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Studies of Spinal Motor Control Networks in Genetically Modified Mouse Models

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Abstract
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Spinal neurons are important in several aspects motor control. For example, the neurons essential for locomotor movements reside in the ventral spinal cord. In this thesis, different motor control functions are being related to neuronal populations defined by their common expression of a gene.

First, a targeted disruption of the gene for vesicular glutamate transporter 2 (*Vglut2/ Slc17a6*) is described. The mutant animals die at birth because of their inability to breathe. The neuronal network in the brainstem, responsible for inspiration, was shown to become non-functional by the targeted deletion of *Vglut2*. To our surprise, it was still possible to induce rhythmic activity with normal left/right alternation in spinal cords isolated from VGLUT2-null embryos. Inconsistent reports of *Vglut1* expression in the spinal cord made us re-evaluate the *Vglut1* and *Vglut2* expressions. While *Vglut2* expression was widespread in the spinal cord, *Vglut1* expression was restricted to a few cells dorsal to the central canal. Taken together, the data suggest that, glutamatergic signaling is mandatory to drive the bilateral breathing, but not needed for coordination of basal alternating spinal locomotor rhythm.

Next, a screen for genes with restricted ventral expression was made. Some of the genes found could be connected to the characteristics of specific neuronal cell populations. For example, fast motor neurons were shown to express the genes *Calca* and *Chodl*. Further, we found the *Chrna2* expression selectively in putative Renshaw cells. It seems likely that the gene product, the alpha2 subunit of the nicotinergic receptor, could be linked to the unique connection of motor neurons to Renshaw cells. We used the *Chrna2* promoter to drive expression of Cre recombinase in a transgenic mouse. The Cre activity was present in most neurons labeled with Renshaw cell markers, which should make it a useful tool for functional studies of this population. The studies presented here show how the genes expressed in subsets of neurons can be used to target populations of neurons for functional studies of neuronal systems.

Keywords: acetyl choline, central nervous system, central pattern generator, Cre recombinase, development, genetic screen, glutamate, interneuron, motor neuron, mouse, mouse genetics, movement, network, neuronal network, nicotinic receptors, physiology, Renshaw cell, rhythm, spinal cord, transmitter

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If the brain were so simple we could understand it, we would be so simple we couldn't.

Lyall Watson

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Wallén-Mackenzie, Å., **Gezelius**, **H.**[#], Thoby-Brisson, M.[#], Enjin, A., Nygård, A., Fujiyama, F., Fortin, G. & Kullander, K. (2006) Glutamatergic signaling mediated by VGLUT2 is required for respiratory rhythm generation but is dispensable for locomotor central pattern generation. *J Neuroscience* 26: 12294–12307.

II **Gezelius**, **H.**, Enjin, A., Lagerström, M., Wallén-Mackenzie, Å. & Kullander, K. (2006) Role of glutamate in locomotor rhythm generating neuronal circuitry. *J. Physiology (Paris)* 100: 297–303.

III Enjin, A., Rabe, N., Nakanishi, S.T., Vallstedt, A., **Gezelius**, **H.**, Memic, F., Lind, M., Hjalt, T., Tourtellotte, W.G., Bruder, C., Eichele, G., Whelan, P.J. & Kullander, K. (2009). Identification of novel spinal cholinergic genetic subtypes disclose *Chodl* and *Pitx2* as markers for fast motor neurons and partition cells. *Submitted manuscript*.

IV **Gezelius**, **H.**, Larhammar, M., Enjin, A., Peuckert, C., Nagaraja, C., Langer, D., Helmchen, F. & Kullander, K. (2009). Conditional genetic labeling of the Renshaw cell population for functional studies of motor control. *Manuscript*.

[#]Equal contribution

Papers not included in this thesis

Wegmeyer, H., Egea, J., Rabe, N., **Gezelius**, **H.**, Filosa, A., Enjin, A., Varoqueaux, F., Deininger, K., Schnütgen, F., Brose, N., Klein, R., Kullander K. & Betz A. (2007) EphA4-dependent axon guidance is mediated by the RacGAP a2-chimaerin. *Neuron* 55: 756–767

Rabe, N., **Gezelius**, **H**.[#], Vallstedt, A.[#], Memeic, F. & Kullander, K. (2009) Netrin-1 dependent spinal interneuron subtypes are required for the formation of left-right alternating locomotor circuitry. *J Neurosci, accepted.*

[#]Equal contribution

Contents

Abbreviations

aCSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP5	2-amino-5-phosphonovaleric acid
β-gal	β-galactosidase
BNPI	brain-specific Na^+-dependent inorganic phosphate transporter
CIN	commissural interneurons
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPG	central pattern generator
CRE	cyclization recombinase
DNA	deoxyribonucleic acid
DNPI	differentiation-associated Na^+/P_i co-transporter
E	embryonic day
eGFP	enhanced green fluorescent protein
ES	embryonic stem (cells)
GABA	γ-aminobutyric acid
L	lumbar segment
mEPSC	miniature excitatory postsynaptic current
MN	motor neuron
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-aspartic acid
P	postnatal day
PBC	pre-Bötzinger complex
PCR	polymerase chain reaction
RC	Renshaw cell
RT-PCR	reverse transcription polymerase chain reaction
TTX	Tetrodotoxin
VACHT	vesicular acetyl choline transporter
VGLUT	vesicular glutamate transporter
VIAAT	vesicular inhibitory amino acid transporter
VMAT	vesicular monoamine transporter
wt	wild type, i.e. not mutated

Introduction

Central pattern generator networks

The central nervous system (CNS) of vertebrates, the most complex organ in the animal kingdom is challenging to study. One way to gain insight into the mechanisms of neuronal signaling is to examine an isolated part of the nervous system. An evident output of the CNS is the control of muscles by the motor neurons (MNs). Neuronal networks capable of creating coordinated rhythmic output, so called central pattern generators (CPGs), are well suited for studies of principles for neuronal network architecture. There are CPGs that control the coordinated output of diverse muscle behaviors, such as breathing, swallowing and walking.

Spinal locomotor networks

The presence of a distinct network in the spinal cord capable of producing the walking behavior of limbed animals, was first demonstrated in a study of decerebrated cats in the beginning of the last century (Graham Brown, 1911). In this study, it was established that the spinal cord itself could generate the output necessary for the rhythmic movements of the limb muscles. To exclude the possibility that the output was due to reflexes, the dorsal roots were cut. Despite the loss of sensory input, these spinal cords were still able to produce a rhythmic coordinated output to the muscles. The complexity and relative inaccessibility of the cat spinal cord have encouraged the use of other model organisms for further detailed studies. The development of *in vitro* models of simple swimming vertebrates has advanced the knowledge of many of the cellular and molecular components present in the spinal networks [reviewed in (Grillner, 2003)]. Based on the knowledge obtained from these relatively simpler vertebrate models, *in vitro* preparations of rodent spinal cord have evolved as a model for studies of locomotor output in mammalian spinal cords. The basic building blocks in the mammalian CPGs are just beginning to be resolved. However, the participating neurons, their characteristics and exact roles are yet to be determined. The studies presented in this thesis use genetic approaches to label or selectively silence subpopulations of neurons. To determine their roles in the spinal motor control networks are studied in the mouse.

Functional locomotor studies in rodents

In an *in vitro* preparation of the rat the spinal cord with intact hind limbs the activation of NMDA receptors can induce a stepping movement of hind limbs (Kudo and Yamada, 1987; Smith and Feldman, 1987). The corresponding activity in the ventral roots can be measured simultaneously, displaying an alternation in activity between the roots at the same level as well as an alternation between flexor and extensor related activity. Such a pattern is normally referred to as locomotor-like activity. In the rat and mouse, the main activity observed in ventral lumbar segment (L)2 root is in phase with the flexor muscles, whereas the main activity in the L5 root is in phase with extensor muscles during drug-induced activity (Kiehn and Kjaerulff, 1996; Whelan et al., 2000). Similar to the rat preparations, locomotor-like rhythms can also be generated by an isolated mouse spinal cord (Jiang et al., 1999; Nishimaru et al., 2000; Whelan et al., 2000). Shifting combinations of NMDA, serotonin and dopamine have been used to elicit the rhythms in the mouse preparations. The *in vitro* preparation of rat and mouse spinal cord has also made it possible to examine the embryonic development of the coordination in rat and mouse (Branchereau et al., 2000; Nishimaru and Kudo, 2000). In the early development, induced rhythms are synchronous in all segments. Later an alternation between left and right side appears but with synchrony within each hemisegment. The coordination of both left and right, as well as flexion and extension, is established the day before birth in both rat (E20.5) and mouse (E18.5).

Regional distribution of rhythmic networks

Several studies in both rat and mouse have established that each half of the spinal cord itself has the capacity to produce rhythmic activity (e.g. (Kudo and Yamada, 1987; Kjaerulff and Kiehn, 1996; Kremer and Lev-Tov, 1997; Whelan et al., 2000). In 1996, Kjaerulff and Kiehn presented an extensive lesion study of the rat spinal cord preparation. One important finding was that the most ventral part of the spinal cord is sufficient to generate and coordinate a locomotor-like rhythm (Kjaerulff and Kiehn, 1996). Furthermore, it was recognized that a rhythm-generating ability is present throughout the spinal cord in the rostro-caudal axis, a finding also confirmed by other studies (Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Kremer and Lev-Tov, 1997; Cazalets et al., 2000). A study of MN activity in a rat E16.5 spinal cord slice preparation report that the smallest unit, necessary for producing a coordinated rhythmic activity, is the ventral quadrant of half a lumbar segment (Nakayama et al., 2004). Recently it has been demonstrated that also the mouse spinal cord can generate a coordinated rhythmic activity with most of the dorsal cord removed (Dyck and Gosgnach, 2009).

Identification of spinal neuronal populations

A neuron can be categorized based on different criteria; the neurotransmitter it uses for signaling, the nature of the axonal projections or by the identity of its developmental origin.

Transmitter phenotype

A traditional way of classifying neurons is to determine the neurotransmitter used for propagating a signal to target cells. The transmitter phenotype of neurons has classically been determined by identification of enzymes responsible for synthesis of the neurotransmitter. This method works well for acetylcholine, γ-aminobutyric acid (GABA) and the monoamines, where the enzymes are exclusively expressed in the neurons using that particular neurotransmitter. However, the amino acids glutamate, aspartate and glycine are present in all cells and for these neurotransmitters there are no specific enzymes located within neurons. An alternative way to identify the neurotransmitter phenotype has emerged by the discovery of vesicular transporter proteins. To date, the vesicular transporters for monoamines, acetylcholine, inhibitory amino acids and glutamate have been identified [VMAT1 and 2: Slc18a1(Liu et al., 1992), Slc18a2, (Takahashi and Uhl, 1997); VACHT: Slc18a3, (Naciff et al., 1997); VIAAT: Slc32a1 (Sagne et al., 1997) and VGLUT1, 2 and 3: Slc17a7 (Bellocchio et al., 2000; Takamori et al., 2000), Slc17a6 (Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Takamori et al., 2001; Varoqui et al., 2002), Slc17a8 (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Takamori et al., 2002)]. By combining the expression of enzymes responsible for producing the neurotransmitter molecules with the expression of vesicular transporters, the neurotransmitter phenotype of most neurons can be determined.

Connections

Neurons can also be classified based on the nature of their axonal projections. The most common designations used to describe the spinal cord neurons are described below.

Efferent – projection out from the spinal cord
Afferent – projection into the spinal cord
Ascending/rostral – projection towards the brain
Descending/caudal –projection towards the tail, from higher centers
Interneurons – neurons connecting within the spinal cord
Commissural – the axon is crossing the midline
Ipsilateral – the axon is not crossing the midline
First-order – axonal connection directly onto a target neuron

Efferent neurons of the spinal cord include the MNs, which connect to muscles, and preganglionic neurons of the autonomous system. The axons of the efferent neurons exit the spinal cord in the ventral roots. Afferent axons transfer information to the spinal cord from sensory neurons located outside the cord. The afferent fibers enter the spinal cord in the dorsal roots. Ascending fibers transfer signals rostrally, toward higher brain centers. Conversely, descending fibers provide incoming signals from higher centers. The term descending can also be used to specify axons with caudal projections. Neurons that only connect within the spinal cord are termed interneurons. The interneurons can be separated into: commissural interneurons (CIN), which project across the midline, and ipsilateral interneurons, which project within the same side of the cord. A further subdivision of the CINs neurons can be made based on the rostro-caudal projection of the axons. Local CINs have their target within the same segment of the spinal cord, whereas ascending CINs project rostrally, descending CINs send their axons caudally and bifurcating CINs have projections traveling in both rostrally and caudally after crossing the midline. Another classification sometimes used reflects the arrangement of the neurons, where a first-order neuron denotes a neuron that has direct contact onto a target neuron. By tradition, most often the target neurons are the MNs, because of the ease to identify them as they have well defined characteristics.

Motor neurons

The MNs form a special class of common for in the CNS. These are the neurons with efferent axonal projections, controlling muscle contractions. One hallmark of all vertebrate MNs is the use of acetylcholine as the neurotransmitter. Within the spinal cord, somatic MNs, which innervate skeletal muscles, are anatomically organized into columns that are correlated to the type of muscles innervated [summarized in (Jessell, 2000)]. The lateral motor columns (LMCs) contain somatic MNs that innervate skeletal muscles of the limbs. The LMCs are further subdivided into pools, where each pool contains MNs that project to a single muscle (Landmesser, 1978a, b). Alpha-MNs, which innervate force-generating extafusal muscle fibers, and gamma-MNs, which innervate the muscle spindle, are two of the types of somatic MNs that form a pool. The alpha-MNs can be further subdivided into fast and slow subsets dependent on the properties of the innervated muscle fiber. However, the cell bodies of slow and fast MNs are intermingled within each motor pool in the spinal cord (Milner et al., 1998). The slow and fast MNs differ in their soma size and electrical properties, features that can be used to identify them (Kernell and Zwaagstra, 1981; Zengel et al., 1985).

Developmental origin

During the development of the spinal cord, the fate of different neurons is specified by the expression of a combination of transcription factors. In early development, only a limited number of progenitor domains are present in the spinal cord, and each domain is determined by the combination of transcription factors expressed (Briscoe and Ericson, 1999; Goulding et al., 2002). Based on the active transcription factors, an early neuronal identity is assigned to the post-mitotic neurons. Later during the development the early post-mitotic neuronal populations can be subdivided and give rise neurons that share some common characteristics (Alvarez et al., 2005). Often the transcription factors are transiently expressed during the development. They can be used to identify neurons by immunolabeling while the protein is expressed, alternatively a genetic technique (described below) can be used to label a population for identification at later stages.

Glutamate in neuronal signaling

Glutamate is the most common excitatory neurotransmitter in the CNS. In order to act as a neurotransmitter, glutamate must be released from presynaptic vesicles in the axon terminal into a synaptic cleft. In the postsynaptic membrane receptors are activated upon binding of glutamate. Membrane transporters rapidly remove the glutamate from the synaptic cleft to terminate the glutamate signal.

Glutamate receptors

There are two classes of glutamate receptors, ionotropic (ion channel) receptors and metabotropic (G protein- coupled) receptors. The ionotropic receptors respond rapidly to glutamate binding by opening an ion channel. The metabotropic receptors are important for modulations of neurons, however, they will not be further discussed here.

Ionotropic glutamate receptors

The activation of an ionotropic glutamate receptor leads to the opening of an ion channel permeable to cations. The electrochemical gradient over the plasma membrane will cause an inflow of Na^+ and Ca^{2+} ions and hence generate a depolarization of the target neuron. There are two groups of ionotropic glutamate receptors, where one group is identified by its activation upon binding of N-methyl-D-aspartic acid (NMDA; called NMDA receptors) and the other group by its selective activation of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or kainate (AMPA/kainate receptors). The two groups of receptors can be independ-

ently modulated by the presence of different substances. Two of the drugs that selectively block the NMDA receptors are 2-amino-5-phosphonovaleric acid (AP5/APV) and MK801. In order to selectively block the AMPA/kainate receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) most often is used. Kynurenate is an antagonist that can be used to block all ionotropic receptors.

Vesicular glutamate transporters

As discussed above, the neurons that use glutamate as a neurotransmitter are most reliably identified by their expression of a vesicular glutamate transporter (VGLUT). Driven by a proton dependent electrochemical gradient, the VGLUT proteins pump glutamate into vesicles. Experiments performed in *Drosophila* indicate that the presence of a single VGLUT protein might be sufficient to fill a vesicle with glutamate (Daniels et al., 2006). It has also been shown that an absence of VGLUT protein results in release of empty vesicles (Fremeau et al., 2004a; Schuske and Jorgensen, 2004; Wojcik et al., 2004; Daniels et al., 2006).

Isoforms

To date there are three isoforms identified and characterized as VGLUT proteins in mammals. The genes encoding the three VGLUTs belong to the solute carrier gene family and are named *Slc17a7/Vglut1*, *Slc17a6/Vglut2* and *Slc17a8/Vglut3* for VGLUT1, VGLUT2 and VGLUT3 respectively[1]. The *Vglut1* gene was identified and cloned already in 1994, but at that time the protein was designated as brain-specific Na^+-dependent inorganic phosphate transporter (BNPI) (Ni et al., 1994). When the protein was characterized as a vesicular glutamate transporter, the name was altered to VGLUT1. Likewise, the VGLUT2 protein was first identified as differentiation-associated Na^+/P_i co-transporter (DNPI) (Aihara et al., 2000). In the mouse, the *Vglut1* and *Vglut2* genes are located close to each other on chromosome 7, whereas the *Vglut3* gene is located on chromosome 10. There is a high sequence similarity between the three isoforms, especially in the 12 transmembrane regions that make up the pore of the protein. No apparent functional difference between the proteins has been established so far (Takamori, 2006), however, the VGLUT3 localization differs in several aspects. All three isoforms have been observed in the axon terminals of neurons. However, whereas VGLUT1 and VGLUT2 are exclusively found in asymmetrical synapses (typical for excitatory neurotransmission), VGLUT3 proteins have been observed both in asymmetrical and symmetrical (modulatory or inhibitory) synapses (Bellocchio et al., 1998; Fremeau et al., 2001; Herzog et

[1] For clarity the gene names *Vglut1* for *Slc17a7*, *Vglut2* for *Slc17a6* and *Vglut3* for *Slc17a8* will be used in this thesis.

al., 2001; Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002). Moreover, there are VGLUT3-containing axons terminating onto proximal dendrites and soma (Fremeau et al., 2002; Schäfer et al., 2002), which is a further sign of inhibitory connectivity. In addition, co-localization of VGLUT3 with markers for other classical neurotransmitters has been established, including GABA (Fremeau et al., 2002; Herzog et al., 2004a), acetylcholine and serotonin (Gras et al., 2002; Schäfer et al., 2002). In contrast to the two other isoforms, VGLUT3 has been found in dendrites and soma of a number of neurons and also in non-neuronal tissues (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Herzog et al., 2004a). The existence of atypical locations of the VGLUT3 raises the possibility that this protein might be involved in functions other than glutamate neurotransmission.

Expression

Although no difference has been identified in the transport characteristics between the VGLUTs, the expression of the genes is restricted to different regions. VGLUT1 and VGLUT2 display a complementary expression in the adult nervous system, with VGLUT1 located in synapses with low probability of release and VGLUT2 in synapses with high probability of release (Marshall et al., 2004). In the brain, the reported expression patterns of the different isoforms are consistent in most regions (Bai et al., 2001; Fremeau et al., 2001; Herzog et al., 2001; Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Hisano, 2003; Herzog et al., 2004a). However, in the spinal cord, the disposition of the different isoforms is unclear since contradicting expression patterns have been reported.

Spinal *Vglut* expression

In the spinal cord of rat and mice, transcripts from the *Vglut1* gene have been reported to be localized in either a confined area dorsal to the central canal (Kullander et al., 2003; Oliveira et al., 2003; Llewellyn-Smith et al., 2007), or in a scattered fashion throughout the grey matter of the spinal cord (Hisano, 2003; Herzog et al., 2004a; Landry et al., 2004). Moreover, while several studies do not observe any VGLUT expression in MNs (Kullander et al., 2003; Oliveira et al., 2003; Myers et al., 2005; Wilson et al., 2005; Llewellyn-Smith et al., 2007), other studies report expression of both VGLUT1 and VGLUT2 in MNs (Hisano, 2003; Herzog et al., 2004a; Landry et al., 2004). Also the reports of *Vglut3* transcripts in the spinal cord are contradictory. Two reports have described the presence of VGLUT3 mRNA in rat spinal cords, by reverse transcription polymerase chain reaction (RT-PCR) (Gras et al., 2002) and *in situ* hybridization (Landry et al., 2004). Also transcripts from the human ortholog of *Vglut3* have been detected in the spinal cord by RT-PCR (Takamori et al., 2002). In contrast, two studies report an absence of VGLUT3 mRNA in rat (Oliveira et al., 2003) and mouse spinal

cords (Schäfer et al., 2002). The inconsistencies in the reports of the spinal cord expressions for the different VGLUTs elicit the need of further studies for clarification.

Genetic *Vglut* mutants

The discovery of the genes that encode the VGLUT proteins opened the possibility to generate mice with a malfunction in presynaptic glutamate vesicle packing. Elimination of a VGLUT will presumably result in silencing of the affected neurons, as they will no longer be able to convey a glutamate-mediated signal to target neurons. Consequently, the function of glutamate signaling from specific populations of neurons within a network can be examined.

VGLUT1-deficient mouse

Mice with a targeted deletion in the *Vglut1* gene have been produced a few years ago (Fremeau et al., 2004b; Wojcik et al., 2004). Bearing in mind the widespread expression of VGLUT1 it was a surprising that these mice are viable at birth. At about two weeks of age, mice homozygous for the non-functional allele display progressive neuro-degeneration, and do not survive longer than three weeks unless given special care (Fremeau et al., 2004b). A problem with coordination is observed in the deteriorating animals. This might be explained by a loss of sensory input, since sensory neurons normally express VGLUT1 (Todd et al., 2003; Hughes et al., 2004). During the first two weeks, however, no movement phenotype was observed [(Fremeau et al., 2004b; Wojcik et al., 2004) and personal communication Wojcik].

VGLUT2-deficient mice

Two different mouse lines carrying a disrupted *Vglut2* gene in all cells have been described (Moechars et al., 2006b). One of these mouse mutants has been produced in our lab and will be described in detail below. Briefly, animals with a complete absence of VGLUT2 protein die shortly after birth. Both newborn pups (Paper I) and embryos dissected out at embryonic day (E) 18.5 (Moechars et al., 2006a) appeared cyanotic, which indicated a breathing failure. This was further supported by the detection of collapsed lung alveoli in mutant embryos (Paper I). Moreover, the VGLUT2 deficient mice did not move and failed to react to forceps stimuli. The lack of reflex response might be an effect of lost VGLUT2-dependent signals from afferent fibers, since Vglut2 transcripts are present in DRG. No apparent developmental abnormalities of the CNS gross morphology were observed (Moechars et al., 2006a; Wallen-Mackenzie et al., 2006). The neuromotor performance and several other behaviors analyzed were not affected in the heterozygous mice with reduced levels of VGLUT2 protein (Moechars et al., 2006a). Analyses of glutamate release in cultured neurons from VGLUT2

deficient mice revealed a strong reduction of the release frequency of thalamic neurons. Also, a graded reduction of the miniature excitatory postsynaptic current (mEPSC) response was shown in neurons from heterozygous and homozygous mice, indicating a relation of quantal size to the amount of VGLUT protein present (Moechars et al., 2006a). More characteristics of the *Vglut2* null mutant mouse will be described below.

VGLUT3-deficient mice

Recently, two different mice with disrupted VGLUT3 function have been described (Gras et al., 2008; Seal et al., 2008). The mice are viable but display deafness and seizures (Seal et al., 2008) or hyperactivity due to a lack of cholinergic neurotransmission in the striatum (Gras et al., 2008). However, when analyzed, neither motor skills, reflexes nor the gait were affected by the targeted disruption (Gras et al., 2008).

Pharmacological block of glutamate signaling in locomotion

In the rodent spinal cord, attempts have been made to elucidate the role of glutamate signaling by the use of drugs that act on different glutamate receptors. In rat spinal cords, the block of either AMPA/kainate receptors with CNQX and/or block of NMDA receptors using AP5 disrupt a serotonin-induced rhythm (Beato et al., 1997). However, in the study by Beato and coworkers, increased levels of serotonin rescued the rhythms if only a single kind of receptor was blocked. The rescue of serotonin-induced AP5-blocked rhythm could not be repeated by increased serotonin in a later study (Cowley et al., 2005). What instead re-established the serotonin-induced rhythm was an elevated K^+ concentration (Cowley et al., 2005). This is in agreement with other experiments where locomotor-like rhythms could be induced by elevated K^+-levels, and subsequent AP5 block was overcome by an additional shift of the K^+ balance (Bracci et al., 1998; Cowley et al., 2005).

In the mouse, similar, but not identical, results has been observed. Blockade of the NMDA receptors in a mouse spinal cord preparation does not abolish a serotonin-induced rhythm (Nishimaru et al., 2000; Whelan et al., 2000). On the other hand, rhythms will be suppressed upon block of all ionotropic receptors by kynurenate (Nishimaru et al., 2000) or by block of AMPA/kainate receptors by CNQX (Whelan et al., 2000). Preliminary data from an *in vitro* preparation of mice lacking functional NMDA receptors further support the suggestion that NMDA receptors may not be critical for rhythm generation and coordination (Smith et al., 1993). These data suggest that in the mouse, AMPA/kainate receptor-mediated signals are critical for generation of locomotor-like rhythms.

Genetically identified excitatory neuronal populations

In the efforts to identify neuronal populations that constitute the locomotor CPG, a combination of genetics and electrophysiology have been used. A synchronous gait have been observed in mice lacking a functional a axon guidance molecule, ephrinB3, normally expressed at the midline, and also in mice missing one of the corresponding receptors, EphA4 (Kullander et al., 2001b; Kullander et al., 2001a; Kullander et al., 2003). This model is one of the first where mutant mice have been extensively studied using the spinal cord *in vitro* preparation. Ventral root recordings on isolated spinal cords from neonatal mice deficient of either ephrinB3 or EphA4 reproduce a hopping pattern in the output rhythms (Kullander et al., 2003). The hopping phenotype is thus probably due to a local defect in the spinal cord, and not caused by misrouting of the descending fibers from higher brain centers, which is also known to happen. Abnormal crossing of axons originating from neurons that normally express EphA4 are present in the lumbar region in spinal cords of EphA4 deficient mice (Kullander et al., 2003). Furthermore, it was recognized that a subset of neurons that express EphA4 is glutamatergic, as revealed by co-expression of VGLUT2. Further investigations have identified a subpopulation of rhythmic active EphA4-expressing neurons with excitatory ipsilateral first-order connections onto MNs (Butt et al., 2005).

An additional excitatory population of neurons in the spinal cord with rhythmic activity during fictive locomotion has also been identified. This is a group of interneurons that express the homeodomain transcription factor *Hb9* (Hinckley et al., 2005; Wilson et al., 2005). Hb9 is also expressed in, and essential for, development of MNs. Hence, a mouse with a knockout of the *Hb9* gene would not be good model for studies of the locomotor CPG. However, expression of the enhanced green fluorescent protein (eGFP) under control of the *Hb9* promoter has made it possible to do visually directed single-cell measurements on genetically identified cells. The Hb9-expressing interneurons exhibit membrane oscillations highly correlated to ventral root bursts during drug-induced locomotor like activity (Hinckley et al., 2005). In addition there are Hb9-expressing interneurons that are rhythmically active during application of rhythm-generating chemicals, even though all synaptic signaling is blocked by the Na^+-channel blocker tetrodotoxin (TTX) (Wilson et al., 2005). Moreover the interneurons with Hb9-expression seem to be involved in walking in living animals. This was indicated by co-immunolocalization of Hb9 and c-Fos, a protein that becomes expressed as a consequence of neuronal activity, in this case a result of walking activity of the animals (Wilson et al., 2005). The rhythmically active $Hb9^+$ interneurons are established to be glutamatergic by presence of VGLUT2, both protein (Hinckley et al., 2005; Wilson et al., 2005) and mRNA (Wilson et al., 2005). In both reports, the rhythmically active $Hb9^+$-interneurons were suggested as

potential candidate neurons for rhythm generation in the spinal locomotor CPG (Hinckley et al., 2005; Wilson et al., 2005).

Renshaw cells

One of the few interneurons that have been physiologically identified in the spinal cord is the Renshaw cell (RC). While the RC act of recurrent inhibition of MNs is known since a long time (Renshaw, 1941), the exact role of these cells in motor control is yet to be established. The recurrent inhibition signaling reflects a strong connectivity between RCs and MNs. Excitatory MN axon collaterals terminate onto RCs, these inhibitory neurons project their axons back to MNs of the same pool (Renshaw, 1946; Eccles et al., 1954; Hultborn et al., 1979) (Figure 1). RCs can be identified in electrophysiological recordings by their initial fast spiking response to a single stimuli applied to MN axons, e.g. by ventral root stimuli (Renshaw, 1946). Based on electrophysiological recordings, the RCs have been located to the ventro-medial region of the ventral horn, in close proximity to the motor axons exit points (Eccles et al., 1954; Thomas and Wilson, 1965).

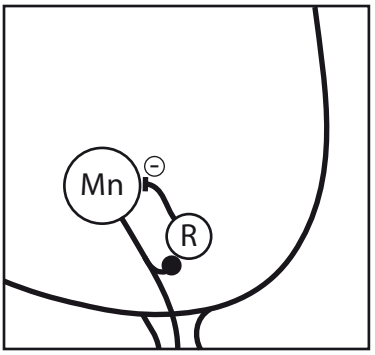

Figure 1. Recurrent inhibition.

Labeling of RCs, filled during single cell recording, followed by immunohistochemical staining have identified gephyrin clustering on the soma as a reliable marker for adult RCs (Alvarez et al., 1997). However, the characteristic large gephyrin clusters first emerge at postnatal day (P) 10 (Geiman et al., 2000), making it an ineffective marker during the development of the locomotor network. Calbindin D28k (calbindin) is another immunohistochemical marker expressed in RCs (Antal et al., 1990; Sanna et al., 1993; Carr et al., 1998), however, in the ventral horn calbindin is also expressed in a few additional cells not displaying the characteristic gephyrin cluster distribution (Carr et al., 1998). In rodents, ventral calbindin immunohistochemical staining, presumably including RCs have been observed in neonates (Geiman et al., 2000) and already at E12.5-14.5 rat and E13.5 mouse embryos (Zhang et al., 1990; Sapir et al., 2004). The calbindin expression have also been observed in electrophysiological identified RCs in neonatal mice (Nishimaru et al., 2005).

The RCs are derived from the V1 IN population (Sapir et al., 2004). The V1 INs is a population of neurons that transiently express the transcription factor Engrailed 1 (En1) as they become post-mitotic during the develop-

ment (Matise and Joyner, 1997). The axons from RCs as well as other V1 neurons project both rostrally and caudally (Matise and Joyner, 1997; Saueressig et al., 1999; Sapir et al., 2004; Alvarez et al., 2005). Most of the calbindin-labeled V1-derived neurons are located in the very ventral portion of the spinal cord where the MN axons exit the spinal cord, and these presumed RCs constitute 9-10% of the V1 IN population (Sapir et al., 2004; Alvarez et al., 2005). Only markers for inhibitory neurotransmission, GAD67 and/or GlyT2, could be observed in axon terminals originating from V1 derived neurons. This indicates that all the V1 INs are inhibitory, and it could be expected that a proportion of the neurons could co-release both glycine and GABA as neurotransmitter (Alvarez et al., 2005).

The RCs have been shown to be rhythmically active during fictive locomotion, both in cat and mouse (McCrea et al., 1980; Noga et al., 1987; Pratt and Jordan, 1987; Nishimaru et al., 2006). Pharmacological or genetic block of cholinergic signaling, as well as genetic targeting of V1 derived neurons, propose that RCs are involved in the control of speed of locomotion (Myers et al., 2005; Gosgnach et al., 2006; Nishimaru et al., 2006). More specific pharmacological or genetic tools are needed to test this hypothesis.

Aims

The overall aim of the studies is to understand how spinal neuronal networks operate. Especially the roles of distinct populations of neurons, which potentially participate in the circuitry, are addressed.

The specific aims of the studies in this thesis are:

Paper I & II
Investigate the importance of glutamatergic neurotransmission in the rhythm generating spinal circuitry, with special focus on the transmission mediated by VGLUT2.

Paper III
Find genes useful as markers for populations of neurons with distinct functional roles in motor control circuitry.

Paper IV
Evaluate one marker found by the method described in paper III, for future studies of the functional role of a distinct population of neurons.

Methodological considerations

The experimental procedures used in this thesis are described in detail in the materials and methods section of each paper. Here, certain aspects of the methods used are summarized and discussed.

Animals

The studies in this thesis are based on analyzes of mutant mice or tissue from mice (Paper I–IV) and rats (Paper II). All animal procedures were approved by the appropriate local ethical committees; Uppsala (C156/4, C167/5, C147/7, C79/9), French Agricultural Ministry and the European Union Council Directive for the Care and Use of Laboratory Animals (number 2889) and University of Calgary Animal Care Committee.

The making of two mouse mutant alleles, loxP-flanked *Vglut2* (*Vglut2*^{f}, Paper I) and *Chrna2-Cre* (Paper IV) are described in this thesis. These mice are mated to mice described elsewhere to generate apt mutant mouse; P*GK-Cre* (Lallemand et al., 1998), *Tau*$^{mGFP\text{-}nlslacZ}$ (kind gift from Silvia Arber,(Hippenmeyer et al., 2005)), *Gt(ROSA)26*$^{Sortm14(CAG\text{-}tdTomato)Hze}$ (tdTomato; Allen Brain Institute). Additionally, *Egr3*$^{-/-}$(Tourtellotte and Milbrandt, 1998) and *Pitx2-Cre* (Liu et al., 2003) mutant mice were used as specified in Paper III. For genotyping of mice, DNA prepared from the tail was subjected to polymerase chain reaction (PCR) analysis or tissue was subject to X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) staining, as specified in each paper. After import to our animal facilities, all animals were maintained on a C57Bl/6 background (Scanbur B&K, Sweden) or by breeding to wild type (wt) littermates. For microarray analyses and in situ hybridization C57/Bl6 mice were used. MN electrophysiology experiments were performed on Swiss Webster mice (Charles River Laboratories, Wilmington, MA)

The Cre - loxP system

The system of Cre-loxP is a genetic tool that can be used to increase the flexibility when working with mutant mice. It is a two-component system that needs both the Cyclization recombinase (Cre) protein and loxP sites to

be functional. The Cre protein mediate a recombination between two identical loxP sites and yield an excision of the DNA sequence between the loxP sites when they are oriented in the same direction (Figure 2A). Often a specific promotor is used to constrain the Cre activity to certain cells (Figure 2D). The loxP sites can be placed in the genome so that they are flanking a gene of interest (floxed gene), this generates a conditional knockout of that gene (figure 2B). Another use is conditional reporter expression, by a construct with a general promotor followed by a floxed stop cassette and then a reporter gene. Before excision of the stop cassette, no expression of reporter is present. However, when Cre is active the reporter gene expression gets activated (Figure 2C). When mating a mouse with a region-specific *Cre* expression with mouse with a floxed gene of interest (or floxed stop reporter gene), offspring carrying both mutations will display a region-specific targeted expression. Currently there are a growing number of mice with specific *Cre* expression, conditionally targeted genes and reporter genes available in the scientific community. In this thesis two new mice are presented, one floxed *Vglut2* mouse (Paper I) and a mouse with Cre activity driven by *Chrna2* promotor sequences (Paper IV).

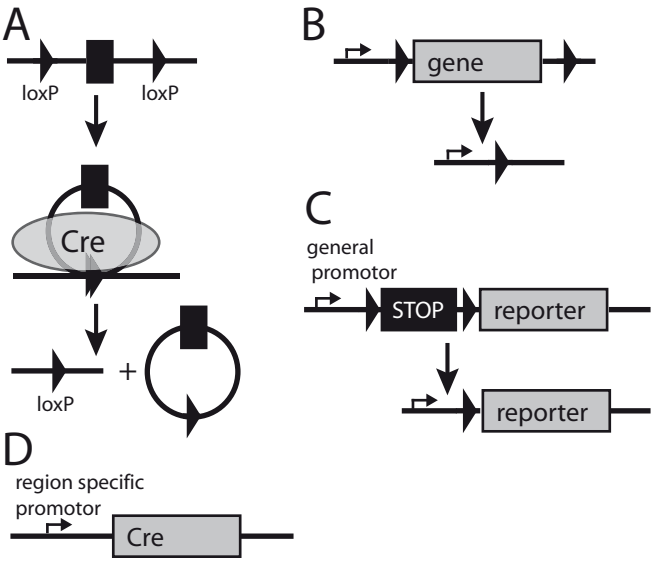

Figure 2. The Cre-loxP system.

Generation of mutant mice

The generation of both of the mutant mice presented in this thesis rely on recombination of a DNA construct into the mouse genomic sequence. In the case of the *Vglut2* recombination occurred in embryonic stem (ES) cells

(described in results below and in Paper I), whereas the *Chrna2-Cre* mutation was introduced in a bacterial artificial chromosome (BAC) plasmid. The BAC DNA was subsequently introduced into the mouse genome.

For the generation of the Cre mouse, a BAC plasmid containing the full *Chrna2* genomic sequence and additional 100 kbase pairs (kb) upstream and 8 kb downstream of the gene (RP23-48P22) was used. The plasmid was transferred to EL250 bacterial cells, protocols and bacteria described at http://recombineering.ncifcrf.gov/. During all the handling, care was taken to not freeze or vigorously shake the BAC to preserve the integrity of the DNA.

In parallel, a plasmid containing *nlsCre* followed by a poly-Adenylation signal (pA) and an FRT-flanked Kanamycin/Neomycin resistance sequence was generated by conventional cloning. The plasmid was used as a template for a PCR reaction with one primer starting with the ATG of the *Cre* and the second primer binding close to the second FRT sequence. Before each primer (5') it was added 50 base pairs, which were homologous to the genomic DNA flanking the first coding exon (starting mid exon2) of the *Chrna2* gene. The PCR was performed with a proofreading Taq-polymerase and a custom-written program to generate a strong single band on an agarose gel. The DNA of the correct length was extracted (QiaEx II, Qiagen, Sweden) following the manufacturers instructions. Care was taken to minimize the exposure of UV light during the procedure.

The purified PCR-product was electroporated into the EL250 bacterial cells containing the BAC plasmid. Successful introduction of the *Cre* construct at the correct location of the *Chrna2* sequence was tested by two PCR reactions on DNA from cultures growing on Kanamycin plates as template. One reaction was corresponding to a correct insert, the other reaction to the wt situation before the insert. The length of the BAC plasmid was tested by restriction enzyme digestion followed by pulse field gel electrophoresis (PFGE, CHEF-mapper, BioRad. The Kanamycin resistance sequence was removed by arabinose-induced FlpE recombinase in the EL250 cells. The integrity of the DNA was tested as before and further confirmed by sequencing of the modified region of the BAC plasmid. The modified genomic BAC DNA sequence was linearized by NotI and purified by PFGE followed by gel extraction and dialysis as described in (Marshall et al., 2004). The DNA was introduced by pronuclear injection, resulting in a random insertion into the mouse genome. Mice with a successful introduction were selected based on the results from a PCR reaction.

Ventral root electrophysiology

The functional output of the locomotor CPG was studied using extracellular electrophysiology on an isolated preparation of mouse spinal cord (Jiang et al., 1999; Nishimaru et al., 2000; Whelan et al., 2000). Such a preparation

can be kept alive for several hours when superfused with an oxygenated buffer. By application of the neurotransmitter chemicals, NMDA, serotonin (5HT) and dopamine, neuronal activities were induced in the spinal cord. The activities were measured in ventral roots, reflecting the MN activity that normally would activate muscles. By measuring on both left and right ventral roots of lumbar level (L) 2 and 5, the flexor and extensor activities and their coordination can be revealed (Whelan et al 2000). Coordination analysis was performed by comparing the onset of two successive bursts within one recorded trace (the period length) to the onset of the first next burst in the analyzed trace (the latency). For each animal 15 cycles with at least five cycles between each comparisons were made and plotted on a circular diagram (Kjaerulff & Kiehn 1996).

In situ hybridization

For detection of cells expressing selected genes, non-radioactive *in situ* hybridization histochemistry (ISH) was used. The method recognizes a transcribed mRNA in the cell, by using a tagged RNA sequence complementary to the selected mRNA (anti-sense probe). An enzyme-coupled antibody then recognizes the tags of the sequence. The cells with transcription of the examined gene are visualized by addition of a substrate that becomes colored by the enzyme. Since mRNA is located in the cell soma, the cell bodies of the cells are visualized. A detected signal informs that the cell is expressing the examined gene, but it does not reveal the location of the protein within the cell. When analyzing the gene expression by ISH we were always looking at several sections, preferably collected from different animals. In the case of *Vglut1*, *Vglut2* and *Vacht* ISHs, the probes used were tested to give expected expression in regions with known patterns of expression.

Immunohistochemistry

Immunofluorescence histochemistry has been used to detect proteins in the studies presented in this thesis. An antibody recognize a protein epitope and bind to it where it is situated in the cell, which is important to keep in mind when analyzing the expression. All antibodies used in this thesis have been tested and described elsewhere, as specified in each paper. Before utilized, the exact working conditions have been evaluated for each antibody. Most of the times, the antibodies used are known markers for a cell population or a cell type. One advantage of fluorescent immunohistochemistry is that several antibodies can be combined, which is good for detecting overlapping expression. In some of the studies a combination of ISH and immunoflourescence was used to reveal the extent of overlapping expression. Depending on the

resolution needed for the analysis, images were obtained with a fluorescence microscope, using an Optigrid system or by a confocal microscope system.

2-Photon microscopy

We have used the technique of 2-photon excitation laser scanning microscopy to be able to visualize fluorescently labeled cells within living tissue. By focusing a pulsed laser beam, simultaneous adsorption of two photons can excite a fluorophore, without excitation of the surrounding that is out of focus. The focused beam is scanned in the tissue and the emitted light is collected. One advantage of the method is that longer wavelengths can be used and that only the area in focus is exposed to the high-energy light. The long wavelengths used (>800nm) naturally have lower energy than normal light used for single-photon excitation, which is important for avoiding photobleaching. The long wavelengths used also enables deeper tissue penetration. Another advantage of the system is that different fluorophores can be detected simultaneously by separation of the emitted light with optical filters. For the 2-photon studies (Paper IV) we have used a reporter mouse with high expression of the fluorophore tdTomato, which is operating in the red range of the spectrum (excitation max 554 nm, emission max 581 nm). There are two main reasons for using that reporter. (1) The long wavelength reduces the energy and enables deep tissue penetration. (2) The best calcium activity dyes known at present are all operating in the green range of the spectrum (Wilson et al., 2007). Despite the advantages described above the method must be used carefully. If focusing the beam for too long time on a small spot, either by zooming in the scan-area or by repeatedly returning to the same locations, the scanned cells can burn because of the highly focused energy. The same could happen if too much laser energy is applied over a wider area. When determining the settings, a balance between image spatial and temporal resolution is desired. We have combined the two by first acquiring an image with high spatial resolution followed by repeated low-resolution imaging at the speeds of 6-7 Hz. With this temporal resolution it is possible to detect active cells but not individual action potentials, which should be good enough for the purpose of the studies discussed in this thesis.

Results and Discussion

Paper I

Based on the expression patterns of *Vglut1–3*, functionally inactive VGLUT2 protein would be expected to ablate most glutamatergic signaling with a spinal origin. To investigate the locomotor CPG and other neuronal circuits, a mouse with a conditional targeted mutation of the *Vglut2* gene was constructed. The control of selective gene targeting was made possible by replacing exon 4–6 in a targeting construct. The construct included exon 4–6 and a neomycin selection marker, all of it flanked by two loxP-sites. Positively selected ES cells (Sv129/R1) were introduced into mice (C57BL/6). The mice that received the mutation ($Vglut2^{f/+}$) were mated on wt (C57BL/6) background and subsequently bred to generate homozygous animals ($Vglut2^{f/f}$). All animals appeared normal and were fertile. The $Vglut2^{f/f}$ mouse was crossed to a transgenic PGK-Cre mouse (Lallemand et al., 1998) to generate a gene disruption (i.e. remove exon 4-6), of *Vglut2* in all cells of the animal ($Vglut2^{f/f;PCre}$ or VGLUT2 KO). The loss of VLGUT2 protein was confirmed by immunofluorescence histochemistry. A lack of VGLUT2 protein resulted in malformation and reduced number of vesicles in asymmetric synapses, observed by electron microscopy in the brainstem region of E18.5 embryos. The VGLUT2 KO animals were born with a Mendelian ratio, but died immediately after birth.

Because of the apparent failure to breath (see Introduction), the respiratory properties of the VGLUT2 KO mice were measured. It was first confirmed that KO animals do not breath, by measurements on surgically delivered E18.5 embryos in a ventilation chamber. Recordings from hindbrain preparations revealed a complete absence of respiratory-related motor output in the $Vglut2^{f/f;PCre}$ animals, a similar effect was seen by application of CNQX to control preparations that is naturally active. The lack of motor output directed the attention to the pre-Bötzinger complex (PBC) in the medulla, which is believed to be an important part of the respiratory CPG (Feldman and Del Negro, 2006). No change was observed in the location or size of the brainstem structures involved in the respiratory rhythm generation. Nonetheless, no natural activity was observed in the PBC area of medulla slices from VGLUT2 KO embryos, not even after application of the excitatory neuromodulator substance P. Furthermore, it was found that the commissural fibers normally synchronizing the two sides failed in communi-

cation. It was also found that the non-functional network activity was not due to loss of glutamate receptors, since local AMPA application could elicit an ipsilateral response. Measurements of individual neurons in the PBC area revealed fast excitatory and slow inhibitory synaptic events in control animals, but only slow inhibitory events in the VGLUT KO animals. Despite the lack of synchronous rhythmic network activity, individual cells were found with rhythmic bursting properties. Taken together, the mouse without functional VGLUT2 protein was unable to synchronize and propagate the rhythms necessary for respiration.

In previous studies it has been suggested that glutamate neurotransmission is an important feature of the locomotor CPG (see Introduction). We therefore wished to examine the output from spinal cords of VGLUT2 deficient mice. *Vglut2* transcript was observed already at E12.5 and was widely distributed in the spinal cord at P0, similar to the expression seen in P4 and adult mice (Kullander et al., 2003). *Vglut1* was exclusively expressed in the dorsal region of the spinal cord at P11, similar to what has been reported in adult murines (Kullander et al., 2003; Oliveira et al., 2003; Llewellyn-Smith et al., 2007). To our surprise, the output from the locomotor CPG of $Vglut2^{f/f;PCre}$ E18.5 spinal cords was similar to the control siblings. In the *in vitro* preparation, equivalent concentrations of the neurostimulatory drugs NMDA, serotonin and dopamine induced coordinated rhythms in both mutant and control spinal cords. No difference could be discerned; neither in coordination nor in the frequency of the rhythms. This finding implies that neurotransmission mediated by VGLUT2 is not required for coordination of locomotor rhythm. Furthermore, the presence of a functional network in the mutants question the importance of the $Vglut2^{+}$ oscillatory neuronal populations, which have been presented as putative rhythm generating populations in the spinal cord [$Hb9^{+}$ interneurons, (Hinckley et al., 2005; Wilson et al., 2005); $EphA4^{+}$ ipsilateral projecting first-order neurons (Kullander et al., 2003; Butt et al., 2005)]. In the $Vglut2^{f/f;PCre}$ mice these neurons should no longer be able to propagate their cyclic activity, at least not through VGLUT2 mediated neurotransmission. Furthermore, a recent study using both 2-photon live imaging and visually guided patch combined with ventral root electrophysiology revealed that the activity in the $Hb9^{+}$ interneurons did not precede the MN output measured in the ventral root (Kwan et al., 2009). This argues against that the $Hb9^{+}$ interneuron population alone is the rhythm generator in the spinal cord. Although not critical, the $Hb9^{+}$ and $EphA4^{+}$ interneurons that express VGLUT2, most likely somehow participate in rhythm formation *in vivo*. The experiments presented in here further support the ideas presented based on pharmacological studies on rat spinal cords (Beato et al., 1997; Bracci et al., 1998; Cowley et al., 2005), namely that the endogenous role of glutamate is to excite the spinal network, excluding a critical role for glutamate mediated signaling in coordination of spinal

rhythmic neurons. However, additional examinations are needed to fully understand the role of glutamate in the spinal locomotor CPG.

Paper II

The unexpected finding of the normal performing locomotor network in mice without functional VGLUT2 protein made us concerned about the discrepancies in spinal cord mRNA expression described for the VGLUTs. Our group has previously reported an expression of *Vglut1* restricted to a discrete area in the dorsal spinal cord in P11 (Paper I) and P20 (Kullander et al., 2003) mouse. A similar restricted pattern has also been reported for adult rat spinal cord by other groups (Oliveira et al., 2003; Llewellyn-Smith et al., 2007). Contrary to these studies, there have been reports of *Vglut1* transcripts present throughout the whole grey matter of adult rat spinal cord (Hisano, 2003; Herzog et al., 2004a; Landry et al., 2004). In these later reports, strong hybridization signals is also observed in large neurons in the ventral spinal cord, presumably MNs. The occurrence of Vglut mRNA in MNs is also what differs between the reports of spinal *Vglut2* expression; the same groups that observe *Vglut1* transcripts in MNs also detect substantial *Vglut2* expression in MNs. One of the studies confirmed the expression in MNs by the co-localization of both *Vglut1* and *Vglut2* with choline acetyltransferase mRNA, a marker for MNs (Herzog et al., 2004b). In order to explain the discrepancies of *Vglut* transcript patterns of expression, we repeated the *in situ* hybridization experiment on spinal cord sections. First, we increased the time of development to pick up low levels of mRNA. This resulted in an over-staining of the neurons already previously detected. However, no low-level expression of *Vglut1* could be detected. Second, the accuracy of the probes we use were tested by making *in situ* hybridizations on brain sections with characterized expression of *Vglut1* and *Vglut2*, respectively. The mRNAs detected with our *Vglut1* and *Vglut2* probes reiterated the distinct patterns of expression previously described in the brain (Fremeau et al., 2001; Herzog et al., 2001; Fremeau et al., 2004b). Third, to further exclude a difference dependent on the probes used in different experiments, we obtained the probes used in the studies by Herzog et al. and Landry et al. When using these rat probes on mouse spinal cords (P11) we nonetheless observed a restricted expression of *Vglut1* as well as an expression pattern of *Vglut2* that was widespread but not present in MNs. Fourth, in order to exclude a species difference we used our mouse probes on adult rat spinal cords. The expression patterns observed still resembled the patterns described by our group and others (Kullander et al., 2003; Oliveira et al., 2003; Wallen-Mackenzie et al., 2006; Llewellyn-Smith et al., 2007). The attempts to clarify the distribution of *Vglut1* and *Vglut2* transcripts all suggest a restricted expression of *Vglut1* in dorsal region of the lumbar spinal cord, in

comparison to the widespread expression observed for *Vglut2*. An absence of *Vglut3* transcripts in the spinal cord has been reported for mouse (Schäfer et al., 2002), and is supported by a study that reported a corresponding nonappearance in the rat (Oliveira et al., 2003). In contrast to these observations, two studies of rat spinal cords have detected *Vglut3* transcripts by RT-PCR (Gras et al., 2002) and *in situ* hybridization (Landry et al., 2004). Given the equivalent patterns of *Vglut1* and *Vglut2* mRNA expression displayed by the mouse and rat, a species difference seems an unlikely explanation for the deviating reports of *Vglut3* mRNA in the spinal cord. So far we have not made a thorough analysis of the VGLUT3 expression in the spinal cord, but our preliminary results supports the finding that the spinal cord is devoid of *Vglut3* expression neurons. The functional CPG in the VGLUT2 deficient animals together with an absence of *Vglut1*- and *Vglut3*-expressing neurons in the ventral spinal cord, the suggested location of spinal locomotor CPG neurons (Kjaerulff and Kiehn, 1996), indicate that vesicular packaging of glutamate is not critical for normal CPG function. The observation of normal movements in VGLUT1 and VGLUT3 deficient mice (Fremeau et al., 2004b; Wojcik et al., 2004; Gras et al., 2008; Seal et al., 2008) further support that neither VGLUT1 nor VGLUT3 mediated signaling is not involved in the basic CPG circuit. Unless another source of glutamate exists in the spinal cord, it seems possible that glutamate is dispensable for the coordination in the spinal locomotor neuronal circuitry.

Paper III

The need for genetic markers of distinct neuronal populations involved in motor control prompted us to perform a screen for genes with high expression in the mouse lumbar ventral spinal cord. Two parallel strategies were used to locate genes with a restricted ventral expression. (1) The ventral mRNA levels were compared to the dorsal by a microarray assay, the expressed sequences with a high ventral ratio of expression were selected for further analysis. (2) Images of ISH performed on sagittal sections from embryonic (E14.5) mice, available at www.genepaint.org, were manually evaluated. Genes with restricted expression in the central or ventral spinal cord were selected for further analysis. Naturally, we found well-characterized genes known to be expressed in the ventral spinal cord. This validated the methods used for selection, however, these genes were not analyzed further. Subsequently, floating ISHs were performed for 341 genes on spinal cord sections from P11 mouse, an age when the animals have started weight-bearing locomotion.

Based on the expression patterns revealed by the ISH, the genes were divided into six different categories (Paper III, supplementary figure 1). Out of 247 genes showing distinct expression, 57 genes looked like cholinergic

MNs expression. In addition, 102 genes were seen in presumable MN, but also more widely throughout the grey matter. Double labeling with *Vacht* further specified 12 genes to be expressed in subsets of the cholinergic cells. Three of these presumable subpopulations markers were analyzed further to address if they were selectively expressed by either fast or slow MNs. One of these genes, Calcitonin/calcitonin-related polypeptide, alpha (*Calca*), has been related to fast motor unit type (Forsgren et al., 1993; Piehl et al., 1993). We found an almost complete overlap of *Calca* expression with Chondrolectin (*Chodl*) and a complementary non-overlapping expression of estrogen related receptor, beta (*Esrrb/ERRβ*) within the lateral motor column. Based on the large soma size and the measured electrophysiological properties, it seems likely that *Chodl* is a novel marker for fast MNs. On the contrary, a expression complementary to fast neurons, the soma size and presence in areas normally devoid of γMNs indicates that *Esrrb* could be is expressed in slow MNs. Technical difficulties have so far hampered the electrophysiological characterization of the *Esrrb*. No recorded cells have been stained after recording, possibly the protein was diluted into the pipette during patch recording. We suggest that *Calca* and *Chodl* could be used as markers for fast MNs, and that *Esrrb* is a putative maker for slow MNs.

One other gene, *Pitx2*, was found in *Vacht* expressing cells, distinct from the motor columns. At P11, it was found to have a very restricted expression in neurons with a cholinergic phenotype located close to the central canal. Based on the axonal projections, the cholinergic phenotype and the size of these cells, we propose that these are a subpopulation of partition cells .

In summary, the screen identified genes expressed in subsets of neurons. Some of the genes labeled neuronal populations with already known markers, but we also found novel markers for selected subpopulations of neurons. Additionally, several genes with annotated functions have been localized to the visualized MNs by the screen. In this paper only the cholinergic neurons identified in the screen have been examined. However, we also found genetic markers specific for populations of non-cholinergic interneurons, one of them is examined in paper IV.

Paper IV

The need for genetic markers specifically expressed in distinct neuronal populations has recently been put forward (Kiehn, 2006; Alvarez and Fyffe, 2007; Stepien and Arber, 2008; Goulding, 2009). In the screen for genes with ventral expression described in paper III, one of the genes displayed a very distinct expression restricted to the ventral rim in the lumbar spinal cord. The gene, *Chrna2*, translates into the alpha 2 subunit of the nicotinergic acetylcholine receptor. The restricted expression observed is in accordance with a previous study, which described a restricted expression in the

adult brain and ventral cervical spinal cord (Ishii et al., 2005). The expression was also observed to be restricted during embryonic development at E14.5, which is an important feature when utilizing the Cre-loxP system. It is well known that the cholinergic MNs have axon collaterals terminating on other MNs and on RCs. No overlap was observed between *Chrna2* and *Vacht* expression, which suggests that *Chrna2* is exclusively expressed by RCs in the spinal cord. A transgenic mouse with *Cre* expression under the control of the *Chrna2* promoter regions was generated with BAC recombineering techniques. Crosses to two different loxP-excision activated reporter mice were made to analyze the distribution of Cre activity. In the most ventral region of the spinal cord, *Chrna2-Cre* activated β-galactosidase (β-gal) expression was to a large extent localized in neurons with calbindin immunoreactivity and large gephyrin clusters. The almost complete overlap with RC markers in the area where the cells known to be situated support the idea that the *Chrna2-Cre* mouse can be used to target RCs. In addition to the identified RCs, β-gal was also observed in a few other cells, some of them were large neurons localized in the MN area. This raises the possibility that Cre is active in a subpopulation of MNs during development. However, further studies are necessary to explicitly identify the nature of these cells. Being aware of the limited additional non-RC Cre activity, the mouse can still be used to address role of RCs in motor control circuitry. For the purpose of live imaging, the Chrna2-Cre was bred to a tdTomato reporter mouse. In the isolated spinal cord of double mutant offspring, individual cells could be detected ventrally throughout the rostro-caudal levels of the spinal cord. The cells were detected both in an epifluorescence microscope and in a 2-photon laser scanning microscope system. This demonstrated that such a mouse could be used both for visually guided identification of the cells and that the population can be assessed in live imaging activity measurements.

Summary

In this thesis different functions are being related to neurons linked by common expression of a gene. In the case of VGLUT2 deficient mice, a large group of neurons were affected. Within this group are the neurons important for the respiratory circuitry in the brainstem. On the other hand, the study on spinal locomotor CPG in these mice, together with the expression patterns, suggested that none of the glutamatergic populations present have a critical role for propagation or coordination of the locomotor rhythm. In the other part of the thesis it is shown that the expression of a single gene can be connected to a subset of neuron with specific characteristics. It was for example found that fast MNs do express both the genes *Calca* and *Chodl*. In the case of the *Chrna2*, it seems likely that the gene product, the alpha2 subunit of the nicotinergic receptor, could be directly linked to the unique connection of MNs to RCs. However, there is no need to know the function of the gene when using the promoter sequence to drive the expression of a foreign gene in a transgenic animal.

Future perspectives

In this thesis, we demonstrate that a mouse without functional VGLUT2 protein can still produce a locomotor-like rhythm. We also present evidence that VGLUT1-expressing neurons are not likely to participate in the spinal locomotor CPG network. A living mouse with selective loss of VGLUT2 in the spinal cord neurons could extend our data that VGLUT2-mediated neuronal communication is not critical for the normal function of the CPG. A freely moving mouse would probably give insight to the role of the highly abundant VGLUT2-expressing neurons in the spinal cord. Such a model could be achieved by generating of a mouse with VGLUT2 removed from the spinal cord, but not from brainstem nuclei. To our knowledge, the Cre-expressing mouse necessary for this experiment does not yet exist, but might be available in the future.

In this thesis, a novel transgenic mouse with Cre activity largely restricted to RCs is presented. The visualization of the cells in living tissue makes it possible to study the activity of the population during fictive locomotion. Together with electrophysiological measurements and MN axons tracings the suggested pool specificity of the RCs (Nishimaru, 2006) can be further elucidated. Also, the Cre activity can be used to selectively ablate or silence the population. This could be achieved by crosses with a reporter mouse with conditional expression of diphtheria-toxin A subunit (DTA) or with mouse that conditionally ablate VIAAT [floxed *Viaat*, (Tong et al., 2008)]. Ultimately, the cells with *Chrna2-Cre* could be acutely silenced in the adult mouse for analysis the function of RCs when they are in their mature form. Such an experiment could be achieved by mating to the mouse with conditional activation of the allatostatin receptor and subsequent administration of allatostatin directly to the spinal cord (Gosgnach et al., 2006; Zhang et al., 2008).

We also want to characterize other potential participating neurons involved in the locomotor CPG networks. For this reason we are analyzing mouse lines carrying mutations in axon guidance molecules, which are giving a similar hopping phenotype as reported for ephrinB3 and EphA4 mutants.

The ability for the locomotor system to rapidly adapt and regain function after a complete spinal transection when combining several treatments was

recently described for rats (Courtine et al., 2009). This study further emphasize the need for better undestanding the spinal network for locomotion. Therapheutical treatments with stimulations electrodes connected to the nervous system are now beginning to be tested and is no longer something only seen in Science-Fiction movies.

Together, all our studies will expectantly bring us closer to an insight of how the neuronal circuit underlying locomotion operates. The belief is that the principles learned from the spinal neuronal network also can be applied to better understand the neuronal circuits of the brain.

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