA4-positive neurons as well as the EphA4-negative

neurons were heterogeneous with respect to firing phase and rhythmicity. Three of the recovered EphA4-positive cells were likely to be motor neurons. The other two groups of EphA4-positive neurons were either highly significant or significant with respect to rhythmicity, while no EphA4-positive neurons were non-rhythmic. The EphA4-negative population contained all kinds of rhythmicity. EphA4-positive neurons tended to fire in the ipsilateral phase, and they were rhythmic during drug-induced locomotion making them possible candidates as constituents of the hindlimb CPG (**figure 3, Paper II**). The cells recorded from were located in the ventro-lateral spinal cord (**figure 2B, Paper II**). They were thus in a position corresponding to that of ipsilateral interneurons as described by Nissen and colleagues (Nissen *et al.*, 2005) which opens the possibility that we were recording from V1 and V2 interneurons.

Spike triggered averaging of the local, ipsilateral L2 DC ventral root recording was used to test whether any of the recorded interneurons projected onto the local ipsilateral motor neurons. In cells that fired spontaneously in the absence of ventral root activity, the spontaneous firing was used for the spike triggered averaging. The remaining silent cells were current injected to elicit firing in the non-locomotor state. The types of responses we observed were either excitatory, inhibitory or no signal. Analysis revealed that a subpopulation of EphA4-positive neurons has predominantly short latency (7.6 ± 2.9ms) excitatory input onto ipsilateral motor neurons and that they fire in phase with the ipsilateral motor neurons. This means that this subpopulation is likely to be monosynaptically connected to the ipsilateral motor neurons and that the EphA4-positive neurons provide excitation to motor neurons (Butt & Kiehn, 2003). Conversely, of all the inhibitory interneurons, only one was EphA4-positive and this cell fired out of phase with the ipsilateral L2. The no signal cells may reflect one of two things: either they were not firing action potentials at all or, alternatively, they projected to a different segment than the ipsilateral L2 segment we were DC recording from.

In conclusion, our data showed that the majority of EphA4-positive neurons are rhythmically active and that a subset of these are ipsilaterally projecting, excitatory interneurons with projections onto motor neurons. This positions them as an identified excitatory interneuron group in the mammalian CPG and suggests that they may be defined by the combination of EphA4 expression and glutamate release.

4.3 PAPER III – V2 INTERNEURON CHARACTERIZATION

In **Paper III** (Lundfald *et al.*, 2007) we asked the question "Can ipsilateral EphA4-expressing excitatory interneurons be related to a group of ventral interneurons already described?" We knew from the literature mentioning unpublished results on the V2 group of interneurons, that V2 interneurons were presumed to project ipsilaterally (Saueressig *et al.*, 1999; Lee & Pfaff, 2001). We also knew from a Society for Neuroscience abstract by Maxwell and colleagues (Maxwell *et al.*, 2003) that Lhx3 cells (the precursors of motor neurons and V2 interneurons) are glutamatergic. Furthermore, the neurons recorded from in **Paper II** were located in the area of ipsilateral interneurons suggesting that they may belong to the V1 or V2 group of interneurons (see above). We therefore set out to investigate whether the EphA4 population overlapped with the V2 interneurons.

We did indeed find an overlap between EphA4 expression and Lhx3 (**figure 6, Paper III**), and therefore went on to look for an overlap between V2 interneurons and EphA4. However, the V2 group of interneurons had never been characterized in detail, so we extended our studies to a detailed characterization of the V2 interneurons in embryonic and postnatal spinal cords. We found that the V2 interneurons at P0 are scattered in a broad band in lamina VII extending from lamina X to the lateral edge of the grey matter (**figure 4, Paper III**). This corresponds with the location of the V2 interneurons in adult mice (Al-Mosawie *et al.*, 2007). However, the V2b group of interneurons (as determined by expression of the transcription factor Gata2/3) was additionally located in the area around the central canal in Lamina X.

We then went on to look at the projection pattern and found that the V2a interneuron group (as determined by expression of the transcription factor Chx10) is indeed ipsilateral both in embryos and in P0 spinal cords (**figure 1-3, Paper III**). While the V2a interneurons project locally (around 1.5 segments) both rostrally and caudally at E13.5, we were only able to verify local caudal projections at P0. The V2b interneuron group was also found to be locally and ipsilaterally projecting in embryos and probably ipsilateral in newborn mice (**figure 1-3, Paper III**). Tracing and reliably labeling V2 interneurons proved difficult. We tried a number of different tracing techniques with varying results and ran into problems of reliably identifying V2b interneurons as the specific marker Gata2/3 is downregulated half way through embryogenesis. However, while we are convinced that the V2 interneurons are ipsilaterally projecting, recent evidence points to V2a interneurons also being ascending at P0 (K. Dougherty and O. Kiehn, personal communication). What may be deduced from our data as well as from the tracing difficulties is that many V2 interneurons are short range or segmental ipsilateral interneurons coordinating activity between local pools of neurons.

The Lhx3-expressing precursor cells of V2 interneurons express glutamate and are considered excitatory but the transmitter content of V2 interneurons had not been investigated, so we performed a series of *in situ* hybridization and immunohistochemistry experiments in P0 spinal cords to look at this. We chose to look in P0 mice to be able to relate our findings to the electrophysiological data obtained from neonatal mice and to avoid the confusion of changing transmitter phenotypes as well as the changing effects of the same neurotransmitters during embryogenesis (Gao *et al.*, 2001; Nakayama *et al.*, 2002; Nabekura *et al.*, 2004). It turned out that at P0 the V2a (Chx10) interneurons are glutamatergic (80% ± 14.5%) as determined by expression of vGluT2. Only 1% were glycinergic while no Chx10 cells were GABAergic (**figure 5A-E+I, Paper III**). On the contrary, the V2b (Gata2/3) cells contained the inhibitory transmitters glycine and GABA, but in different proportions depending on the location. In lamina VII, 83% ± 8.3% were glycinergic while 23% ± 5.1% were GABAergic. In Lamina X the opposite was observed with 64% ± 4.3% of the Gata2/3 cells being GABAergic while only 4% ± 7.2% of the Gata2/3 neurons were glycinergic. Only 3.1% ± 3.2% (lamina VII) and 1% ± 0.7% (lamina X) of the Gata2/3 cells were glutamatergic (**figure 5F-H+J, Paper III**). In short: V2a interneurons are excitatory while V2b interneurons are inhibitory. This seems to be paralleled in zebrafish, for while the ipsilateral and excitatory nature of Alx interneurons (the Chx10

homolog) has already been described (Kimura *et al.*, 2006), the same group of people now reports the zebrafish V2b interneurons to be ipsilateral and inhibitory (Kimura *et al.*, 2008a) something which has also very recently been described by Batista and colleagues (Batista *et al.*, 2008). Knowing that V2a and V2b interneurons are generated in equal numbers (Peng *et al.*, 2007; Batista *et al.*, 2008; Kimura *et al.*, 2008b) these results are also in accordance with those described from adult mice where approximately 50% of the total V2 interneuron population was glutamatergic (Al-Mosawie *et al.*, 2007).

In immunostainings for both EphA4 and V2 transcription factors at ages from E13.5 up until P0, we found that 67-87% of all Chx10 cells expressed EphA4 while 49-68% of all Gata2/3 cells were positive for EphA4, demonstrating that V2 interneurons, for the most, part express EphA4 (**figure 7, Paper III**). This corresponds well with the fact that both V2 interneuron groups are ipsilateral and that their EphA4 expression will make sure that they stay ipsilaterally during development.

As described above, we found in **Paper I** that EphA4-expressing cells are involved in normal locomotion and that some normally EphA4-expressing cells will cross the midline aberrantly in EphA4 KO mice. We also found that there are glutamatergic EphA4 cells, and hypothesized that the cells that cross aberrantly are excitatory. In **Paper II** we recorded from a local (segmental) population of EphA4-expressing cells that were ipsilateral, glutamatergic and had mono- or polysynaptic input onto motor neurons. Naturally, we wondered if these ipsilateral EphA4-expressing, glutamatergic cells could belong to a group of known interneurons. While the results so far in **Paper III** did not conflict with this possibility, we had not answered the question of whether the abnormally crossing EphA4 cells in EphA4 KO animals (**Paper I**) could overlap with the V2 interneurons that are ipsilateral and excitatory. Tracing experiments combined with IHC on newborn *ephA4*$^{LacZ-/-}$ mice were performed to answer this. We traced both from the ventral side and the dorsal side along the entire lumbar spinal cord but never saw V2a interneurons that crossed the midline in EphA4 dysfunctional mice (**figure 9, Paper III**). This means that the abnormally crossing processes from EphA4 cells observed in **Paper I** cannot originate from the V2a pool of interneurons but must belong to another ipsilateral excitatory cell group. We have excluded the possibility that this cell group is the Hb9-expressing (also ipsilateral and excitatory) cells as they do not express EphA4 (**figure 8, Paper III**).

We were really surprised that we were not able to link *ephA4*$^{LacZ-/-}$ aberrant crossover to the V2a interneuron group as all accumulated evidence indicated an overlap between the two populations. We tried in many different ways and asked ourselves if we had missed something in our tracing studies. It is fully possible that the axonal diameter or damage upon making the slit for dye application precludes uptake of the tracers used. However, as the same tracing methods were used in both **Paper I** and **III**, we feel confident that we would have picked up a possible overlap between the populations. There are two possible explanations for why we could not show that processes from EphA4-expressing V2 interneurons cross the midline in *ephA4*$^{LacZ-/-}$ animals. It is possible either that they do cross, and we could not show it due to problems with dye uptake as discussed above, or they do not cross, leading to

speculations that EphA4-ephrinB3 signaling is not the only clue by which axons are guided to their correct destination.

In conclusion, our data shows that V2 interneurons are located at a position which suggests them to be members of the mammalian CPG. We demonstrated that V2a interneurons are glutamatergic while V2b interneurons contain the inhibitory transmitters glycine and GABA. Furthermore, we establish the V2 population as segmental ipsilaterally projecting interneurons, and that a vast majority of all V2 interneurons express EphA4. Lastly, we show that V2 interneurons and motor neurons do not account for all EphA4-expressing neurons in the ventral spinal cord and that the aberrant crossing of processes from EphA4-positive neurons in *ephA4*$^{LacZ-/-}$ mice does not originate from the V2a population, leading us to hypothesize that there is an as yet unknown excitatory, ipsilateral and EphA4-expressing component of the mammalian CPG.

4.4 PAPER IV – V2A INTERNEURONS SIGNIFICANT FOR LOCOMOTION

In zebrafish, the Chx10 homolog, Alx, is expressed in V2 interneurons that are glutamatergic and project ipsilaterally directly onto motor neurons (Kimura *et al.*, 2006). Since we recorded from similar glutamatergic and ipsilaterally projecting onto motor neurons (but EphA4-expressing) cells in **Paper II**, there is the possibility of an overlap between the two groups of cells (and a possibility that the V2 group of interneurons have similar function in fish and mammals). However, we never tested this directly as our hypothesis was developed long after the finishing of **Paper II** and direct proof would involve repeating the experiments looking for Chx10 instead of EphA4 (with a significant risk of not being able to detect Chx10 as transcription factors may diffuse out of the cell/become diluted after >30 minutes of recording (Hinckley *et al.*, 2005b)). We have, however, investigated the role of V2a interneurons in locomotion using a mouse strain in which Chx10 cells are ablated by killing them specifically with DTA (**Paper IV,** Crone *et al.*, 2008a). This transgenic Chx10-DTA mouse was produced in a collaboration project with researchers in Kamal Sharma's laboratory in Chicago (**figure 1, Paper IV**).

In these mice the V2a cells are indeed selectively ablated in this mouse. The only interneuron class to change in numbers was the Chx10 cells. Furthermore the number of vGluT2 positive interneurons was reduced by about 30% in the Chx10 DTA mouse, indicating that Chx10 cells constitute about 30% of the total number of vGluT2 positive interneurons in the neonatal ventral spinal cord (**figure 1, Paper IV**).

To investigate the role of the Chx10 cells, we performed ventral root recordings from L2 and L5 in newborn (P0) Chx10-DTA animals. We observed that the V2a interneurons are not needed for intrinsic rhythmogenic activity in isolated spinal cord preparations (**figure 2, Paper IV**). However, we found that the variability of the burst amplitude and the cycle period was significantly increased in the Chx10-DTA animals as compared to wildtype mice (concentration matched experiments). These results suggest that V2a interneuron stabilize the locomotor rhythm and pattern by inputs to the rhythm-generating neurons and the motor neurons, respectively (**figure 3, Paper IV**). That V2a interneurons are active during locomotion is confirmed by those of Al-

Mosawie and colleagues (Al-Mosawie *et al.*, 2007), who use c-fos labeling to show that V2 interneurons are indeed active during locomotion.

When we started to analyze the coordination of the flexor-extensor output in the Chx10-DTA mice, we observed, much to our surprise, that the left-right coordination was disturbed (**figure 4, Paper IV**) to the extent that it looked as if the two sides were uncoupled, while the flexor-extensor (iL2 to iL5) coordination was normal. We, of course, wondered how this is possible when the Chx10 neurons are clearly ipsilateral (as shown in **Paper III**) and we had a closer look at the anatomy of the commissural interneurons to see if they were intact. Midline staining revealed that the number and location of CINs is identical in wildtype and Chx10-DTA animals at P0 (**figure 5, Paper IV**), and this was supported by two sets of electrophysiological data. In the first experiment, it was shown that the reflex responses were normal in the Chx10-DTA mice. This was done by stimulating the dorsal L3 root while recording intracellularly from contralateral motor neurons. Wildtype and Chx10-DTA mice had indistinguishable excitatory and inhibitory connections, indicating that the integrity of the CINs was intact (Edgley *et al.*, 2003) (**figure 5, Paper IV**). This was confirmed in tests where the dorsal L2 root was activated and the motor neuron response recorded from the ipsilateral and contralateral L2 roots as well as the contralateral L5 ventral root (**supplementary figure S1, Paper IV**). In these experiments, the CINs were also found to be normal. In the other set of data we added blockers of the inhibitory transmitters to spinal cords from wildtype and Chx10-DTA mice and found that the excitatory CINs could indeed drive synchronous left-right motor activity (as described by Bracci *et al.*, 1996; Cowley & Schmidt, 1997; Hinckley *et al.*, 2005a) in both types of animals (**supplementary figure S1, Paper IV**). On the basis of the anatomical and physiological data, we concluded that the CIN system in Chx10-DTA mice is normal.

Although the CINs in Chx10-DTA animals are normal, the coordination between left and right is disturbed. We took this as a signal that the Chx10 neurons projected ipsilaterally to CINs which then drive the coordination. If the input from the Chx10 cells to the CINs disappears, the coordination between left and right will be disturbed. We therefore looked for anatomical evidence that Chx10 cells would project to ipsilateral CINs. We traced Chx10::CFP, Lhx3::YFP and Sox14::GFP (Sox14 expression largely overlaps with Chx10 expression, see **supplementary figure S2, Paper IV**, also mentioned in Poh *et al.*, 2002) mice in the midline with a fluorescent dye and found in all mice that FP-labeled processes contacted retrogradely labeled CINs in the ventral spinal cord. Furthermore, many of the FP-labeled processes contacting CINs also expressed vGluT2 (**figure 6 and supplementary figure S3, Paper IV**). Together these data provide anatomical evidence to support our idea that V2 interneurons can directly excite CINs in the ventral lumbar spinal cord. Additional evidence for this was provided when we found that V2a interneuron processes (identified on the basis of the Chx10::CFP labeling) containing vGluT2 could be found in close apposition with the soma or processes of commissural V0 interneurons (as determined by expression of Evx1 and rhodamine dextran to reveal CINs). We also observed that V2a interneurons contact other CINs than the Evx1 positive ones, indicating that they may provide excitatory drive onto other CINs as well (**figure 6, Paper IV**).

It is possible to neurally evoke normal, alternating locomotion by activating either sensory afferents or by stimulating reticulospinal neurons in the brainstem (Lev-Tov & Delvolve, 2000; Gordon & Whelan, 2006b; Zaporozhets *et al.*, 2006). We wanted to assess the contribution of the V2 interneurons to neurally evoked locomotion and first stimulated reticulospinal neurons in the brainstem-spinal cord *in vitro* preparation (**figure 7, Paper IV**). Wildtype animals showed normal, alternating locomotion as previously described, but in the Chx10-DTA animals stimulation elicited transient and uncoordinated motor activity. However, when inducing locomotion in the same preparations by use of 5-HT and NMDA, the locomotor-like activity was rhythmic. This is consistent with V2a interneurons acting as spinal mediators of descending brainstem input critical for locomotor induction *in vivo*. However, Chx10 is also present in reticulospinal neurons of the brainstem (Cepeda-Nieto *et al.*, 2005) and the result could therefore also reflect the loss of descending inputs to the spinal locomotor system. Similarly, locomotion failed to be induced in Chx10-DTA spinal cords by stimulation of sacral sensory afferents, and when stimulating thoracic dorsal roots, only a tonic, non-rhythmic activity was observed. This is in contrast with the rhythmic, locomotor-like responses elicited by the same kind of stimulation in wildtype spinal cords (**figure 7, Paper IV**). These results are in accordance with those of Al-Mosawie and colleagues (Al-Mosawie *et al.*, 2007) which show that V2 interneurons receive primary afferent input. Our results therefore support our idea that V2a interneurons are spinal mediators of neurally evoked locomotion.

In conclusion, we have produced a transgenic mouse in which Chx10 neurons are specifically ablated. In the absence of Chx10 interneurons, the spinal cord fails to exhibit consistent left–right alternation. Locomotor burst activity shows increased variability, but flexor-extensor coordination is unaffected. Evidence for a direct excitatory input of V2a interneurons onto commissural interneurons was provided by anatomical tracing studies. Among the CINs contacted were the molecularly defined V0 (Evx1 positive) interneurons which drive left-right alternation. This points to an essential role for V2a interneurons in the control of left-right alternation and implies that bilateral coordination consists of at least two components; commissural interneurons and a class of ipsilateral interneurons that activate commissural pathways. These findings were summarized in an extensive wiring diagram that also encompasses the previous wiring diagram for the segmental CINs (**figure 8, Paper IV**).

4.5 OVERALL DISCUSSION

On the large scale, Chx10 expressing interneurons are located throughout the spinal cord column and it is logical that they may have different roles depending on location. They are clearly involved in the locomotion patterning at the level of the lumbar limb CPG (**Paper IV)**, while they may act as locomotion initiators in the brainstem (Cepeda-Nieto *et al.*, 2005) and as respiratory neurons in the respiratory CPG in the brainstem (S.A. Crone, S. Droho, J.C. Viemari, A. Mrejeru, J.M. Ramirez and K. Sharma, personal communication).

4.5.1 Flexor-extensor speculations

With the addition of complexity by adding limbs to locomotion, the need for flexor-extensor control comes on top of the necessary coordination between left and right. It is possible that the range of the ipsilateral V2 projections is too short to coordinate between flexor and extensor muscles, a fact that is supported by the length of the projections described in **Paper III** and the locomotor results in **Paper IV**. Instead coordinators for this may be looked for among the long range ascending and descending CINs described by Butt and Kiehn (Butt & Kiehn, 2003) and among other excitatory ipsilateral interneurons or as yet unidentified inhibitory neurons. That the V2a interneurons are not the EphA4 KO aberrantly projecting neurons is maybe not so strange in retrospect since it has emerged from the papers in this thesis that the EphA4 interneurons which are excitatory and ipsilaterally projecting to motor neurons are located ventrolaterally in the spinal cord (**Paper II**), while V2a interneurons are located deeper in the ventral spinal cord (**Paper IV**). We cannot rule out that EphA4 ipsilateral interneurons have a flexor-extensor role, but we are unable to conclude this as the cells we recorded from in **Paper II** were only related to the firing of the motor neurons in the local (flexor) segment.

While we never found a V2a interneuron to motor neuron projection, others have reported V2a excitatory and ipsilateral connections to motor neurons in zebrafish and mouse (Kimura *et al.*, 2006; Al-Mosawie *et al.*, 2007). Does this mean that V2a interneurons may have several roles in locomotion? It would certainly be nice to know the physiological connectivity pattern of Chx10 cells, something that could be investigated with the development of Chx10-FP mice. But until we know more, we will just have to speculate based on the findings so far.

4.5.2 Flexible components and speed

Existence of networks that can be reconfigured or recruited during locomotion is likely regarding the different types of gait produced by limbed animals at different speeds. The need for a flexible network is therefore evident. Such flexibility would also involve a flexible component across the midline in order to change the gait pattern from alternating the hindlimbs to (almost) synchrony such as it happens in the horse in the switch from walk to gallop. Possible candidate neurons of activity modulation in relation to speed are the switch cells described by Butt and Kiehn (Butt & Kiehn, 2003). These cells switched from polysynaptic inhibition at rest to monosynaptic excitation during locomotion. Could lumbar Chx10 interneurons be another flexibility component which kicks in at higher speeds to secure left-right alternation?

In the zebrafish, Alx (the homolog to Chx10) interneurons are excitatory and project ipsilaterally to motor neurons (Kimura *et al.*, 2006). A number of these neurons were shown to be involved in strong movements (associated with escape and fast swimming), and these neurons were located in a more dorsal position of the spinal cord and fired before or at the same time as the ipsilateral motor neurons. Another Alx interneuron population, active in weaker movements, was located more ventral and fired throughout the swimming episode in phase with the ventral root (Kimura *et al.*, 2006; McLean *et al.*, 2007). This indicates different roles for V2 interneurons depending on the type of movement performed. The Alx neurons correspond to the

ipsilateral and excitatory circumferential descending (CiD) interneurons also described in zebrafish (Higashijima *et al.*, 2004; Batista *et al.*, 2008). They are divided into subgroups with smaller CiDs located more ventral to the large CiDs (Hale *et al.*, 2001). The large CiDs are involved in escape swimming behavior (Ritter *et al.*, 2001) and are recruited during fast swimming while the smaller CiDs are active during intermediate swimming frequencies (McLean *et al.*, 2007). Findings of this caliber can begin to bring anatomically, genetically and electrophysiologically defined groups of interneurons together, and allow us to draw direct parallels between the fish swimming CPG and the mammalian walking CPG, possibly revealing evolutionarily conserved features of locomotion.

Recently, preliminary evidence from Chx10-DTA mice have shown that, also in mammals, the nature of the movement may determine whether or how V2a interneurons are brought into play (Crone *et al.*, 2008b; Zhong *et al.*, 2008). These studies showed that the left-right coordination in the Chx10-DTA *in vitro* preparation varied with the cycle frequency. At low cycle frequencies the left-right phase was alternating ("walking") while it became disrupted (in a manner similar to that described in **Paper IV**) with higher cycle frequency and eventually became synchronous ("hopping") at high cycle frequencies (this was true for various 5-HT/NMDA concentrations) (Zhong *et al.*, 2008). This was shown to have physiological relevance in the intact animals where it was shown that Chx10-DTA mature mice on a treadmill would walk normally at low speeds while becoming increasingly less alternating with higher speeds (Crone *et al.*, 2008b). At high locomotion speeds, they shifted to a synchronous "hopping" gait. Together these findings imply the existence of additional excitatory interneurons which can drive the left-right alternating system at low speeds. They also suggest that different mammalian locomotor network neurons are called into play depending on speed, similar to what has been described for the zebrafish CPG (McLean *et al.*, 2007).

4.5.3 Excitatory rhythm generation

From our results in **Paper IV** and as demonstrated by Al-Mosawie and colleagues (Al-Mosawie *et al.*, 2007), it seems clear that the Chx10 interneurons are not responsible for the rhythm generation *per se*. Instead, they seem to stabilize rhythm while at the same time ensure left-right alternation. As mentioned in the introduction, excitatory interneurons are necessary for rhythm generation (Grillner, 2003; Kiehn *et al.*, 2008) and there are apparently many more excitatory ipsilateral interneurons than the Chx10 interneurons. However, the responsible neurons for rhythm generation remain to be indentified in the mammalian spinal cord. Although excitatory interneurons are necessary for rhythm generation, a recent study by Wallen-Mackenzie and colleagues on the locomotor phenotype of vGluT2 KO mice suggested that vGluT2-mediated glutamatergic transmission in the spinal cord is dispensable for normal locomotor-like activity (Wallen-Mackenzie *et al.*, 2006) thus indicating that excitatory EphA4 and Chx10 neurons are not necessary for normal alternating locomotion. However, there are several other vesicular glutamate transport proteins, and thus the loss of vGluT2 alone may not eliminate the vesicular accumulation of glutamate in ipsilateral excitatory interneurons. Furthermore, the early loss of vGluT2

may trigger compensatory developmental mechanisms in order to reorganize spinal network activity as described by Myers and colleagues (Myers *et al.*, 2005). Thus, preliminary experiments from the Kiehn laboratory, using a different vGluT2 KO mouse (from Richard D. Palmiter's laboratory), show that the rhythm evoked in these vGluT2 KO mice can be blocked by glutamatergic blockers, although at higher concentrations than needed to block the rhythm in the normal spinal cord (A. Talpalar and O. Kiehn, personal communication).

So much for the drifting phenotypes of Chx10-DTA animals, but what is the mechanism behind the hopping EphA4 KO animals? As aberrantly crossing axons seem to stay within the segment where they aberrantly cross (Coonan *et al.*, 2001; Kadison *et al.*, 2006) there are two possibilities. One possibility is that the observed effect on locomotion (hopping) is a direct effect due to axons aberrantly connecting to neurons within the same segment but on the contralateral side. The other possibility is that hopping is the indirect result of lacking drive onto ipsilateral networks responsible for left-right alternation. It is, of course, also possible that the these two possibilities has a combined effect. Thus, the hopping phenotype may be explained by EphA4 cells no longer projecting to ipsilateral CINs (analogous to Chx10 cells in **Paper IV**) and motor neurons (as described in **Paper II**), but instead connecting to as yet unidentified neurons on the contralateral side to bind the two halves in synchrony.

While there are many similarities between mammalian and aquatic vertebrate CPG systems, there also seem to be some differences. In the latter, a relatively homogenous group of ipsilaterally projecting, descending interneurons generates a fast locomotor rhythm as well as provides excitatory input to motor neurons and CINs (McLean *et al.*, 2007). In mammals, these tasks may be divided between different classes of excitatory interneurons. Recent evidence from zebrafish suggests that multipolar commissural descending (MCoD) interneurons, which are excitatory (Higashijima *et al.*, 2004), commissural descending (Hale *et al.*, 2001) interneurons active in swimming (and which express the transcription factor Exv2 (Satou *et al.*, 2008)) may be the sole source of rhythm generation at low speeds of swimming (McLean *et al.*, 2007). In contrast, mammalian CINs are not generating the rhythm, as hemicord preparations from both wildtype and Chx10-DTA animals are rhythmically active (**Paper IV**, see also Kudo & Yamada, 1987; Kremer & Lev-Tov, 1997; Whelan *et al.*, 2000). Instead, mammalian CINs may be involved in setting the speed of locomotion as the frequency goes down when only one side of the cord is induced to locomote (Kjaerulff & Kiehn, 1997). In the rostro-caudal axis, expression of Hox10 proteins and other transcription factors in the lumbar spinal cord divides the V2a (and probably all other) interneurons into subpopulations. Further dissection of spinal cord interneuron populations together with continued research in several species will help us to understand the molecular mechanisms underlying physiological locomotor behavior.

5 CONCLUDING REMARKS

The work presented in this thesis is a small contribution towards the tremendous task of understanding mammalian locomotor networks. It is a dream to be able to identify a neuron on the basis of a handful of protein markers. A certain combination of markers can thus tell us about the neuron's location in the spinal cord (ventral or dorsal, rostral or caudal, medial or lateral), transmitter phenotype (inhibitory or excitatory), projection pattern (commissural or ipsilateral, ascending or descending or segmental), connectivity (to which type of cell), firing frequency and firing phase in the locomotion cycle. Thus we will have an idea of the neuron's function in the locomotor network on the basis of a handful of expressed proteins. To fulfill this dream it will be necessary to further divide the different neuronal classes on the basis of proteins expressed later in development. It will require a lot of screening and possibly laser microdissection combined with microarray analysis. Furthermore, a lot of electrophysiology experiments on transgenic mice are needed in order to reveal the actual physiological roles of the classes defined. We still have a long way to go, but we also already know a lot about locomotor networks in a wide variety of species and, collectively, science has a wide range of tools to use. This will help us to increase our understanding of how locomotor networks are constructed in vertebrates.

It is important to keep in mind that live animal locomotion is not dependent on the CPG activity alone but also includes complex integration of the locomotor pattern with important sensory feedback of muscle, pressure and postural information as well as input from brain structures such as the cerebellum and the basal ganglia. An even bigger challenge is to integrate how this information affects the locomotion pattern but it will also give us a fantastic insight into how the organism is constructed. It is the hope of every spinal cord scientist to add a piece to the big puzzle so that we, some day, will be able to help people with spinal cord injuries to regain some function which will help to make their everyday lives a little more comfortable. With the accumulated knowledge so far, there is hope that we may achieve this goal in the not-so-distant future.

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7 REFERENCES

Akay, T., Acharya, H.J., Fouad, K. & Pearson, K.G. (2006) Behavioral and electromyographic characterization of mice lacking EphA4 receptors. *J Neurophysiol*, **96**, 642-651.

Al-Mosawie, A., Wilson, J.M. & Brownstone, R.M. (2007) Heterogeneity of V2-derived interneurons in the adult mouse spinal cord. *Eur J Neurosci*, **26**, 3003-3015.

Alvarez, F.J., Jonas, P.C., Sapir, T., Hartley, R., Berrocal, M.C., Geiman, E.J., Todd, A.J. & Goulding, M. (2005) Postnatal phenotype and localization of spinal cord V1 derived interneurons. *J Comp Neurol*, **493**, 177-192.

Arenkiel, B.R., Peca, J., Davison, I.G., Feliciano, C., Deisseroth, K., Augustine, G.J., Ehlers, M.D. & Feng, G. (2007) In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. *Neuron*, **54**, 205-218.

Batista, M.F., Jacobstein, J. & Lewis, K.E. (2008) Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Dev Biol*.

Bear, M.F., Connors, B.W. & Paradiso, M.A. (1996) *Neuroscience : exploring the brain*. Williams & Wilkins, Baltimore.

Beg, A.A., Sommer, J.E., Martin, J.H. & Scheiffele, P. (2007) alpha2-Chimaerin is an essential EphA4 effector in the assembly of neuronal locomotor circuits. *Neuron*, **55**, 768-778.

Bracci, E., Ballerini, L. & Nistri, A. (1996) Localization of rhythmogenic networks responsible for spontaneous bursts induced by strychnine and bicuculline in the rat isolated spinal cord. *J Neurosci*, **16**, 7063-7076.

Briscoe, J., Pierani, A., Jessell, T.M. & Ericson, J. (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell*, **101**, 435-445.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T.M., Rubenstein, J.L. & Ericson, J. (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature*, **398**, 622-627.

Brown, T.G. (1911) The intrinsic factors in the act of progression in the mammal. *Soc. Lond. B Biol. Sci.*, **84**, 308-319.

Brownstone, R.M. & Wilson, J.M. (2008) Strategies for delineating spinal locomotor rhythm-generating networks and the possible role of Hb9 interneurones in rhythmogenesis. *Brain Res Rev*, **57**, 64-76.

Buchanan, J.T. (2001) Contributions of identifiable neurons and neuron classes to lamprey vertebrate neurobiology. *Prog Neurobiol*, **63**, 441-466.

Burrill, J.D., Moran, L., Goulding, M.D. & Saueressig, H. (1997) PAX2 is expressed in multiple spinal cord interneurons, including a population of EN1+ interneurons that require PAX6 for their development. *Development*, **124**, 4493-4503.

Butt, S.J., Harris-Warrick, R.M. & Kiehn, O. (2002a) Firing properties of identified interneuron populations in the mammalian hindlimb central pattern generator. *J Neurosci*, **22**, 9961-9971.

Butt, S.J. & Kiehn, O. (2003) Functional identification of interneurons responsible for left-right coordination of hindlimbs in mammals. *Neuron*, **38**, 953-963.

Butt, S.J., Lebret, J.M. & Kiehn, O. (2002b) Organization of left-right coordination in the mammalian locomotor network. *Brain Res Brain Res Rev*, **40**, 107-117.

Butt, S.J., Lundfald, L. & Kiehn, O. (2005) EphA4 defines a class of excitatory locomotor-related interneurons. *Proc Natl Acad Sci U S A*, **102**, 14098-14103.

Callaway, E.M. (2005) A molecular and genetic arsenal for systems neuroscience. *Trends Neurosci*, **28**, 196-201.

Cepeda-Nieto, A.C., Pfaff, S.L. & Varela-Echavarria, A. (2005) Homeodomain transcription factors in the development of subsets of hindbrain reticulospinal neurons. *Mol Cell Neurosci*, **28**, 30-41.

Coonan, J.R., Bartlett, P.F. & Galea, M.P. (2003) Role of EphA4 in defining the position of a motoneuron pool within the spinal cord. *J Comp Neurol*, **458**, 98-111.
Coonan, J.R., Greferath, U., Messenger, J., Hartley, L., Murphy, M., Boyd, A.W., Dottori, M., Galea, M.P. & Bartlett, P.F. (2001) Development and reorganization of corticospinal projections in EphA4 deficient mice. *J Comp Neurol*, **436**, 248-262.
Copp, A., Cogram, P., Fleming, A., Gerrelli, D., Henderson, D., Hynes, A., Kolatsi-Joannou, M., Murdoch, J. & Ybot-Gonzalez, P. (2000) Neurulation and neural tube closure defects. *Methods Mol Biol*, **136**, 135-160.
Cowley, K.C. & Schmidt, B.J. (1994a) A comparison of motor patterns induced by N-methyl-D-aspartate, acetylcholine and serotonin in the in vitro neonatal rat spinal cord. *Neurosci Lett*, **171**, 147-150.
Cowley, K.C. & Schmidt, B.J. (1994b) Some limitations of ventral root recordings for monitoring locomotion in the in vitro neonatal rat spinal cord preparation. *Neurosci Lett*, **171**, 142-146.
Cowley, K.C. & Schmidt, B.J. (1997) Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J Neurophysiol*, **77**, 247-259.
Crone, S.A., Quinlan, K.A., Zagoraiou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo, T., Setlak, J., Jessell, T.M., Kiehn, O. & Sharma, K. (2008a) Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. *Neuron*, In Press.
Crone, S.A., Zhong, G., Harris-Warrick, R. & Sharma, K. (2008b) The gait of mice lacking V2a interneurons is dependent upon speed of locomotion. *Soc. Neurosci. Abstr.*
Cui, W.W., Low, S.E., Hirata, H., Saint-Amant, L., Geisler, R., Hume, R.I. & Kuwada, J.Y. (2005) The zebrafish shocked gene encodes a glycine transporter and is essential for the function of early neural circuits in the CNS. *J Neurosci*, **25**, 6610-6620.
Delcomyn, F. (1980) Neural basis of rhythmic behavior in animals. *Science*, **210**, 492-498.
Dimitrijevic, M.R., Gerasimenko, Y. & Pinter, M.M. (1998) Evidence for a spinal central pattern generator in humans. *Ann N Y Acad Sci*, **860**, 360-376.
Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick, T., Bartlett, P.F., Murphy, M., Kontgen, F. & Boyd, A.W. (1998) EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. *Proc Natl Acad Sci U S A*, **95**, 13248-13253.
Douglas, J.R., Noga, B.R., Dai, X. & Jordan, L.M. (1993) The effects of intrathecal administration of excitatory amino acid agonists and antagonists on the initiation of locomotion in the adult cat. *J Neurosci*, **13**, 990-1000.
Eberhart, J., Swartz, M., Koblar, S.A., Pasquale, E.B., Tanaka, H. & Krull, C.E. (2000) Expression of EphA4, ephrin-A2 and ephrin-A5 during axon outgrowth to the hindlimb indicates potential roles in pathfinding. *Dev Neurosci*, **22**, 237-250.
Eberhart, J., Swartz, M.E., Koblar, S.A., Pasquale, E.B. & Krull, C.E. (2002) EphA4 constitutes a population-specific guidance cue for motor neurons. *Dev Biol*, **247**, 89-101.
Edgerton, V.R., Courtine, G., Gerasimenko, Y.P., Lavrov, I., Ichiyama, R.M., Fong, A.J., Cai, L.L., Otoshi, C.K., Tillakaratne, N.J., Burdick, J.W. & Roy, R.R. (2008) Training locomotor networks. *Brain Res Rev*, **57**, 241-254.
Edgley, S.A., Jankowska, E., Krutki, P. & Hammar, I. (2003) Both dorsal horn and lamina VIII interneurones contribute to crossed reflexes from feline group II muscle afferents. *J Physiol*, **552**, 961-974.
Eide, A.L., Glover, J., Kjaerulff, O. & Kiehn, O. (1999) Characterization of commissural interneurons in the lumbar region of the neonatal rat spinal cord. *J Comp Neurol*, **403**, 332-345.
Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. & Jessell, T.M. (1997a) Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. *Cold Spring Harb Symp Quant Biol*, **62**, 451-466.
Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M. & Briscoe, J. (1997b) Pax6 controls progenitor

cell identity and neuronal fate in response to graded Shh signaling. *Cell*, **90**, 169-180.
Fawcett, J.P., Georgiou, J., Ruston, J., Bladt, F., Sherman, A., Warner, N., Saab, B.J., Scott, R., Roder, J.C. & Pawson, T. (2007) Nck adaptor proteins control the organization of neuronal circuits important for walking. *Proc Natl Acad Sci U S A*, **104**, 20973-20978.
Fetcho, J.R., Higashijima, S. & McLean, D.L. (2008) Zebrafish and motor control over the last decade. *Brain Res Rev*, **57**, 86-93.
Gao, B.X., Stricker, C. & Ziskind-Conhaim, L. (2001) Transition from GABAergic to glycinergic synaptic transmission in newly formed spinal networks. *J Neurophysiol*, **86**, 492-502.
Geiman, E.J., Zhang, Y., Narayan, S. & Goulding, M. (2006) Anatomical characterization of V3 interneuron subpopulations in the neonatal mouse spinal cord. *Soc. Neurosci. Abstr.*, 252.210/V255.
Gianino, S., Stein, S.A., Li, H., Lu, X., Biesiada, E., Ulas, J. & Xu, X.M. (1999) Postnatal growth of corticospinal axons in the spinal cord of developing mice. *Brain Res Dev Brain Res*, **112**, 189-204.
Glover, J.C., Petursdottir, G. & Jansen, J.K. (1986) Fluorescent dextran-amines used as axonal tracers in the nervous system of the chicken embryo. *J Neurosci Methods*, **18**, 243-254.
Gordon, I.T. & Whelan, P.J. (2006a) Deciphering the organization and modulation of spinal locomotor central pattern generators. *J Exp Biol*, **209**, 2007-2014.
Gordon, I.T. & Whelan, P.J. (2006b) Monoaminergic control of cauda-equina-evoked locomotion in the neonatal mouse spinal cord. *J Neurophysiol*, **96**, 3122-3129.
Gosgnach, S., Lanuza, G.M., Butt, S.J., Saueressig, H., Zhang, Y., Velasquez, T., Riethmacher, D., Callaway, E.M., Kiehn, O. & Goulding, M. (2006) V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature*, **440**, 215-219.
Goulding, M. & Pfaff, S.L. (2005) Development of circuits that generate simple rhythmic behaviors in vertebrates. *Curr Opin Neurobiol*, **15**, 14-20.
Greferath, U., Canty, A.J., Messenger, J. & Murphy, M. (2002) Developmental expression of EphA4-tyrosine kinase receptor in the mouse brain and spinal cord. *Mech Dev*, **119 Suppl 1**, S231-238.
Grillner, S. (1981) Control of locomotion in bipeds, tetrapods and fish. In Brooks, V.B. (ed) *Handbook of Physiology: Section 1, The Nervous System II. Motor Control*. American Physiological Society, Bethesda, Md., pp. 1179 -1236.
Grillner, S. (2003) The motor infrastructure: from ion channels to neuronal networks. *Nat Rev Neurosci*, **4**, 573-586.
Grillner, S., Wallen, P., Saitoh, K., Kozlov, A. & Robertson, B. (2008) Neural bases of goal-directed locomotion in vertebrates--an overview. *Brain Res Rev*, **57**, 2-12.
Guertin, P.A. (2004) Role of NMDA receptor activation in serotonin agonist-induced air-stepping in paraplegic mice. *Spinal Cord*, **42**, 185-190.
Guertin, P.A. (2005) Paraplegic mice are leading to new advances in spinal cord injury research. *Spinal Cord*.
Hale, M.E., Ritter, D.A. & Fetcho, J.R. (2001) A confocal study of spinal interneurons in living larval zebrafish. *J Comp Neurol*, **437**, 1-16.
Hanson, M.G. & Landmesser, L.T. (2003) Characterization of the circuits that generate spontaneous episodes of activity in the early embryonic mouse spinal cord. *J Neurosci*, **23**, 587-600.
Hanson, M.G. & Landmesser, L.T. (2004) Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron*, **43**, 687-701.
Hargrave, M., Karunaratne, A., Cox, L., Wood, S., Koopman, P. & Yamada, T. (2000) The HMG box transcription factor gene Sox14 marks a novel subset of ventral interneurons and is regulated by sonic hedgehog. *Dev Biol*, **219**, 142-153.
Higashijima, S., Schaefer, M. & Fetcho, J.R. (2004) Neurotransmitter properties of spinal interneurons in embryonic and larval zebrafish. *J Comp Neurol*, **480**, 19-37.

Hinckley, C., Seebach, B. & Ziskind-Conhaim, L. (2005a) Distinct roles of glycinergic and GABAergic inhibition in coordinating locomotor-like rhythms in the neonatal mouse spinal cord. *Neuroscience*, **131**, 745-758.
Hinckley, C.A., Hartley, R., Wu, L., Todd, A. & Ziskind-Conhaim, L. (2005b) Locomotor-like rhythms in a genetically distinct cluster of interneurons in the mammalian spinal cord. *J Neurophysiol*, **93**, 1439-1449.
Hinckley, C.A. & Ziskind-Conhaim, L. (2006) Electrical coupling between locomotor-related excitatory interneurons in the mammalian spinal cord. *J Neurosci*, **26**, 8477-8483.
Iizuka, M., Kiehn, O. & Kudo, N. (1997) Development in neonatal rats of the sensory resetting of the locomotor rhythm induced by NMDA and 5-HT. *Exp Brain Res*, **114**, 193-204.
Issberner, J.P. & Sillar, K.T. (2007) The contribution of the NMDA receptor glycine site to rhythm generation during fictive swimming in Xenopus laevis tadpoles. *Eur J Neurosci*, **26**, 2556-2564.
Ivanova, A., Signore, M., Caro, N., Greene, N.D., Copp, A.J. & Martinez-Barbera, J.P. (2005) In vivo genetic ablation by Cre-mediated expression of diphtheria toxin fragment A. *Genesis*, **43**, 129-135.
Iwasato, T., Katoh, H., Nishimaru, H., Ishikawa, Y., Inoue, H., Saito, Y.M., Ando, R., Iwama, M., Takahashi, R., Negishi, M. & Itohara, S. (2007) Rac-GAP alpha-chimerin regulates motor-circuit formation as a key mediator of EphrinB3/EphA4 forward signaling. *Cell*, **130**, 742-753.
Jankowska, E. (2008) Spinal interneuronal networks in the cat: elementary components. *Brain Res Rev*, **57**, 46-55.
Jessell, T.M. (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet*, **1**, 20-29.
Kadison, S.R., Makinen, T., Klein, R., Henkemeyer, M. & Kaprielian, Z. (2006) EphB receptors and ephrin-B3 regulate axon guidance at the ventral midline of the embryonic mouse spinal cord. *J Neurosci*, **26**, 8909-8914.
Kandel, E.R., Schwartz, J.H. & Jessell, T.M. (2000) *Principles of neural science*. McGraw-Hill, Health Professions Division, New York.
Karunaratne, A., Hargrave, M., Poh, A. & Yamada, T. (2002) GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev Biol*, **249**, 30-43.
Kiehn, O. (2006) Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci*, **29**, 279-306.
Kiehn, O. & Kjaerulff, O. (1996) Spatiotemporal characteristics of 5-HT and dopamine-induced rhythmic hindlimb activity in the in vitro neonatal rat. *J Neurophysiol*, **75**, 1472-1482.
Kiehn, O., Quinlan, K.A., Restrepo, C.E., Lundfald, L., Borgius, L., Talpalar, A.E. & Endo, T. (2008) Excitatory components of the mammalian locomotor CPG. *Brain Res Rev*, **57**, 56-63.
Kiehn, O., Sillar, K.T., Kjaerulff, O. & McDearmid, J.R. (1999) Effects of noradrenaline on locomotor rhythm-generating networks in the isolated neonatal rat spinal cord. *J Neurophysiol*, **82**, 741-746.
Kimura, Y., Okamura, Y. & Higashijima, S. (2006) alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. *J Neurosci*, **26**, 5684-5697.
Kimura, Y., Satou, C. & Higashijima, S.-I. (2008a) V2a and V2b neurons are generated by the final divisions of pair-producing progenitors. *Soc. Neurosci. Abstr.*
Kimura, Y., Satou, C. & Higashijima, S.I. (2008b) V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. *Development*.
Kinney, G.G., Sur, C., Burno, M., Mallorga, P.J., Williams, J.B., Figueroa, D.J., Wittmann, M., Lemaire, W. & Conn, P.J. (2003) The glycine transporter type 1 inhibitor N-[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine potentiates NMDA receptor-mediated responses in vivo and produces an antipsychotic profile in rodent behavior. *J Neurosci*, **23**, 7586-7591.

Kjaerulff, O. & Kiehn, O. (1996) Distribution of networks generating and coordinating locomotor activity in the neonatal rat spinal cord in vitro: a lesion study. *J Neurosci*, **16**, 5777-5794.
Kjaerulff, O. & Kiehn, O. (1997) Crossed rhythmic synaptic input to motoneurons during selective activation of the contralateral spinal locomotor network. *J Neurosci*, **17**, 9433-9447.
Kremer, E. & Lev-Tov, A. (1997) Localization of the spinal network associated with generation of hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. *J Neurophysiol*, **77**, 1155-1170.
Kudo, N. & Yamada, T. (1987) N-methyl-D,L-aspartate-induced locomotor activity in a spinal cord-hindlimb muscles preparation of the newborn rat studied in vitro. *Neurosci Lett*, **75**, 43-48.
Kullander, K., Butt, S.J., Lebret, J.M., Lundfald, L., Restrepo, C.E., Rydstrom, A., Klein, R. & Kiehn, O. (2003) Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. *Science*, **299**, 1889-1892.
Kullander, K., Croll, S.D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T.M., Klein, R., Yancopoulos, G.D. & Gale, N.W. (2001a) Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. *Genes Dev*, **15**, 877-888.
Kullander, K. & Klein, R. (2002) Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol*, **3**, 475-486.
Kullander, K., Mather, N.K., Diella, F., Dottori, M., Boyd, A.W. & Klein, R. (2001b) Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. *Neuron*, **29**, 73-84.
Lackmann, M. & Boyd, A.W. (2008) Eph, a protein family coming of age: more confusion, insight, or complexity? *Sci Signal*, **1**, re2.
Lanuza, G.M., Gosgnach, S., Pierani, A., Jessell, T.M. & Goulding, M. (2004) Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. *Neuron*, **42**, 375-386.
Leber, S.M. & Sanes, J.R. (1995) Migratory paths of neurons and glia in the embryonic chick spinal cord. *J Neurosci*, **15**, 1236-1248.
Lee, S.K. & Pfaff, S.L. (2001) Transcriptional networks regulating neuronal identity in the developing spinal cord. *Nat Neurosci*, **4 Suppl**, 1183-1191.
Leighton, P.A., Mitchell, K.J., Goodrich, L.V., Lu, X., Pinson, K., Scherz, P., Skarnes, W.C. & Tessier-Lavigne, M. (2001) Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature*, **410**, 174-179.
Lev-Tov, A. & Delvolve, I. (2000) Pattern generation in non-limb moving segments of the mammalian spinal cord. *Brain Res Bull*, **53**, 671-675.
Lim, R., Hoang, P. & Berger, A.J. (2004) Blockade of glycine transporter-1 (GLYT-1) potentiates NMDA receptor-mediated synaptic transmission in hypoglossal motorneurons. *J Neurophysiol*, **92**, 2530-2537.
Liu, Q.R., Lopez-Corcuera, B., Mandiyan, S., Nelson, H. & Nelson, N. (1993) Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. *J Biol Chem*, **268**, 22802-22808.
Lundfald, L., Restrepo, C.E., Butt, S.J., Peng, C.Y., Droho, S., Endo, T., Zeilhofer, H.U., Sharma, K. & Kiehn, O. (2007) Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. *Eur J Neurosci*, **26**, 2989-3002.
Maxwell, D.J., Hartley, R., Arber, S., Pierani, A., Jessell, T.M. & Todd, A.J. (2003) Transmitter phenotype and axonal projections of neurons originating from ventral progenitor domains in the mouse spinal cord. *Soc. Neurosci. Abstr.*, 186.185/I102.
McClive, P., Pall, G., Newton, K., Lee, M., Mullins, J. & Forrester, L. (1998) Gene trap integrations expressed in the developing heart: insertion site affects splicing of the PT1-ATG vector. *Dev Dyn*, **212**, 267-276.
McCrea, D.A., Pratt, C.A. & Jordan, L.M. (1980) Renshaw cell activity and recurrent effects on motoneurons during fictive locomotion. *J Neurophysiol*, **44**, 475-488.
McLean, D.L., Fan, J., Higashijima, S., Hale, M.E. & Fetcho, J.R. (2007) A topographic map of recruitment in spinal cord. *Nature*, **446**, 71-75.

McLean, D.L., Merrywest, S.D. & Sillar, K.T. (2000) The development of neuromodulatory systems and the maturation of motor patterns in amphibian tadpoles. *Brain Res Bull*, **53**, 595-603.
Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M.K., Burrill, J. & Goulding, M. (2001) Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron*, **29**, 385-399.
Murai, K.K. & Pasquale, E.B. (2004) Eph receptors, ephrins, and synaptic function. *Neuroscientist*, **10**, 304-314.
Myers, C.P., Lewcock, J.W., Hanson, M.G., Gosgnach, S., Aimone, J.B., Gage, F.H., Lee, K.F., Landmesser, L.T. & Pfaff, S.L. (2005) Cholinergic input is required during embryonic development to mediate proper assembly of spinal locomotor circuits. *Neuron*, **46**, 37-49.
Nabekura, J., Katsurabayashi, S., Kakazu, Y., Shibata, S., Matsubara, A., Jinno, S., Mizoguchi, Y., Sasaki, A. & Ishibashi, H. (2004) Developmental switch from GABA to glycine release in single central synaptic terminals. *Nat Neurosci*, **7**, 17-23.
Nakayama, K., Nishimaru, H. & Kudo, N. (2002) Basis of changes in left-right coordination of rhythmic motor activity during development in the rat spinal cord. *J Neurosci*, **22**, 10388-10398.
Nishimaru, H., Takizawa, H. & Kudo, N. (2000) 5-Hydroxytryptamine-induced locomotor rhythm in the neonatal mouse spinal cord in vitro. *Neurosci Lett*, **280**, 187-190.
Nissen, U.V., Mochida, H. & Glover, J.C. (2005) Development of projection-specific interneurons and projection neurons in the embryonic mouse and rat spinal cord. *J Comp Neurol*, **483**, 30-47.
Nornes, H.O. & Carry, M. (1978) Neurogenesis in spinal cord of mouse: an autoradiographic analysis. *Brain Res*, **159**, 1-6.
Peng, C.Y., Yajima, H., Burns, C.E., Zon, L.I., Sisodia, S.S., Pfaff, S.L. & Sharma, K. (2007) Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron*, **53**, 813-827.
Pierani, A., Brenner-Morton, S., Chiang, C. & Jessell, T.M. (1999) A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell*, **97**, 903-915.
Pierani, A., Moran-Rivard, L., Sunshine, M.J., Littman, D.R., Goulding, M. & Jessell, T.M. (2001) Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron*, **29**, 367-384.
Plachez, C. & Richards, L.J. (2005) Mechanisms of axon guidance in the developing nervous system. *Curr Top Dev Biol*, **69**, 267-346.
Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T. & Hargrave, M. (2002) Patterning of the vertebrate ventral spinal cord. *Int J Dev Biol*, **46**, 597-608.
Pratt, C.A. & Jordan, L.M. (1987) Ia inhibitory interneurons and Renshaw cells as contributors to the spinal mechanisms of fictive locomotion. *J Neurophysiol*, **57**, 56-71.
Quinlan, K.A. & Kiehn, O. (2007) Segmental, synaptic actions of commissural interneurons in the mouse spinal cord. *J Neurosci*, **27**, 6521-6530.
Ritter, D.A., Bhatt, D.H. & Fetcho, J.R. (2001) In vivo imaging of zebrafish reveals differences in the spinal networks for escape and swimming movements. *J Neurosci*, **21**, 8956-8965.
Rossignol, S., Chau, C., Brustein, E., Giroux, N., Bouyer, L., Barbeau, H. & Reader, T.A. (1998) Pharmacological activation and modulation of the central pattern generator for locomotion in the cat. *Ann N Y Acad Sci*, **860**, 346-359.
Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T.M. & Rubenstein, J.L. (2000) Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev*, **14**, 2134-2139.
Sapir, T., Geiman, E.J., Wang, Z., Velasquez, T., Mitsui, S., Yoshihara, Y., Frank, E., Alvarez, F.J. & Goulding, M. (2004) Pax6 and engrailed 1 regulate two distinct aspects of renshaw cell development. *J Neurosci*, **24**, 1255-1264.

Satou, C., Kimura, Y. & Higashijima, S.-I. (2008) Analysis of spinal V0 neurons in zebrafish. *Soc. Neurosci. Abstr.*
Saueressig, H., Burrill, J. & Goulding, M. (1999) Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. *Development*, **126**, 4201-4212.
Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H. & Pfaff, S.L. (1998) LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell*, **95**, 817-828.
Skarnes, W.C., Moss, J.E., Hurtley, S.M. & Beddington, R.S. (1995) Capturing genes encoding membrane and secreted proteins important for mouse development. *Proc Natl Acad Sci U S A*, **92**, 6592-6596.
Smith, E., Hargrave, M., Yamada, T., Begley, C.G. & Little, M.H. (2002) Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord. *Dev Dyn*, **224**, 231-237.
Smith, J.C. & Feldman, J.L. (1987) In vitro brainstem-spinal cord preparations for study of motor systems for mammalian respiration and locomotion. *J Neurosci Methods*, **21**, 321-333.
Stanford, W.L., Cohn, J.B. & Cordes, S.P. (2001) Gene-trap mutagenesis: past, present and beyond. *Nat Rev Genet*, **2**, 756-768.
Stein, E. & Tessier-Lavigne, M. (2001) Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. *Science*, **291**, 1928-1938.
Stokke, M.F., Nissen, U.V., Glover, J.C. & Kiehn, O. (2002) Projection patterns of commissural interneurons in the lumbar spinal cord of the neonatal rat. *J Comp Neurol*, **446**, 349-359.
Supplisson, S. & Bergman, C. (1997) Control of NMDA receptor activation by a glycine transporter co-expressed in Xenopus oocytes. *J Neurosci*, **17**, 4580-4590.
Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. & Kaneko, T. (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol*, **467**, 60-79.
Tanabe, Y. & Jessell, T.M. (1996) Diversity and pattern in the developing spinal cord. *Science*, **274**, 1115-1123.
Tessier-Lavigne, M. & Goodman, C.S. (1996) The molecular biology of axon guidance. *Science*, **274**, 1123-1133.
Wahl, S., Barth, H., Ciossek, T., Aktories, K. & Mueller, B.K. (2000) Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol*, **149**, 263-270.
Wallen-Mackenzie, A., Gezelius, H., Thoby-Brisson, M., Nygard, A., Enjin, A., Fujiyama, F., Fortin, G. & Kullander, K. (2006) Vesicular Glutamate Transporter 2 Is Required for Central Respiratory Rhythm Generation But Not for Locomotor Central Pattern Generation. *J Neurosci*, **26**, 12294-12307.
Vallstedt, A., Muhr, J., Pattyn, A., Pierani, A., Mendelsohn, M., Sander, M., Jessell, T.M. & Ericson, J. (2001) Different levels of repressor activity assign redundant and specific roles to Nkx6 genes in motor neuron and interneuron specification. *Neuron*, **31**, 743-755.
Wegmeyer, H., Egea, J., Rabe, N., Gezelius, H., Filosa, A., Enjin, A., Varoqueaux, F., Deininger, K., Schnutgen, F., Brose, N., Klein, R., Kullander, K. & Betz, A. (2007) EphA4-dependent axon guidance is mediated by the RacGAP alpha2-chimaerin. *Neuron*, **55**, 756-767.
Wenner, P., O'Donovan, M.J. & Matise, M.P. (2000) Topographical and physiological characterization of interneurons that express engrailed-1 in the embryonic chick spinal cord. *J Neurophysiol*, **84**, 2651-2657.
Whelan, P., Bonnot, A. & O'Donovan, M.J. (2000) Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. *J Neurophysiol*, **84**, 2821-2833.
Wilson, J.M., Hartley, R., Maxwell, D.J., Todd, A.J., Lieberam, I., Kaltschmidt, J.A., Yoshida, Y., Jessell, T.M. & Brownstone, R.M. (2005) Conditional rhythmicity

of ventral spinal interneurons defined by expression of the Hb9 homeodomain protein. *J Neurosci*, **25**, 5710-5719.
Yokoyama, N., Romero, M.I., Cowan, C.A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L.F. & Henkemeyer, M. (2001) Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. *Neuron*, **29**, 85-97.
Zafra, F., Aragon, C. & Gimenez, C. (1997) Molecular biology of glycinergic neurotransmission. *Mol Neurobiol*, **14**, 117-142.
Zaporozhets, E., Cowley, K.C. & Schmidt, B.J. (2006) Propriospinal neurons contribute to bulbospinal transmission of the locomotor command signal in the neonatal rat spinal cord. *J Physiol*, **572**, 443-458.
Zeilhofer, H.U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., Bosl, M.R. & Fritschy, J.M. (2005) Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *J Comp Neurol*, **482**, 123-141.
Zhang, F., Aravanis, A.M., Adamantidis, A., de Lecea, L. & Deisseroth, K. (2007a) Circuit-breakers: optical technologies for probing neural signals and systems. *Nat Rev Neurosci*, **8**, 577-581.
Zhang, Y., Geiman, E., Narayan, S., Gosgnach, S., Akay, T., Lanuza, G., Velasques, T. & Goulding, M. (2007b) Functional roles of spinal V3 interneurons in maintaining the rhythmicity and stability of locomotor outputs. *Soc. Neurosci. Abstr.*, 406.405/TT431.
Zhong, G., Crone, S., Harris-Warrick, R. & Sharma, K. (2008) The locomotor pattern in the spinal cord of mice lacking V2a interneurons is dependent upon the frequency of the locomotor rhythm. *Soc. Neurosci. Abstr.*
Zhou, Y., Yamamoto, M. & Engel, J.D. (2000) GATA2 is required for the generation of V2 interneurons. *Development*, **127**, 3829-3838.
Zhuang, B. & Sockanathan, S. (2006) Dorsal-ventral patterning: a view from the top. *Curr Opin Neurobiol*, **16**, 20-24.