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MOLECULAR CODING AND PHYSIOLOGICAL ROLES OF EXCITATORY NEURONS IN MOTOR CONTROL

Vanessa Ribeiro Caldeira

Karolinska Institutet

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Cover art by Mattias Karlen

Original idea from Peter Löw

This graphic work captures three of the main elements of this thesis. Our attempt at identifying the molecular basis of locomotion is represented by the DNA double helix road. The road signs symbolize locomotor commands and frequency, the cardinal representation of locomotor rhythm and the main point of inquiry in this thesis. And our animal model is represented in the center as the main protagonist of this work.

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"Nature shows us only the tail of the lion. But there is no doubt in my mind that the lion belongs with it even if he cannot reveal himself to the eye all at once because of his huge dimension."

Albert Einstein
From "The Curious History of Relativity", Jean Eisenstaedt, 2006

MOLECULAR CODING AND PHYSIOLOGICAL ROLES OF EXCITATORY NEURONS IN MOTOR CONTROL

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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ABSTRACT

Locomotion is a complex motor action that provides humans and other animals with the ability to move through the environment. In vertebrate locomotion, supraspinal centers convey the initiating or terminating command signals to the spinal cord which in turn generates the rhythm and pattern of muscle activities underlying locomotor activity.

In supraspinal centers, the intermingled configuration of neuronal populations has made it difficult to identify cell populations responsible for locomotor initiating and terminating signals with standard electrophysiological methods. These questions can be addressed, however, with the combinatorial use of mouse genetics to manipulate discrete groups of neurons and electrophysiological and behavioral studies to address their function in motor control.

Although a lot of progress has been made in deciphering the organization of the mammalian spinal locomotor networks through the use of early developmental markers, the present molecular classification of interneurons does not capture rhythm-generating neurons. It has become apparent that the interneuron composition of the spinal cord is quite complex and that the cardinal classes of interneurons are actually comprised of a highly diverse set of transcriptionally distinct neuronal types that cover diverse physiological functions. There is, therefore, a strong need for the identification of fine-grained molecular markers for spinal interneurons overall and glutamatergic spinal interneurons in particular since glutamatergic neurons are thought to be the drive for rhythmic motor output.

The work in this thesis addresses these questions and attempts at either ascribing functions to specific groups of neurons or providing a molecular database for future in-depth investigations.

In *Paper IV* of this thesis, we studied the roles of a subset of glutamatergic neurons in the supraspinal control of locomotion. We identified brainstem V2a neurons as a glutamatergic excitatory descending pathway that is involved in the arrest of ongoing locomotion. In *Paper I*, we investigated the mechanisms underlying the abnormal locomotor pattern observed in mice with a disrupted EphA4 signaling pathway. We linked the hopping-like locomotor phenotype observed in EphA4 signaling mutants to the aberrant crossing of spinal glutamatergic neurons. In *Paper II*, we investigated the

role of a subset of glutamatergic interneurons in rhythm generation in an attempt to elucidate the identity of rhythm-generating neurons. We showed that, although it is unlikely they are the sole rhythm-generating neurons, glutamatergic Hb9::Cre-derived interneurons contribute to rhythm generation in the mouse. In *Paper III*, we took it a step further and investigated the transcriptome profile of spinal glutamatergic neurons with the aim of identifying discrete molecular populations to which we can ascribe some of the locomotor functions that still remain elusive such as rhythm generation. Our findings provide a comprehensive overview of the transcription factors, ion channels and metabotropic receptors expressed in spinal glutamatergic neurons.

Overall, the work in each of the constituent papers of this thesis has broadened our understanding of glutamatergic neurons, their molecular and functional diversity and at the same time brought us a step closer to deciphering the functional organization of locomotor networks in the mouse.

LIST OF SCIENTIFIC PAPERS

I. Borgius L, Nishimaru H, **Caldeira V**, Kunugise Y, Löw P, Reig R, Itohara S, Iwasato T, Kiehn O. (2014) Spinal Glutamatergic Neurons Defined by EphA4 Signaling Are Essential Components of Normal Locomotor Circuits. *J Neurosci* 34(11):3841-3853.

II. **Caldeira V**, Dougherty KJ, Borgius L, Kiehn O. (2016) Spinal Hb9::Cre-derived Excitatory Interneurons Contribute to Rhythm Generation in the Mouse. *Scientific Reports* (*Manuscript under review*).

III. **Caldeira V**, Borgius L, Löw P, Kiehn O. Transcriptome Analysis of Spinal Excitatory Neurons. *Manuscript*.

IV. Bouvier J, Caggiano V, Leiras R, **Caldeira V**, Bellardita C, Balueva K, Fuchs A, Kiehn O. (2015) Descending Command Neurons in the Brainstem that Halt Locomotion. *Cell* 163(5):1191-1203.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (Serotonin)
cGi	Caudal gigantocellular reticular nucleus
ChAT	Choline AcetylTransferase
Chx10	Ceh-10 Homeodomain-Containing Homolog
CNs	Commissural Neurons
Cre	Cyclization recombinase
EphA4	Eph receptor A4
FACS	Fluorescence Activated Cell Sorting
FPKM	Fragments per kilobase of exon per million fragments mapped
GABA	Gamma-Aminobutyric acid
GFP	Green Fluorescent Protein
Hb9	Homeobox gene Hb9
INs	Interneurons
Isl1	LIM homeodomain transcription factor 1
Lhx3	LIM homeobox protein 3
Lhx9	LIM homeobox protein 9
Mc	Magnocellular reticular nucleus
MLR	Mesencephalic locomotor region
mRNA	Messenger Ribonucleic acid
NMDA	N-Methyl-D-aspartic acid
PnC	Caudal pontine reticular nucleus
rGi	Rostral gigantocellular reticular nucleus
Shox2	Short stature homeobox 2
Vglut2	Vesicular Glutamate Transporter 2
YFP	Yellow Fluorescent Protein

1 INTRODUCTION

Locomotion is the motor action that provides humans and other animals with the ability to move through the environment. Although complex it is an innate behavior that takes no learning to be executed and is already laid down in the nervous system before birth. In vertebrates, the command signal for locomotion is integrated in supraspinal centers, which convey the initiating or terminating signals to the spinal cord (Dubuc et al., 2008; Grillner and Georgopoulos, 1996; Mori, 1987; Roberts et al., 2008; Ryczko and Dubuc, 2013; Takakusaki et al., 2003), whereas the timing and sequence of muscle activities underlying locomotion originate from neuronal networks in the spinal cord (Goulding, 2009; Grillner, 2006).

A lot of effort has been placed into dissecting supraspinal commands for locomotion as well as elucidating the functional organization of spinal locomotor systems. In two relatively simple vertebrate nervous systems, the tadpole and the lamprey, the spinal locomotor networks controlling swimming are understood in great detail (Grillner, 2003; Roberts et al., 2008). From the use of cats as the prevailing experimental model throughout the 20th century some progress was also made regarding a functional understanding of spinal locomotor networks in mammals. A forward jump in understanding locomotor circuits in mammals came, however, from recent experiments using rodents, in particular the mouse, as an experimental model. This model system allowed the use of developmental genetics and the manipulation of molecularly defined groups of supraspinal and spinal neurons to address their function in motor control and locomotor activity, respectively (Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2006; McLean and Dougherty, 2015; Whelan, 2010).

1.1 LOCOMOTOR CONTROL

The mesencephalic locomotor region in the midbrain and neurons in the reticular formation in the lower brainstem are two of the neuronal structures directly involved in initiating locomotion in vertebrates (Armstrong, 1988; Georgopoulos and Grillner, 1989; Jordan et al., 2008; Ryczko and Dubuc, 2013).

The mesencephalic locomotor region serves as a control unit; it integrates locomotor commands from higher brain structures and receives inputs from the basal ganglia and hypothalamus (Ryczko and Dubuc, 2013). Increased activity in this region will gradually translate to an increase in locomotor speed.

Many neurons in the mesencephalic locomotor region are excitatory and release both glutamate and acetylcholine as neurotransmitters (Ryczko and Dubuc, 2013). They are thought to project to and activate neurons in the reticular formation in the brainstem either directly or indirectly via cholinergic neurons in the brainstem, thereby providing the final command signal to initiate locomotion (Dubuc et al., 2008).

Although the exact identity of lower brainstem reticular formation neurons is not fully understood, there seem to be two systems involved in the initiation signal, a glutamatergic locomotor pathway in all vertebrates and a serotonergic locomotor pathway in mammals (Jordan et al., 2008). In addition, it has also been suggested that the sustained descending activity of lower brainstem reticular formation neurons may determine the duration of locomotor episodes (Deliagina et al., 2000; Drew et al., 1986).

However, besides the initiation and maintenance signals, there have been indications that lower brainstem reticular formation neurons may be implicated in a dedicated stop command that allows for a timed locomotor arrest according to behavioral needs. In the Xenopus tadpole, GABAergic descending pathways immediately terminate swimming upon head contact with obstacles (Perrins et al., 2002). Similarly in the cat, electrical stimulation of the rostral medullary and/or caudal pontine reticular formations may lead to general motor inhibition (Mori, 1987; Takakusaki et al., 2003).

Excitatory and inhibitory brainstem descending neurons are intermingled in the reticular formation and mesencephalic locomotor region (Esposito et al., 2014; Holstege, 1991; Ryczko and Dubuc, 2013). Therefore, the classical methods of electrical stimulation or pharmacological inactivation of specific areas in the brainstem cannot distinguish between different populations of cells, thereby making it difficult to identify specific cell populations involved in locomotor initiation or termination signals. However, implementation of developmental genetics methods in combination with selective manipulation of discrete molecular defined groups of reticular neurons may increase the fidelity and precision of the intervention. Recently, a group of neurons in the zebrafish caudal-most brainstem defined by the expression of the transcription factor Chx10, the brainstem V2a neurons, was shown to project to the spinal cord and participate in the initiation and maintenance of locomotion (Kimura et al., 2013). In the mouse, brainstem V2a neurons were shown to project to the spinal cord (Bretzner and Brownstone, 2013; Cepeda-Nieto et al., 2005), but no

direct link has been described between their activation and locomotor initiation, maintenance or termination. In *Paper IV* we addressed this question and investigated the role of brainstem V2a neurons in the control of locomotion. This study led to a new and surprising role for brainstem V2a neurons; it demonstrated that brainstem V2a neurons are involved in the control of the episodic nature of locomotion, in that they provide a behaviorally relevant stop signal.

1.2 THE GENERAL ORGANIZATION OF LOCOMOTOR NETWORKS IN THE MAMMALIAN SPINAL CORD

Neuronal networks in the spinal cord are responsible for some of the key features that characterize limbed locomotion in mammals, namely rhythm generation and pattern formation. Neuronal networks responsible for walking are distributed rostro-caudally along the ventral, lower thoracic (Th12-Th13) and lumbar (L1-L6), spinal cord (Kiehn, 2006; Kjaerulff and Kiehn, 1996).

Rhythm-generating neurons are supposed to drive other neurons in the network into rhythmicity and provide either direct or indirect rhythmic excitation to motor neurons. Initially, lesion and pharmacological studies suggested that these neurons are ipsilaterally projecting and excitatory (Kiehn et al., 2008). Later, optogenetic activation an inactivation experiments showed that excitatory spinal neurons are both necessary and sufficient for rhythm generation (Hagglund et al., 2010; Hagglund et al., 2013; Kiehn, 2006; Kiehn et al., 2008).

Pattern formation in mammals relies on the precise and coordinated temporal activation of flexor and extensor muscles across the same or different joints in a limb or between limbs as well as left-right coordination.

The flexor and extensor components of locomotor networks seem to be reciprocally connected via ipsilaterally projecting inhibitory interneurons and are thought to be directly driven by distinct populations of rhythm-generating neurons (Endo and Kiehn, 2008). Although the nature of these inhibitory network components is only partially known, there is strong evidence that reciprocally connected inhibitory Ia-interneurons (Hultborn et al., 1976; Hultborn and Lundberg, 1972) are essential for the coordination of their activity (Britz et al., 2015; Deliagina and Orlovsky, 1980; Geertsen et al., 2011; Pratt and Jordan, 1987; Talpalar et al., 2011; Zhang et al., 2014). However, it is unlikely that Ia interneurons are the sole contributors to flexor-extensor alternation.

Left-right alternation is dependent on the action of either inhibitory commissural neurons acting on contralateral motor neurons or excitatory commissural neurons acting on contralateral premotor inhibitory neurons (Jankowska, 2008; Kiehn, 2006). This dual inhibitory system may be driven either directly by rhythm-generating neurons or indirectly by other excitatory neurons in the spinal cord, and is thought to be involved in the regulation of alternation at different speeds of locomotion (Talpalar et al., 2013).

1.3 MOLECULAR NEUROBIOLOGY OF LOCOMOTOR NETWORKS IN THE MOUSE SPINAL CORD

The combination of mouse genetics and electrophysiological studies allowed designated populations of neurons to be assigned to key network functions in the mammalian spinal cord and has thereby paved the way to our increasing understanding of the spinal locomotor circuitry (Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2006, 2011, 2016a; McLean and Dougherty, 2015; Stepien and Arber, 2008; Whelan, 2010). However, essential elements of the locomotor network structure still need to be determined.

Pattern generation. By relying on the classification of spinal interneurons into 11 cardinal groups _ventral, V0-V3, and dorsal, dI1-dI6, domains_ (Goulding and Pfaff, 2005; Jessell, 2000) in order to identify and selective manipulate subsets of these interneurons, we have been able to elucidate their role in the spinal locomotor networks. Through ablation and loss-of-function studies of these neuronal subclasses, the neuronal circuits underlying left-right (Figure 1A) (Crone et al., 2008; Jankowska, 2008; Kiehn, 2011; Quinlan and Kiehn, 2007; Talpalar et al., 2013) and flexor-extensor (Figure 1B) (Britz et al., 2015; Talpalar et al., 2011; Zhang et al., 2014) coordination in mammals have been determined in great detail.

Rhythm generation. However, although optogenetic, pharmacological and lesion studies have provided strong evidence that the rhythmic drive in the locomotor circuit comes from activity in ipsilaterally projecting excitatory glutamatergic neurons in the mouse spinal cord (Hagglund et al., 2010; Hagglund et al., 2013; Kiehn, 2006; Kiehn et al., 2008; Kjaerulff and Kiehn, 1996), the identity of rhythm-generating neurons still remains elusive. Ablation of entire classes of ipsilaterally projecting excitatory interneurons, V2a-INs, V3-INs and dI3 Isl1-INs, had no effect on rhythm generation (Bui et al., 2013; Crone et al., 2008; Kiehn et al., 2010; Zhang et al., 2008).

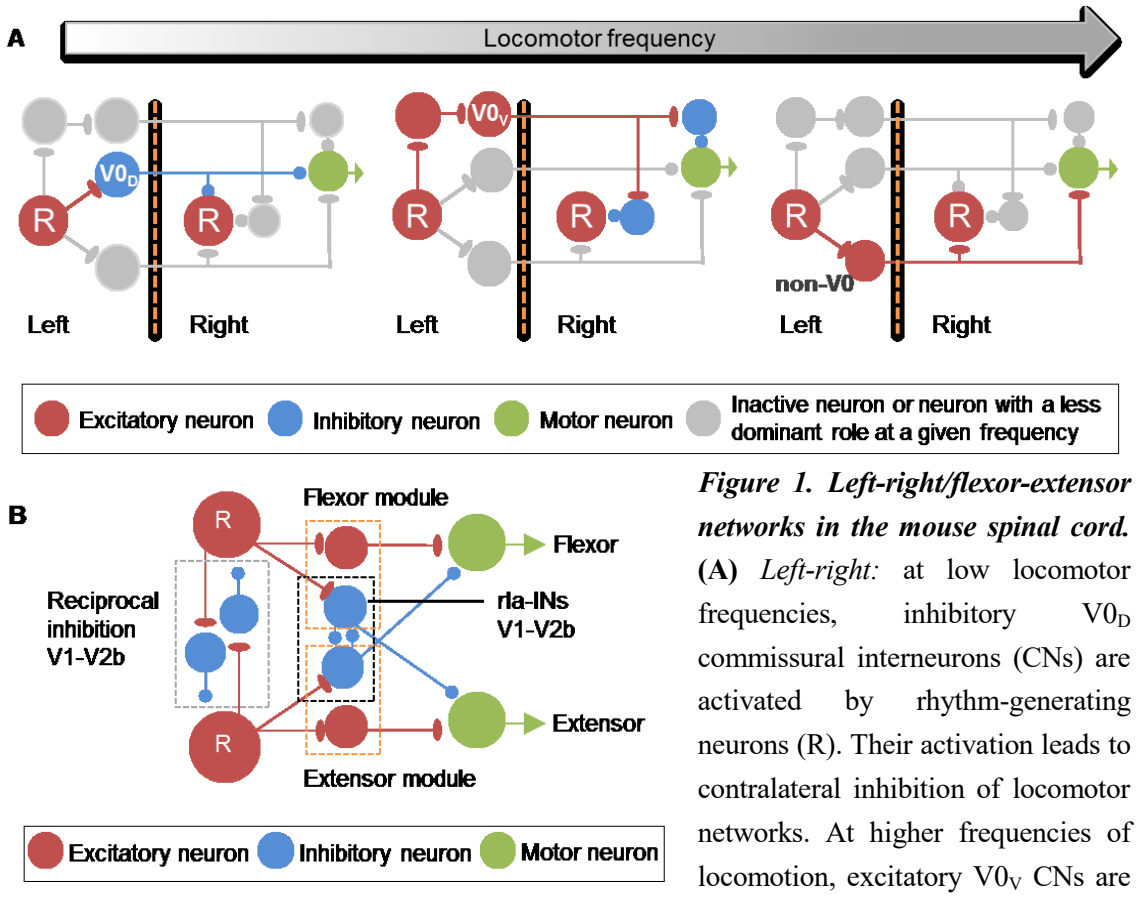


Figure 1. Left-right/flexor-extensor networks in the mouse spinal cord. **(A)** *Left-right:* at low locomotor frequencies, inhibitory $V0_D$ commissural interneurons (CNs) are activated by rhythm-generating neurons (R). Their activation leads to contralateral inhibition of locomotor networks. At higher frequencies of locomotion, excitatory $V0_V$ CNs are recruited. Their activation causes contralateral inhibition of locomotor networks via local inhibitory neurons (blue). At very high frequencies of locomotion left-right synchrony is secured by excitatory non-V0 neurons (red). **(B)** *Flexor-extensor:* a module comprising excitatory neurons and rIa-INs (dashed boxes) receives input from excitatory rhythm-generating circuits and provides rhythmic excitation and inhibition to flexor and extensor antagonist, respectively. Inhibitory neurons belonging to the V1 and V2b classes of neurons (blue box) provide reciprocal inhibition between flexor and extensor rhythm generators. rIa-Ins also belong to the V1 and V2b classes. A single neuron in the diagrams represents a group of neurons. Adapted from (Kiehn, 2016b).

The only instance in which rhythm generation was partially affected was when a subset of Shox2 interneurons were functionally inactivated (Dougherty et al., 2013). The transcription factor Shox2 was identified in a genetic screening of spinal interneurons and although its expression defines a group of glutamatergic interneurons in the ventral and intermediate spinal cord, Shox2 interneurons do not sprout from one single cardinal class of interneurons, instead, they span several of the dorsal and ventral cardinal classes (Dougherty et al., 2013).

It has become apparent that the interneuron composition of the spinal cord is quite complex and that the cardinal classes of interneurons are actually comprised of a highly diverse set of transcriptionally distinct neuronal types that cover diverse physiological functions (Kiehn, 2016b).

Search for rhythm-generating circuits: a closer look at spinal glutamatergic neurons. In order to identify rhythm-generating neurons as well as increase our understanding of the constituent elements of spinal locomotor networks, we adopted different approaches; we investigated the mechanisms behind an aberrant locomotor phenotype, the roles of a specific group of neurons in locomotion, and the transcriptome profile of glutamatergic neurons in the spinal cord. These approaches are outlined in *Papers I-III* of this thesis work.

In *Paper I* we studied the mechanism behind the aberrant locomotor phenotype observed in EphA4 mutants. Previous studies have shown that EphA4 signaling is essential for the spatiotemporal organization of neuronal circuits (Beg et al., 2007; Coonan et al., 2001; Dottori et al., 1998; Kullander et al., 2003; Restrepo et al., 2011). In the absence of this signaling pathway, axons from both brain and spinal neurons display aberrant midline crossing which is accompanied by a pronounced change in motor behavior; alternating gaits are replaced by a rabbit-like hopping gait. EphA4 is, however, broadly expressed in both excitatory an inhibitory neurons in the spinal cord and supraspinal areas. Taking advantage of mouse genetics, we could attribute this gait switch to the loss of EphA4 signaling in excitatory neurons in the spinal cord proper. Our findings suggest that excitatory EphA4-expressing neurons are not only essential components of the mammalian locomotor network but also possibly involved in rhythm generation. However, because both EphA4-expressing neurons and glutamatergic neurons are very heterogeneous populations, we were not able to further pinpoint the identity of these cells.

In *Paper II* we sought to elucidate the identity of rhythm-generating neurons by investigating whether glutamatergic Hb9::Cre-derived interneurons are involved in rhythm generation in the mouse spinal cord. Glutamatergic Hb9::Cre-derived interneurons include the canonical Hb9 interneurons. Canonical Hb9 interneurons were identified by Ziskind Conhaim's and Rob Brownstone's groups in the early 2000's (Hinckley et al., 2005; Wilson et al., 2005). They are located in the ventral spinal cord but only in segments Th12 to L3, and became of great interest because of their rhythmogenic properties (Ziskind-Conhaim et al., 2010). However, because their firing pattern did not fit with that of a rhythm-generating role, doubt was raised about their role as candidate rhythm-generating neurons (Kwan et al., 2009). Using a specific genetic approach to silence synaptic transmission in glutamatergic Hb9::Cre-derived interneurons, we verified that the functional removal of glutamatergic Hb9::Cre-derived interneurons from the network led to a significant decrease in

locomotor frequency without any effect on pattern formation. These data suggest that glutamatergic Hb9::Cre-derived interneurons might be involved in rhythm generation. However, since canonical Hb9 interneurons account for a very negligible part of the Hb9::Cre-derived population, it is unlikely that they alone are responsible for the observed decrease in locomotor frequency.

Lastly, we decided to take a closer look at the transcriptional character of interneurons in the mouse postnatal spinal cord. In *Paper III* we investigated the molecular identity of glutamatergic neurons under the premise that by determining the transcriptional profile of glutamatergic spinal interneurons, we might be able to fragment them into discrete molecular and possibly functional subtypes and thereby dissect locomotor functions that remain elusive, such as rhythm generation, as well as assign groups of glutamatergic neurons to other motor functions. For this, we resorted to RNA-sequencing (RNA-seq) to investigate the transcriptome profile of spinal excitatory cells. RNA-seq has the crucial advantage of allowing the investigation of the complete set of transcripts in a cell at a specific developmental stage with very low background signal, because sequences can be unambiguously mapped to unique regions of the genome. Additionally, it has a large dynamic range of expression levels over which transcripts can be detected. Through the analyses carried out in this paper, we provided a comprehensive account of the transcription factors expressed in glutamatergic spinal neurons as well as transcription factors enriched in glutamatergic subgroups in the mouse spinal cord. Additionally, we also provided a detailed account of ion channels and metabotropic receptors expressed in spinal glutamatergic neurons.

2 AIMS

I. TO IDENTIFY THE MOLECULAR IDENTITY AND THE MECHANISMS BEHIND THE HOPPING-LIKE LOCOMOTOR PHENOTYPE OBSERVED IN EPHA4 SIGNALING MUTANTS

II. TO INVESTIGATE WHETHER GLUTAMATERGIC HB9::CRE-DERIVED INTERNEURONS ARE INVOLVED IN RHYTHM GENERATION

III. TO PROVIDE A COMPREHENSIVE TRANSCRIPTOME PROFILE OF GLUTAMATERGIC NEURONS IN THE POSTNATAL MOUSE SPINAL CORD

IV. TO INVESTIGATE THE ROLE OF BRAINSTEM V2A NEURONS IN THE CONTROL OF LOCOMOTION

3 METHODOLOGICAL CONSIDERATIONS

All methods and materials pertinent to the work included in this thesis were detailed in their respective articles (*Papers I-IV*). In this section we will therefore confine ourselves to discussing some of the methodological choices and constraints that underlie the work presented in this thesis.

3.1 RNA-SEQUENCING

The premise of the work carried out in *Paper III* is that a comprehensive molecular profile of glutamatergic neurons in the postnal spinal cord might fragment this heterogeneous glutamatergic population into discrete molecular and possibly functional subtypes, and thereby help elucidate the identity of rhythm-generating neurons in particular and the wiring of spinal locomotor networks as a whole.

We focused our studies on P3 spinal cords because, while our ultimate goal was to identify post-natal markers for glutamatergic spinal interneurons, we were also interested in a time point in which the expression of some of the hallmark developmentally regulated transcription factors was not extinct (Benito-Gonzalez and Alvarez, 2012).

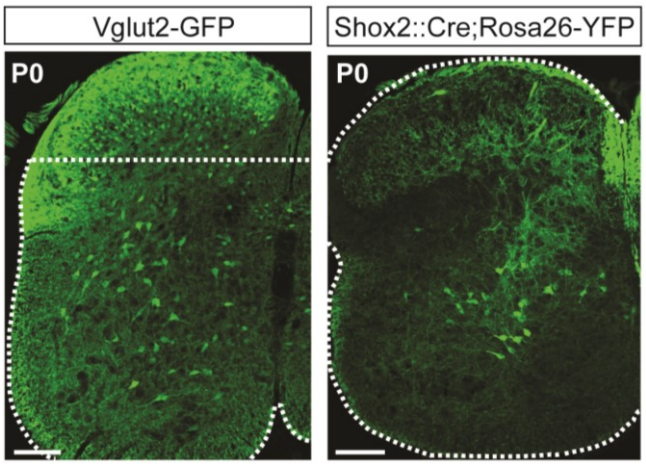


Figure 2. Spatial distribution of FACS collected cells in the mouse lumbar spinal cord. Distribution of GFP^+/YFP^+ cells (green) in the Vglut2-GFP (left) and Shox2::Cre;Rosa26-YFP (right) lumbar spinal cords, respectively. Dashed lines encircle the area from which GFP^+ neurons were isolated: lamina IV-X. Scale bar: 100 µm.

In order to isolate our populations of interest (glutamatergic cells, $Vglut2^+$ and $Shox2^+$), we used fluorescence activated cell sorting (FACS) to collect GFP or YFP labelled cells from Vglut2-GFP and Shox2::Cre; Rosa26-YFP spinal cords, respectively. Since the core of the spinal locomotor network is located in the ventral spinal cord (Kiehn, 2006; Kjaerulff and Kiehn, 1996), we only collected GFP^+ cells from lamina IV to X in the Vglut2-GFP spinal cord (Figure 2, left). For this, we surgically cut away the dorsal horn along the length of the lumbar spinal cord (L1-L6). Great care was taken to make reproducible cuts between spinal cords using external landmarks to reduce

the experimental variability of tissue sampling between cords. For Shox2 spinal cords the entire lumbar spinal cord was used since there are no dorsally located Shox2^{+} cells (Figure 2, right). To avoid RNA degradation, surgery was performed as fast as possible and followed by a time optimized preparation of the tissue for FACS.

We had to optimize our FACS settings in order to ensure we captured representative samples of our cells of interest with minimal contamination of false positives. To that end, we ran littermate negative controls prior to every round of FACS in order to set collection gates that excluded negative cells and increased sort purity, which is defined by the percentage of cells harvested that are target cells (Figure 3). On average, we achieved 97% purity for GFP^{-} cells and 83% purity for GFP^{+} cells.

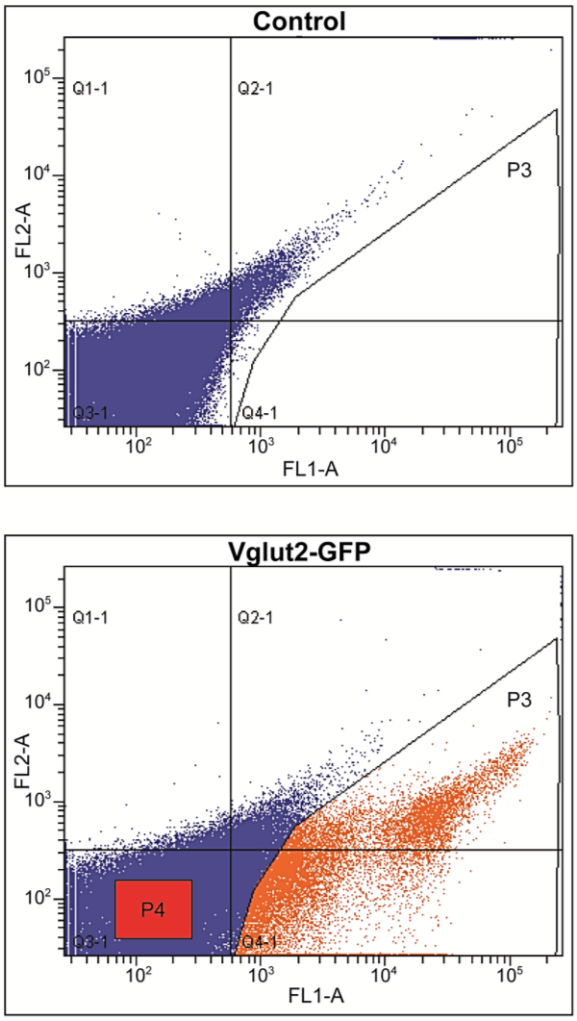


Figure 3. FACS: gate setting. Although only one fluorophore was present (GFP), we used fluorescence channels 1 (FL1, GFP) and 2 (FL2, GFP spillover for which we compensate) to enhance visualization. (**TOP**) FL1/FL2 dot plot showing wild-type cells (blue, no fluorophore) collected from control spinal cords. Prior to every FACS run, spinal cord cells from *Vglut2-GFP* negative littermate controls were used to set the collection gate P3. (**BOTTOM**) FL1/FL2 dot plot indicating the collection gate for GFP^{+} cells (P3, orange) as well as the collection gate for GFP^{-} cells (P4, red). Both GFP^{+} and GFP^{-} cells were collected from Vglut2-GFP spinal cords; these cells were reanalyzed to assess purity of sorted fractions.

To investigate the transcriptome of these FACS collected cells, we performed RNA-sequencing and had to optimize the process of cDNA synthesis and amplification. To that end, we used the SMARTer^{TM} PCR cDNA Synthesis Kit (Clontech) while carefully controlling for RNA purity and integrity. RNA yield was measured on a Qubit$^{®}$ Fluorometer (Qubit$^{®}$ 2.0, Invitrogen) and quality assessment was carried out

on Agilent's 2200 TapeStation System (Agilent Technologies). RNA samples with RNA integrity number equivalent (RIN^e) ≥ 8 were used for cDNA synthesis.

We used normalized mean raw read counts to identify differentially expressed genes with DESEq2 (Love et al., 2014). DESEq2 is a software package that runs in the R platform. It estimates variance-mean dependence in count data from high-throughput sequencing assays and tests for differential expression using negative binomial distribution.

We did not use any read count cut offs because we do not have the necessary parameters to determine how low a read count needs to be for it to be considered biologically irrelevant. Instead, we adopted the default cut off method implemented by DESEq2, which meant the read counts ≤ 3 were excluded. We did implement, however, a fold-change cut off ($\log_2FC \geq 0.59$ or $FC \geq 1.5$) in order to narrow down the number of differentially expressed genes while focusing on transcripts with the largest differences in expression levels.

Additionally, since this study identified a large number of differentially expressed genes, rather than tackle genes whose functions are less obvious from a neuronal point of view, we resorted to network analysis based on knowledge derived from scientific literature to identify biologically relevant molecules in the mouse nervous system amongst the differentially expressed transcripts. We performed Ingenuity Pathway Analysis (IPA) and extracted analysis-ready molecules from Ingenuity networks, under the assumption that highly-interconnected cellular networks are likely to represent significant biological functions.

Regarding transcript expression levels, instead of using fpkm (fragments per kilobase of exon per million fragments mapped) values we decided to use mean normalized read counts to rank transcripts' expression in four categories: very high, high, medium or low. We did so because we believe that working with raw data reduces biases. Although it is pretty difficult to determine the biological implications of a given read count value, we reckon that it might serve as a reference guideline for expression analysis studies.

3.2 TRANSGENIC LINES LIMITATIONS

Because transgenic mice can have specific recombination patterns that don't necessarily mimic wild-type gene expression, knowledge of transgene expression

and/or recombination patterns in our areas of interest is necessary for the correct interpretation of experimental data as well as choice of controls and experimental design.

When looking to determine whether the hopping phenotype observed upon inactivation of the EphA4 signaling was due to spinal intrinsic network defects, *Paper I*, we resorted to the *Hoxb8::Cre* mouse since the *Hoxb8* gene is exclusively expressed in the spinal cord. However, although transgene expression in *Hoxb8::Cre* mice is observed throughout the thoracic, lumbar and sacral segments, it only commences at cervical segment 4 (Figure 4). Given this expression pattern, it is unlikely that forelimb locomotor networks are fully captured by transgene recombination in *Hoxb8::Cre* mice. Therefore, it is not surprising that while full *EphA4 KO* mice display quadrupedal hopping at all frequencies of locomotion, *Hoxb8::Cre; EphA4*$^{\Delta/Flox}$ mice display a hindlimb hopping gait instead.

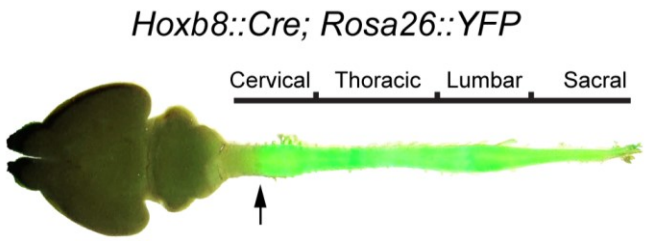


Figure 4. Hoxb8 expression detected with YFP in Hoxb8::Cre;Rosa26-YFP mice. Recombination is restricted to the spinal cord with rostro-caudal limit at the 4th cervical root (C4), indicated by an arrow.

Although 98.7% of the GFP^{+} cells in the *Vglut2-GFP* mouse are glutamatergic (N = 3, 18 sections, *Vglut2* mRNA *in situ* hybridization quantified in lamina VII-X) (unpublished data), transgene expression in these mice does not seem to capture all glutamatergic cells in the spinal cord. When comparing spinal cords from *Vglut2-GFP* mice to those of the well characterized *Vglut2::Cre* mouse line (Borgius et al., 2010), this becomes more apparent (Figure 5A). Although 96% of the GFP^{+} cells also co-express Cre protein in Vglut2-GFP; Vglut2::Cre spinal cords (Figure 5A'), around 20% of the Cre^{+} cells are not GFP^{+} (Figure 5A"). In *Paper III*, we used GFP expression in the *Vglut2-GFP* mouse to isolate glutamatergic from non-glutamatergic neurons. Therefore, we had to implement an analysis method that corrected for glutamatergic cell contamination within the GFP^{-} population.

Similarly, in *Paper II* we were faced with gene expression patterns that were mouse-line specific. Although *Hb9::Cre* (Yang et al., 2001) and *Hb9-GFP* (Wichterle et al., 2002) mice were generated with an analogous strategy to the *Hb9-LacZ* mice (Arber et al., 1999), reporter expression in *Hb9-LacZ* mice is restricted to motor neurons

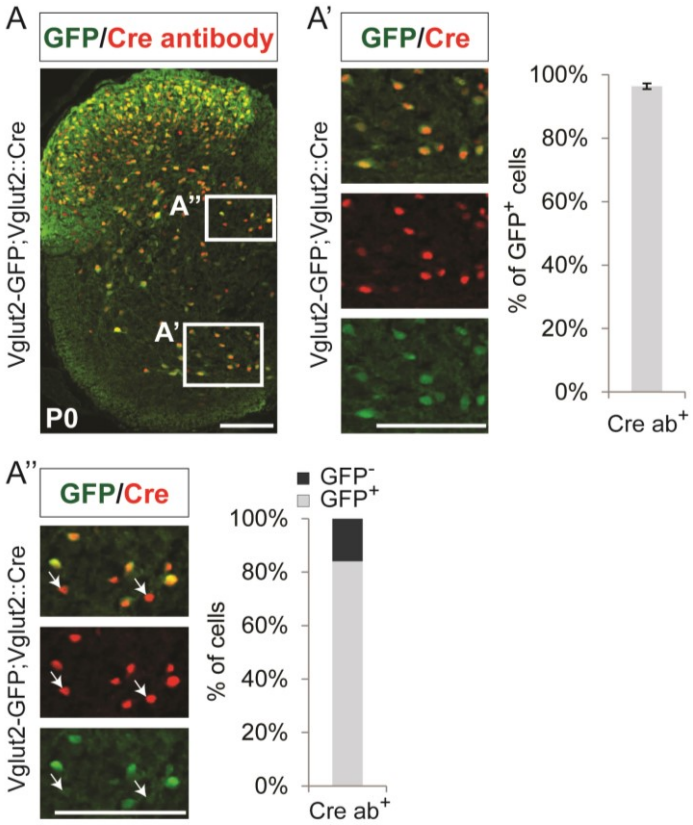


Figure 5. Transgene Specificity in Vglut2-GFP mice assessed by crosses with the well characterized Vglut2:: Cre mouse. (**A**) Co-expression of GFP (green) and Cre antibody (red, Cre ab) in the *Vglut2-GFP; Vglut2::Cre* mouse spinal cord at P0. **(A' and A")** Magnifications of white boxed areas as well as bar graphs showing the percent of GFP^+ cells that are Cre ab^+ **(A')**, and the percent of Cre ab^+ cells that are $GFP^{+/-}$ **(A")** in *Vglut2-GFP; Vglut2::Cre* mice. Arrowheads indicate lack of overlap. Scale bars: 100 µm.

whereas in the *Hb9::Cre;Rosa26-FP* and *Hb9-GFP* mouse lines a ventral population of interneurons, which includes the canonical Hb9 interneurons, is also marked (Figure 6A and B). Additionally, a dorsal population of cells is also captured in the *Hb9::Cre;Rosa26-FP* mouse line (Figure 6A). Therefore, we had to adjust our experimental design accordingly; we used conditional genetics to functionally remove our cells of interest from the network.

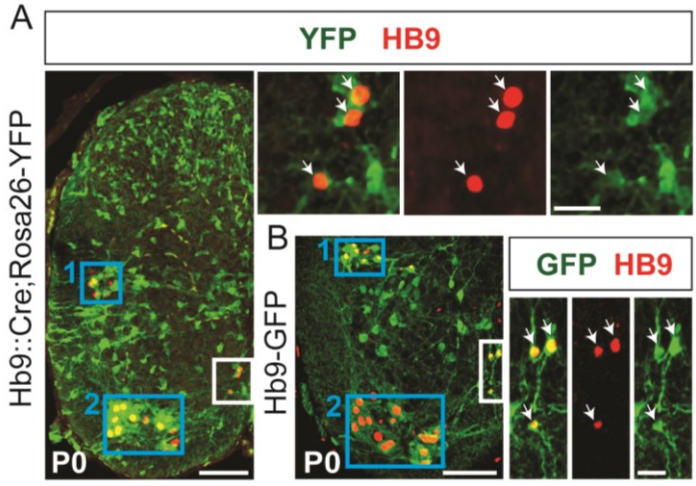


Figure 6. Transgene expression in Hb9::Cre and Hb9-GFP mice. (**A and B**) Distribution of Hb9::Cre-derived INs (green), as marked by YFP expression in *Hb9::Cre;Rosa26-YFP* mice (**A**), and distribution of GFP neurons (green) in the ventral spinal cord of *Hb9-GFP* mice (**B**). Canonical Hb9 INs, as marked by HB9 protein expression (red) in medial lamina VIII, are highlighted in the white boxed area (**A and B**). Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between YFP/GFP (green) and canonical Hb9 INs (Hb9 antibody, red). Preganglionic neurons (blue box 1) and motor neurons (blue box 2) also express HB9 protein (**A and B**). Scale bars: 100 µm and 25 µm.

3.3 THE MOUSE MODEL: *IN VITRO* VERSUS *IN VIVO*

The mouse has been extensively used as an experimental model not only because its behavior can be studied *in vivo* in several locomotor paradigms, but also because the nervous system from newborn mice can be isolated and studied *in vitro* (Figure 7) (Bonnot et al., 2002; Cazalets et al., 2000; Clarac et al., 2004; Goulding, 2009; Kiehn et al., 2000; Kiehn and Kullander, 2004; Kiehn et al., 2008; Nishimaru and Kudo, 2000). The rhythmic pattern of activity induced in the *in vitro* preparation closely resembles the pattern of locomotor activity in the intact adult animal (Kiehn et al., 1996), which shows that the network controlling locomotion is already in place in the spinal cord at birth. Moreover, isolated brainstem-spinal cord preparations, which preserve the immediate essential supraspinal control areas, are also possible with this animal model.

While in some instances we can ascribe functions to specific populations of neurons based on the combinatorial use of mouse genetics and adequate behavioral studies, we many times need to resort to an *in vitro* investigation in order to dissect the mechanism behind a behavioral output. This can be more clearly appreciated in the work described in *Paper I* and *Paper IV*.

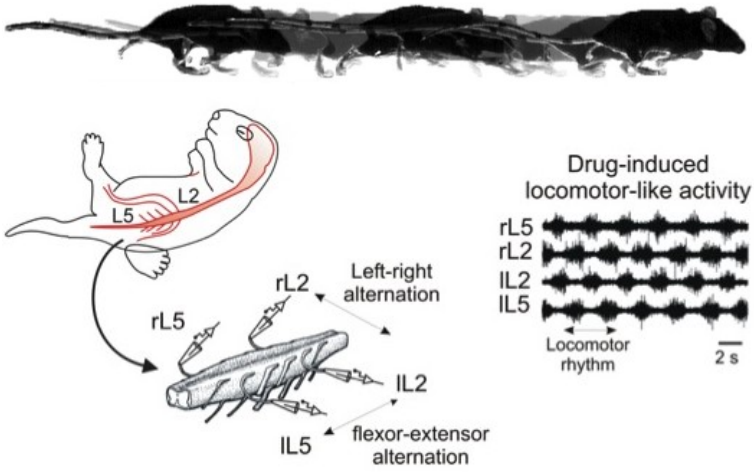


Figure 7. High-speed freely moving mouse silhouette and schematic of rodent in vitro spinal cord preparation.

The behavioral studies on *Emx1::Cre* and *Hoxb8::Cre ; EphA4* mutant mice, carried out in *Paper I*, demonstrated that the hopping-like locomotor phenotype observed in EphA4 signaling mutant mice was due to network defects in spinal neurons. Similarly, this hopping behavior was ascribed to glutamatergic neurons by studying over-ground locomotion in *Vglut2::Cre; EphA4* mutant mice. However, to confirm the spinal origin of these excitatory neurons and determine the actual mechanism behind this phenotype, it was necessary to investigate the isolated spinal cord. Locomotor-like activity in the *Vglut2::Cre; EphA4* mutant spinal cord reproduced the locomotor phenotype observed *in vivo*, thereby confirming the observed phenotype was due to

spinal intrinsic mechanisms. Retrograde labeling of lumbar spinal interneurons showed that glutamatergic neurons aberrantly crossed through the ventral commissure in EphA4 signaling mutant mice. Additionally, *in vitro* locomotor experiments also demonstrated that aberrant crossing of axons in the ventral commissure is sufficient to elicit a hopping phenotype.

In *Paper IV*, optogenetic activation of brainstem V2a neurons in combination with behavioral studies showed that the main functional phenotype of brainstem V2a neurons is the arrest of locomotion in freely moving mice. Furthermore, *in vivo* kinematic analysis revealed that brainstem V2a neuron-mediated locomotor arrest is different from that of a defensive freezing behavior (Brandao et al., 2008; Yilmaz and Meister, 2013). However, in order to pinpoint which brainstem region triggered the stop, or to determine if the stop signal was integrated in the spinal cord or if it was due to V2a collaterals recruiting inhibitory descending pathways in the brainstem, or to determine where V2a descending axons terminate in the spinal cord, or even to investigate if V2a activation affects the rhythm layers or the pattern layers of spinal locomotor networks, we had to perform studies in the isolated spinal cord. These questions could not be even remotely addressed with *in vivo* experiments because, even with high genetic specificity and positional precision, there would be confounding factors due to the extensive interconnectivity in the central nervous system as well as the interactions between different behavioral states in which motor output is just the measure of integrated brain activity.

Therefore, because we are not able to fully understand and/or dissect a behavior without investigating its underpinning mechanisms, the combination of *in vivo* and *vitro* studies is essential.

4 RESULTS AND DISCUSSION

Glutamatergic neurons are thought to be the drive for rhythmic motor output (Hagglund et al., 2010; Hagglund et al., 2013; Kiehn, 2006; Kiehn et al., 2008). In this thesis work, we took a closer look at glutamatergic neurons and not only explored their many roles on motor function but also investigated the molecular makeup of this very heterogeneous population. Throughout the work in this thesis we have: 1) identified the miswiring of spinal glutamatergic neurons as a possible mechanism behind the hopping-like locomotor phenotype observed in EphA4 signaling mutants; 2) identified and described a group of neurons in the mouse spinal cord, the glutamatergic Hb9::Cre-derived interneurons, which might be involved in rhythm generation; 3) provided a comprehensive list of transcription factors, channels and receptors expressed in glutamatergic neurons in the mouse postnatal spinal cord; and 4) identified brainstem V2a neurons as a glutamatergic descending pathway that is involved in the arrest and/or modulation of ongoing locomotion.

Although seemingly unrelated topics if not for the glutamatergic nature of the cells investigated, the work in each of the constituent papers of this thesis has brought us a little closer to understanding the molecular nature of rhythm-generating neurons and/or the mechanisms involved in rhythm generation.

4.1 *PAPER I:* SPINAL GLUTAMATERGIC NEURONS DEFINED BY EPHA4 SIGNALING ARE ESSENTIAL COMPONENTS OF NORMAL LOCOMOTOR CIRCUITS

In this study, we demonstrate that the hopping-like phenotype in EphA4 signalling mutant mice is due to aberrant crossing of spinal neurons. Additionally, our findings indicate that this hopping-like locomotor phenotype might be due to the aberrant midline crossing of spinal glutamatergic neurons in the ventral commissure. Collectively, our data suggest that EphA4 signalling in excitatory spinal neurons is important for the correct wiring of locomotor circuitries.

4.1.1 The hopping phenotype is generated by changes in the spinal circuitry

Inactivation of the EphA4 signaling (*EphA4* KOs, *EphrinB3* KOs, or α-*Chn* KOs) leads to axon guidance defects with an increased overcrossing of both supraspinal and spinal neurons, and results in a hopping-like locomotor phenotype (Beg et al., 2007; Coonan et al., 2001; Dottori et al., 1998; Kullander et al., 2003; Restrepo et al., 2011). One hypothesis regarding the mechanism behind the hopping phenotype in EphA4 signaling mutants is that bilateral innervation of the spinal cord due to aberrantly

crossing corticospinal tract (CST) axons might lead to left-right synchrony (Asante et al., 2010).

To verify if aberrant crossing of CST axons was sufficient to elicit a hopping phenotype, we selectively inactivated EphA4 signaling in *Emx1*-expressing neurons in the *Emx1::Cre; EphA4*$^{\Delta/Flox}$ mouse. *Emx1* is a marker for pyramidal neurons of the cerebral cortex (Chan et al., 2001) (Figure 8A). We observed that although there was an increase in midline re-crossing of CST axons both in the cervical enlargement and in the lumbar spinal cord in *Emx1::Cre; EphA4*$^{\Delta/Flox}$ mice (Figure 8B-E), when running freely on a runway *Emx1::Cre; EphA4*$^{\Delta/Flox}$ mice displayed an alternating gait (Figure 8G) similar to that of wild-type mice.

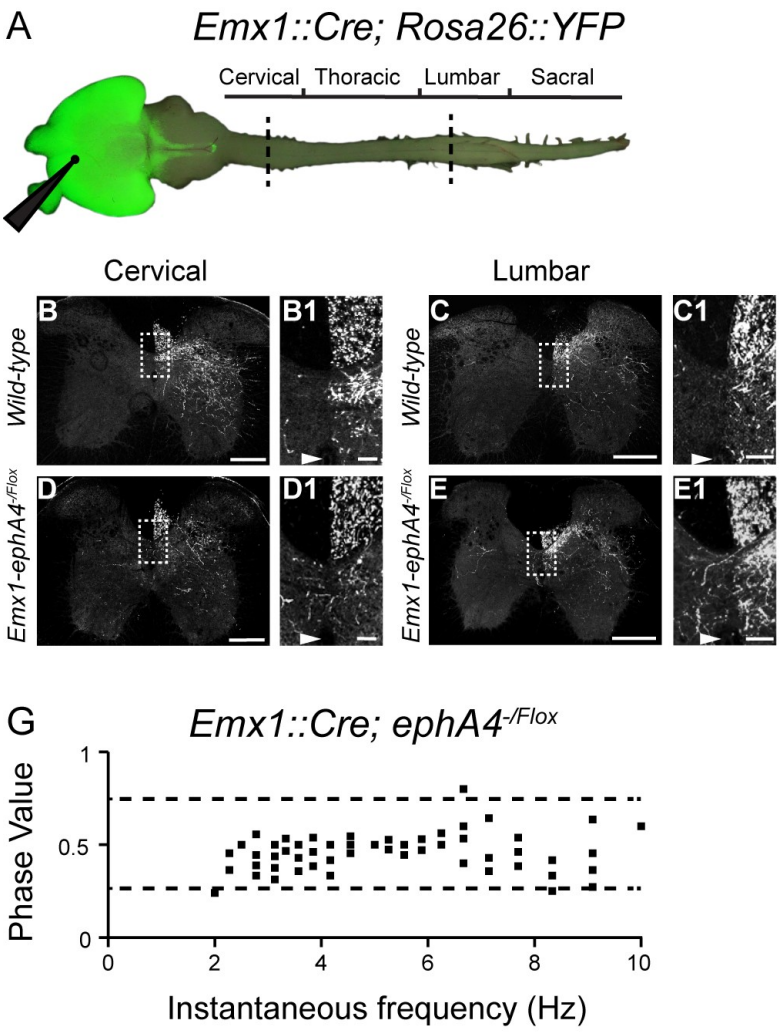


Figure 8. Normal over-ground locomotor pattern in Emx1::Cre; EphA4$^{\Delta/Flox}$ mice. **(A)** *Emx1* expression visualized by YFP in the *Emx1::Cre; Rosa26-YFP* mouse. The black arrowhead indicates site of injection of biotinylated dextran amine (BDA). Dashed lines indicate transverse section sites in **B-E**. **(B-E)** Transverse spinal cords sections of wild-type and *Emx1::Cre;EphA4*$^{\Delta/Flox}$ mice showing anterograde labeled CST axons in the cervical **(B, D)** and lumbar enlargements **(C, E). (B1-E1)** Magnification of white boxed areas in **B-E**. White arrowheads indicate the central canal. (**G**) Frequency-phase relationship of the locomotor gait in *Emx1::Cre; EphA4*$^{\Delta/Flox}$ mice.

These data indicate that although inactivation of EphA4 signaling in CST neurons leads to bilateral innervation of the spinal cord, these re-crossing axons have seemingly no effect on over-ground locomotor pattern. However, it cannot be ruled

out that these bifurcating CST axons might exert some effect on skilled locomotion where cortical influence is much stronger (Drew et al., 2002).

Conversely, we also demonstrated that spinal cord specific deletion of EphA4 signaling is sufficient to generate an over-ground hopping gait, since we could reproduce the hopping-like gait with *Hoxb8::Cre; EphA4$^{\Delta/Flox}$* mice. *Hoxb8* is expressed exclusively in the spinal cord; its expression commences at cervical segment 4 and is observed throughout the thoracic, lumbar and sacral segments.

Altogether these data indicate that the hopping phenotype observed in EphA4 signaling mutants is due to spinal cord intrinsic mechanisms.

4.1.2 Identification of glutamatergic neurons as responsible for the hopping phenotype

Another hypothesis regarding the mechanisms behind the hopping phenotype in EphA4 KOs is that inactivation of EphA4 signaling leads to a reconfiguration of the spinal locomotor network due to aberrant midline crossing of excitatory neurons that would otherwise project ipsilaterally (Beg et al., 2007; Butt et al., 2005; Iwasato et al., 2007; Kullander et al., 2003; Restrepo et al., 2011; Wegmeyer et al., 2007). To address this question, we firstly evaluated the consequences of inactivating EphA4 signaling selectively in glutamatergic neurons. To that end, we used the *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mouse. We observed that *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mice mimicked the over-ground locomotor phenotype observed in the full *EphA4 KO* mouse (Figure 9A). When running freely on a runway they displayed a quadrupedal hopping gait at all observed frequencies of locomotion with occasional alternation at locomotor frequencies below and

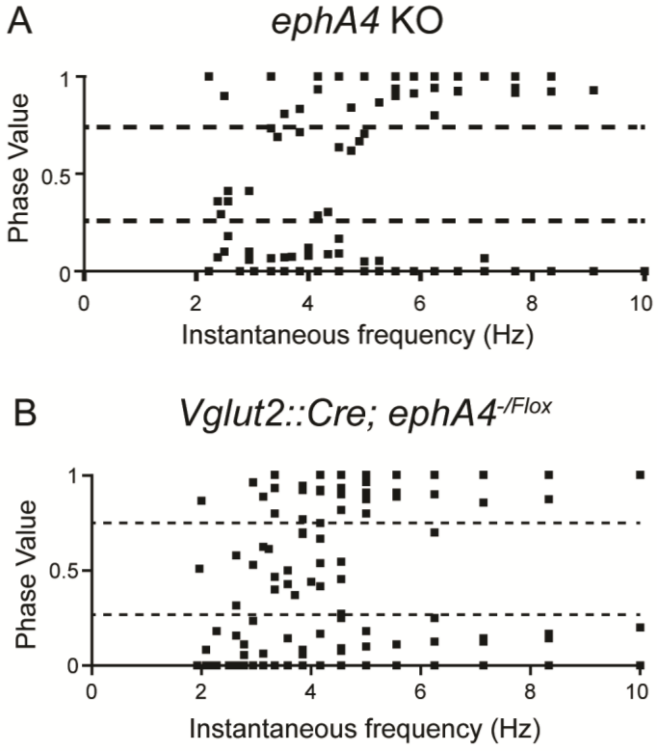


Figure 9. Locomotor pattern in freely moving full EphA4 KO and Vglut2::Cre; EphA4$^{\Delta/Flox}$ mice. (**A**) Frequency-phase relationship of the locomotor gait in *EphA4 KO* mice. (**B**) Frequency-phase relationship of the locomotor gait in *Vglut2::Cre; ephA4$^{\Delta/Flox}$* mice.

around 4Hz (Figure 9B).These data indicate that the neurotransmitter phenotype of the neurons affected in EphA4 signaling mutants is glutamatergic.

4.1.3 Aberrant midline crossing in the ventral commissure generates hopping in *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mice

We then set out to investigate if there was an increase in the midline crossing of glutamatergic neurons in *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mice compared to *Vglut2::Cre* control mice. To that end, we resorted to retrograde labeling of neurons in the isolated Vglut2::Cre; EphA4$^{\Delta/Flox}$ and Vglut2::Cre spinal cords (Figures 10A-F1). We observed an overall increase in the proportion of axons crossing in the ventral commissure of Vglut2::Cre; EphA4$^{\Delta/Flox}$ spinal cords compared to controls which was reflected in an increase in the number of glutamatergic (Vglut2^{+}) overcrossing neurons (Figure 10G).

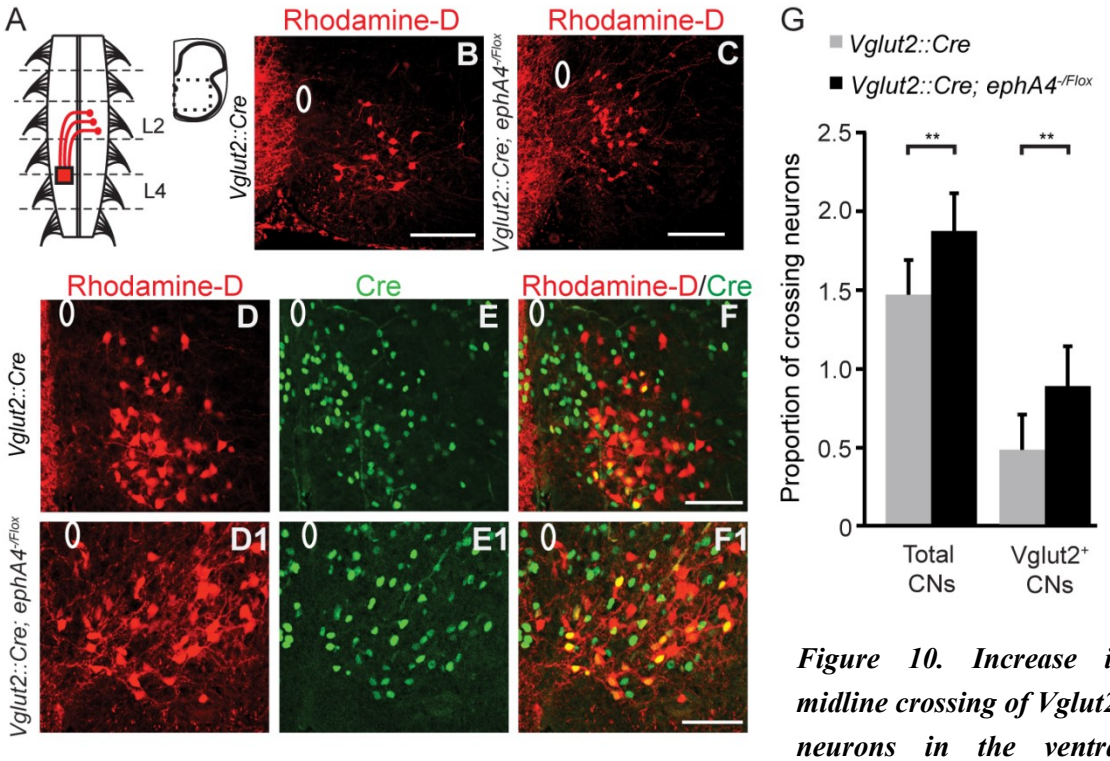


Figure 10. Increase in midline crossing of Vglut2^{+} neurons in the ventral spinal cord of Vglut2::Cre; EphA4$^{\Delta/Flox}$ mice. (**A**) Schematic picture illustrating the site for the rhodamine tracer application (red box). (**B** and **C**) Transverse L2 sections showing rhodamine dextran back-labeling of neurons and axons in *Vglut2::Cre* (**B**) or *Vglut2::Cre; ephA4$^{\Delta/Flox}$* mice (**C**). (**D-F1**) Transverse L2 sections showing increase in rhodamine dextran back-labeled neurons (red) that are Vglut2-positive (green, Cre antibody) in *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mice compared with *Vglut2::Cre* controls. (**G**) Bar graph showing the percent of rhodamine dextran labeled commissural neurons (CNs) (left), and only Vglut2^{+} CNs (right) in *Vglut2::Cre* and *Vglut2::Cre; EphA4$^{\Delta/Flox}$* mice. Error bars represent ± SD. ** indicate $p < 0.001$. White circles indicate the position of the central canal. Scale bars: 200 µm (**B, C**); 100 µm (**D-F1**).

Collectively these data indicate that the hopping phenotype observed upon inactivation of EphA4 signaling is due to aberrant crossing of spinal and not CST neurons. Additionally, our findings indicate that aberrant crossing in the ventral commissure in the spinal cord might lead to a hopping-like locomotor phenotype and suggest the neurotransmitter phenotype of these aberrant crossing neurons is possibly glutamatergic.

4.2 *PAPER II:* SPINAL HB9::CRE-DERIVED EXCITATORY INTERNEURONS CONTRIBUTE TO RHYTHM GENERATION IN THE MOUSE

In this study, we have targeted excitatory Hb9::Cre-derived interneurons (INs) in the *Hb9::Cre* mouse in order to functionally remove them from the network and investigate the consequences of their removal on motor output. Our findings suggest that excitatory Hb9::Cre-derived INs might be involved in mammalian locomotor rhythm generation.

Hb9::Cre-derived INs include the canonical Hb9 INs. We define canonical Hb9 INs as the small subset of neurons clustered in medial lamina VIII in the lower thoracic and upper lumbar mouse spinal cord. These interneurons retain endogenous HB9 protein expression postnatally and also co-express GFP protein under the *Hb9* promoter in *Hb9-GFP* mice (type I cells referred in (Hinckley et al., 2005; Wilson et al., 2005)). These canonical Hb9 INs have been suggested to be part of the kernel for rhythm generation in the mammalian locomotor network (Hinckley et al., 2005; Hinckley and Ziskind-Conhaim, 2006; Wilson et al., 2007; Wilson et al., 2005). However, since canonical Hb9 INs make up a negligible portion of the excitatory Hb9::Cre-derived INs (< 3%), we believe it is unlikely that the canonical Hb9 INs alone are responsible for the observed phenotype.

4.2.1 1/3 of the Hb9::Cre-derived interneurons are glutamatergic

We first demonstrated that the transmitter phenotype of Hb9::Cre-derived INs is mixed by examining overlap of reporter expression in *Hb9::Cre;Rosa26-tdTomato;Vglut2-GFP*, *Hb9::Cre;Rosa26-tdTomato;Glyt2-GFP, and Hb9::Cre;Rosa26-tdTomato;Gad67-GFP* mice, in order to identify Hb9::Cre-derived glutamatergic (Figure 11A), glycinergic (Figure 11B), and GABAergic (Figure 11C) neurons, respectively. We observed that approximately 1/3 are excitatory (33% ± 2% Vglut2) and 2/3 are inhibitory (34% ± 1% GAD67, and 33% ± 1% GlyT2) (N = 3 animals per condition, 48 sections) (Figure 11D).

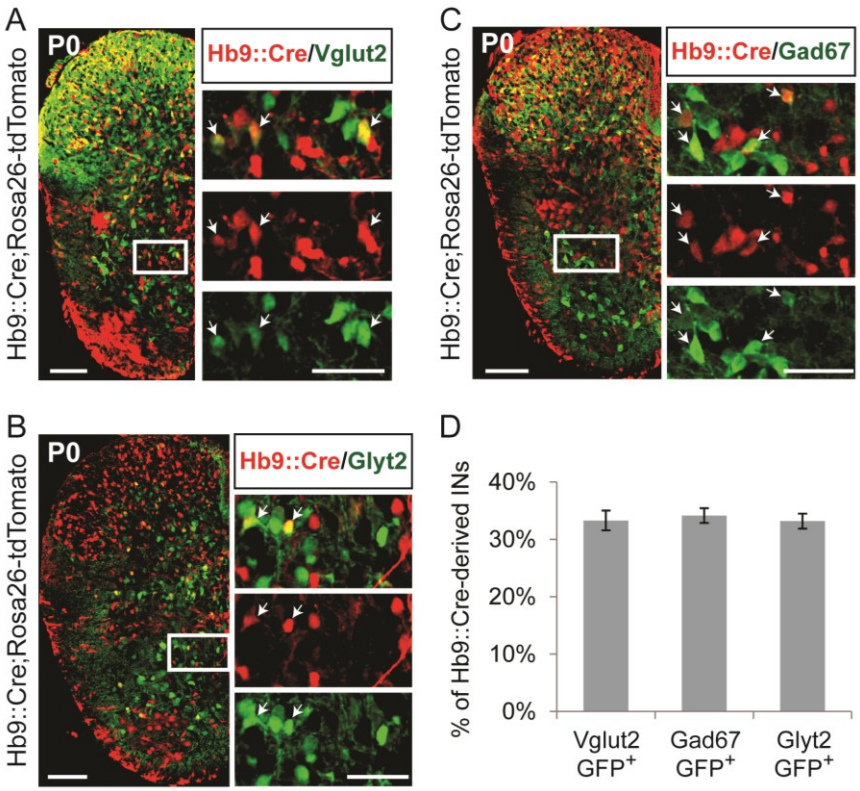


Figure 11. One third of the Hb9::Cre-derived INs are excitatory. (**A**, **B** and **C**) Transverse L1-L3 hemi-sections of P0 spinal cords show co-expression of td-Tomato protein (red) (*Hb9::Cre; Rosa26-tdTomato* mice) with GFP (green) from *Vglut2*- (**A**), *Glyt2*- (**B**), and *Gad67*- (**C**) -*GFP* mice. White boxed area images are magnified on the right. Arrowheads indicate overlap between Hb9::Cre-derived INs (tdTomato protein, red) and Vglut2, Glyt2, or Gad67-GFP neurons (GFP protein, green). Scale bars represent 100 µm for the transverse hemi-sections and 50 µm for the magnified boxes on the right. **(D)** Percent of Hb9::Cre-derived INs that are glutamatergic (Vglut2-GFP$^+$, 33% ± 2%), GABAergic (Gad67-GFP$^+$, 34% ± 1%), and glycinergic (Glyt2-GFP$^+$, 33% ± 1%) in Hb9::Cre;Rosa26-tdTomato spinal cords.

4.2.2 Silencing of synaptic transmission in excitatory Hb9::Cre-derived interneurons leads to lower fictive locomotor frequency

To determine the functional impact of the loss of excitatory synaptic transmission in Hb9::Cre-derived INs, we performed ventral root recordings in spinal cords isolated from *Hb9::Cre-Vglut2$^{\Delta/\Delta}$* and control mice (Figure 12A). We verified that locomotor frequencies in Hb9::Cre-Vglut2$^{\Delta/\Delta}$ cords were 26 - 31% lower than that of controls during drug-evoked locomotor-like activity (Figure 12B). In contrast, there was no significant difference in pattern generation, including left-right and flexor-extensor alternation (Figure 12C).

We also observed a similar effect on locomotor frequency upon neurally-evoked locomotor-like activity. Thus the bursting frequency was reduced in Hb9::Cre-Vglut2$^{\Delta/\Delta}$ cords by 15 - 21% compared to controls at stimulation strengths of 250 µA - 1 mA (Figure 12D).

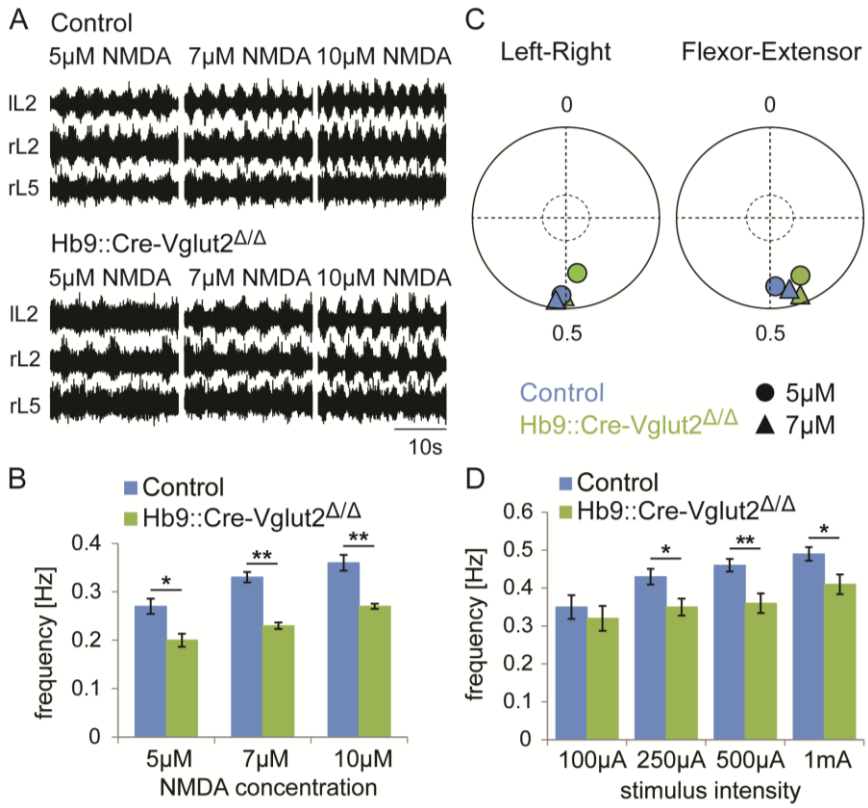


Figure 12. Silencing of glutamatergic output in Hb9:: Cre-derived INs leads to reduced frequency of drug and neural evoked locomotor-like activity. (**A**) Traces from ventral root recordings of drug-evoked locomotor-like activity in spinal cords from control (upper traces) and *Hb9::Cre-Vglut2*$^{\Delta/\Delta}$ (lower traces) mice at three NMDA concentrations. All include 5-HT (8 μM). (**B**) Frequency of locomotor-like activity as a function of NMDA concentration on a constant background of 5-HT (8 μM) in control (blue) and Hb9::Cre-Vglut2$^{\Delta/\Delta}$ (green) spinal cords. Error bars represent ± SEM. * indicates $p < 0.001$ and ** indicates $p < 0.0001$. (**C**) Circular plots of mean left-right (lL2-rL2) and flexor-extensor (rL2-rL5) coordination in control (blue) and Hb9::Cre-Vglut2$^{\Delta/\Delta}$ (green) spinal cords at 5 and 7 μM NMDA and 8 μM 5-HT. Each point corresponds to the mean vector value for each drug concentration. Individual vector values were generated from 50 locomotor cycles in each spinal cord. The inner circle indicates significance level of $p = 0.05$. (**D**) Frequency of neural-evoked (descending fiber-evoked) locomotor-like activity as function of stimulation amplitude. Error bars represent ± SEM. * indicates $p < 0.05$ and ** indicates $p < 0.005$.

Importantly, *Hb9* is expressed by motor neurons (Arber et al., 1999) and mammalian motor neurons release glutamate in addition to acetylcholine from central collaterals (Mentis et al., 2005; Nishimaru et al., 2005; Talpalar et al., 2011). Since locomotion can be initiated by ventral root stimulation (Mentis et al., 2005; Pujala et al., 2016), we also showed that Vglut2-mediated glutamatergic synaptic transmission in motor neurons does not account for the reduced frequency of locomotion observed in Hb9::Cre-Vglut2$^{\Delta/\Delta}$ spinal cords.

The cardinal feature that should characterize rhythm-generating neurons is that their selective manipulation should have a direct impact on locomotor frequency. By silencing the output of glutamatergic Hb9::Cre-derived INs and studying motor output

in an isolated spinal cord preparation, we have shown that excitatory Hb9::Cre-derived INs are likely part of the locomotor rhythm generator, as demonstrated by the reduced locomotor frequency.

4.2.3 Hb9::Cre-derived interneurons constitute a population distinct from the Shox2-nonV2a

To further characterize the excitatory Hb9::Cre-derived IN population, we turned to the developmentally expressed transcription factors found in excitatory neurons, and looked for overlap with the exclusively excitatory and ipsilateral IN markers, *Isl1, Chx10* and *Shox2* (Figures 13A and 13B). We found that Hb9::Cre-derived INs seldomly co-expressed Isl1 (6% ± 0.2%, N = 2, 22 sections) (Figure 13C).

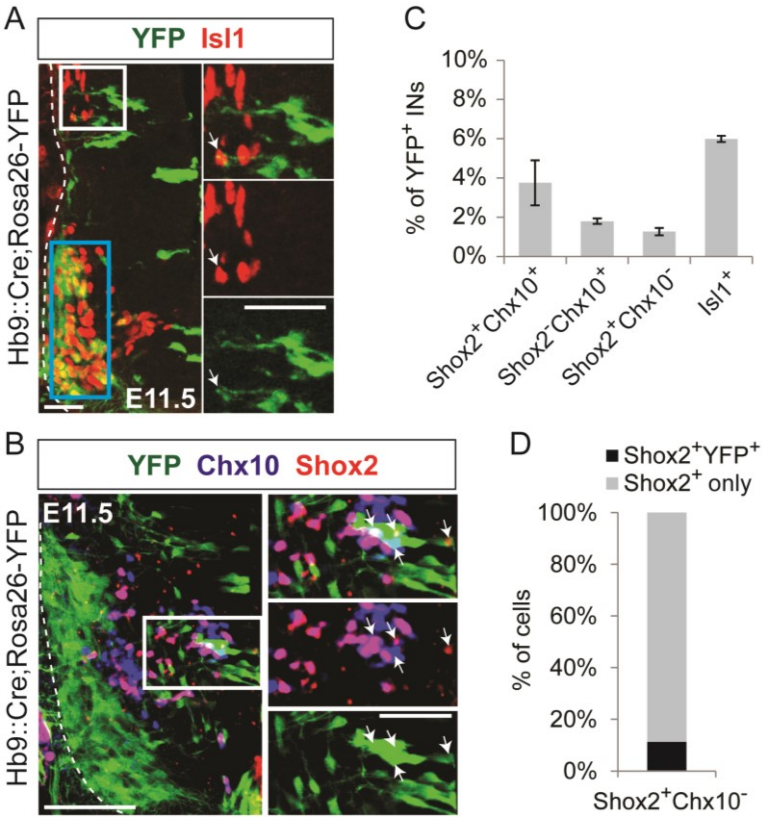


Figure 13. Hb9::Cre-derived INs do not overlap with the Shox2 non-V2a population. (**A**) Co-expression of YFP (green) and Isl1 antibody (red) in the *Hb9::Cre; Rosa26-YFP* mouse spinal cord at E11.5. Motor neurons are also labeled by Isl1 antibody (blue box). Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between Isl1 (red) and Hb9::Cre-derived INs (green). Scale bars: 100 µm and 50 µm. (**B**) Co-expression of YFP (green), Shox2 antibody (red) and/or Chx10 antibody (blue) in the *Hb9::Cre;Rosa26-YFP* mouse ventral spinal cord at E11.5. Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate overlap between Hb9::Cre-derived INs (green) and $Shox2^{+}Chx10^{-}$ (red), $Shox2^{-}Chx10^{+}$ (blue) or $Shox2^{+}Chx10^{+}$ (pink) INs. Scale bars: 100 µm and 50 µm. (**C**) Quantification of overlap in (**A**) and (**B**). Bar graph showing percent of overlap between Hb9::Cre-derived INs (YFP^{+}) and Shox2 V2a ($Shox2^{+}Chx10^{+}$), $Shox2^{OFF}$ V2a ($Shox2^{-}Chx10^{+}$), Shox2 non-V2a ($Shox2^{+}Chx10^{-}$), and Isl1 ($Isl1^{+}$) INs. Error bars represent ± SEM. (**D**) Percent of the Shox2 non-V2a IN population ($Shox2^{+}Chx10^{-}$) that overlaps with Hb9::Cre-derived INs (YFP^{+}, darker grey) in the *Hb9::Cre;Rosa26-YFP* mouse spinal cord at E11.5. Error bars represent ± SEM.

Chx10 is the marker for V2a neurons and the expression of *Shox2* and *Chx10* can be used to categorize excitatory neurons in the ventral spinal cord. Shox2 non-V2a INs ($Shox2^{+}Chx10^{-}$) is the only subgroup of excitatory neurons that has been shown to contribute to rhythm generation in mammals, whereas Shox2 V2a INs ($Shox2^{+}Chx10^{+}$) and $Shox2^{OFF}$ V2a INs ($Shox2^{-}Chx10^{+}$) seem to be involved in pattern generation (Dougherty et al., 2013). We found that Hb9::Cre-derived INs rarely overlapped with the Shox2 V2a (4% ± 0.1%), $Shox2^{OFF}$ V2a (2% ± 0.1%) and Shox2 non-V2a (1.3% ± 0.2%) populations (N = 2, 22 sections) (Figure 13C). Moreover, we also verified that Hb9::Cre-derived INs make up less than 12% ± 2% of the Shox2 non-V2a population (Figure 13D).

Altogether, these data indicate excitatory Hb9::Cre-derived INs are a population of neurons distinct from the Shox2 non-V2a that is likely to be involved in the generation of locomotor rhythm.

4.2.4 Considerations about canonical Hb9 interneurons

Although excitatory Hb9::Cre-derived INs include the canonical Hb9 INs, canonical Hb9 INs only make up 3% of the excitatory Hb9::Cre-derived interneuron population. Therefore, it is unlikely that the frequency phenotype observed in Hb9::Cre-$Vglut2^{\Delta/\Delta}$ spinal cords is due to canonical Hb9 INs alone.

It would have been interesting to selective silence glutamatergic transmission exclusively in the canonical Hb9 INs in order to determine their role in locomotor rhythm. However, this would have required the use of a mouse line that does not exist, namely an inducible (e.g. tamoxifen) Hb9::Cre line. With accurate timing, we might have been able to only eliminate Vglut2 from the canonical population of Hb9 cells. But unfortunately such a mouse line is not available.

4.3 *PAPER III:* TRANSCRIPTOME ANALYSIS OF SPINAL EXCITATORY NEURONS

To dissect the molecular makeup of glutamatergic neurons in the mouse ventral spinal cord, we compared the postnatal expression profile of the main glutamatergic group of neurons in the spinal cord, *Vglut2*-expressing neurons, to that of non-glutamatergic neurons as well as to that of the only glutamatergic subgroup linked to rhythm generation, the Shox2 interneurons (Dougherty et al., 2013). Our findings provide a comprehensive overview of the transcription factors expressed in glutamatergic neurons in the mouse spinal cord, and thereby offer tools for both the identification of

glutamatergic subgroups as well as the dissection of functions that still remain elusive such as rhythm generation.

4.3.1 Transcriptional landscape of spinal glutamatergic interneurons: genes up and down regulated in Vglut2-GFP$^+$ cells

Firstly, we compared the transcriptome content of glutamatergic neurons to that of non-glutamatergic cells in order to determine which genes were enriched in glutamatergic cells in comparison to all other cell groups as well as eliminate non-glutamatergic and non-neuronal transcripts from the investigated population.

To identify genes that were differentially expressed between glutamatergic and non-glutamatergic cells, RNA-sequencing was performed on mRNA from GFP$^+$ and GFP$^-$ cells isolated from Vglut2-GFP spinal cords, lamina IV-X, and followed by DESEq2 analysis (Love et al., 2014). Genes were considered differentially expressed if their adjusted p-value (padj) was lower than 0.05 and their absolute $\log_2$ fold change was higher than 0.59 ($\left|\log_2 \text{Fold change } \textit{Vglut2-GFP}^+ \text{ vs } \textit{GFP}^-\right| \geq 0.59$; padj ≤ 0.05). Mean normalized read counts amongst expressed transcripts ranged from 843.397 to 1.16 (Figure 14A). DESEq2 analysis revealed that of the 22.967 transcripts expressed in Vglut2-GFP spinal cord cells, 3.458 (15%) were up regulated ($\log_2$FC ≥ 0.59; padj ≤ 0.05), and 3.419 (15%) were down regulated ($\log_2$FC ≤ -0.59; padj ≤ 0.05) in Vglut2-GFP$^+$ cells (Figure 14A and B).

We focused this analysis on the differentially expressed transcription factors identified as the most biologically significant analysis-ready molecules for the mouse nervous system according to the Ingenuity Pathway Analysis (IPA).

From the 196 differentially expressed transcription factors identified with IPA, 74 of them were up regulated (top 24, Figure 14C), and 122 of them were down regulated (top 24, Figure 14D) in Vglut2-GFP$^+$ cells. We identified well known glutamatergic developmental markers amongst the transcripts up regulated in Vglut2-GFP$^+$ cells, namely *Sim1*, *Evx2, Isl1, Lhx3*, *Chx10, Shox2, Lbx1 and Lmx1b* (Figure 14C). Amongst the transcription factors down regulated in Vglut2-GFP$^+$ (Figure 14D), we identified transcripts involved in vascular development such as *Meox2* (Douville et al., 2011), in oligodendrocyte differentiation such as *Sox6* (Baroti et al., 2016), and in establishing a mesenchymal cell phenotype such as *Twist1* and *Zeb1* (Katoh and Katoh, 2009) as well as transcripts involved in determining the identity of spinal

inhibitory interneurons such as *Gata3*, a marker for the ventral inhibitory interneuron group V2b (Zhang et al., 2014).

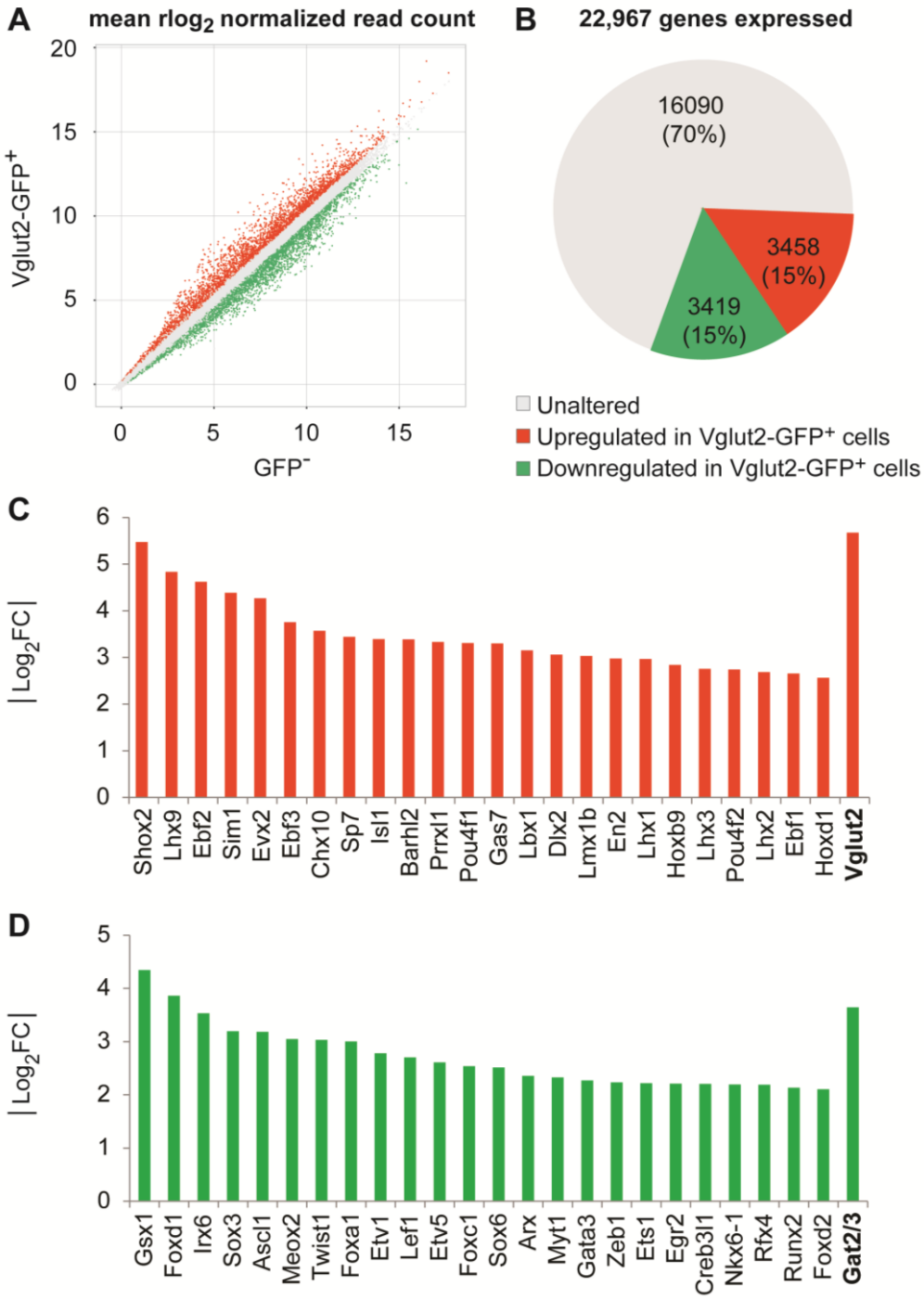


Figure 14. Transcriptome analyses of spinal Vglut2-GFP⁺ cells. (**A**) Scatterplot illustrating relative gene expression levels of differentially expressed transcripts up (red) and down regulated (green) in GFP⁺ cells as wells as transcripts with uniform expression (grey) between GFP⁺ and GFP⁻ cells from P3 spinal cords of *Vglut2-GFP* mice. (**B**) Pie chart of DESeq2 analysis. DESeq2 comparison of GFP⁺ and GFP⁻ transcriptomes revealed a total of 22.967 genes expressed in Vglut2-GFP cells, of which 3.458 were up regulated (red) and 3.419 down regulated (green) in GFP⁺ cells. (**C** and **D**) Top 24 transcription factors up regulated (≥ 5.89-fold enrichment with padj ≤ 0.05) (**C**), and down regulated (≥ 4-fold down regulation with padj ≤ 0.05) (**D**) in GFP⁺ cells.

We also identified the vesicular glutamate transporter 2 (Vglut2) as the main up-regulated neurotransmitter transporter, and the GABA transporter 2/3 (Gat2/3) as the main down-regulated transporter in Vglut2-GFP$^+$ cells (Figure 14C and D, respectively).

All together, these data validate our experimental approach, in that we have successfully isolated glutamatergic neurons in the ventral spinal cord, and provide a comprehensive overview of the molecular identity of glutamatergic neurons in the mouse postnatal spinal cord.

4.3.2 Transcriptional diversity of spinal glutamatergic interneurons: distribution of differentially expressed genes between Vglut2-GFP$^+$ and Shox2::Cre; Rosa26-YFP$^+$ spinal neurons

We then compared the transcriptome profile of Vglut2-GFP$^+$ cells, lamina IV-X, to that of the Shox2::Cre; Rosa26-YFP$^+$ interneurons excluding all transcripts that were considered differentially expressed and downregulated in glutamatergic neurons in our previous analysis (*Vglut2 GFP*$^+$ vs *GFP*$^-$ analysis).

RNA-sequencing was performed on mRNA isolated from sorted GFP$^+$ and YFP$^+$ cells, from Vglut2-GFP and Shox2::Cre;Rosa26-YFP spinal cords, respectively, and followed by DESEq2 analysis (Love et al., 2014). Mean normalized read counts amongst expressed transcripts ranged from 302.426 to 2.8 (Figure 15A). DESEq2 analysis revealed that of the 16.183 genes expressed in Vglut2-GFP$^+$ and Shox2::Cre; Rosa26-YFP$^+$ spinal cord cells, 955 (6%) transcripts were up regulated or enriched ($\left|\log_2 FC\right| \geq 0.59$; padj ≤ 0.05) in GFP$^+$ cells, and 1.118 (7%) transcripts were up regulated or enriched ($\left|\log_2 FC\right| \geq 0.59$; padj ≤ 0.05) in YFP$^+$ cells (Figure 15A and B).

From the 51 differentially expressed transcription factors identified with IPA, transcripts for 32 of them were up regulated in Shox2::Cre;Rosa26-YFP$^+$ cells (top 10, Figure 15C), and transcripts for 19 of them were up regulated in Vglut2-GFP$^+$ cells (top 10, Figure 15D).

More than 1/3 (7/19, 37%) of the transcription factors enriched in Vglut2-GFP$^+$ cells in this analysis had not been identified in our previous differential analysis (*Vglut2-GFP*$^+$ vs *GFP*$^-$ analysis), the other 2/3 had already been found up regulated in

glutamatergic cells. Similarly, more than 2/3 (22/32, 69%) of the transcription factors enriched in Shox2::Cre; Rosa26-YFP$^+$ cells were also unique to this analysis.

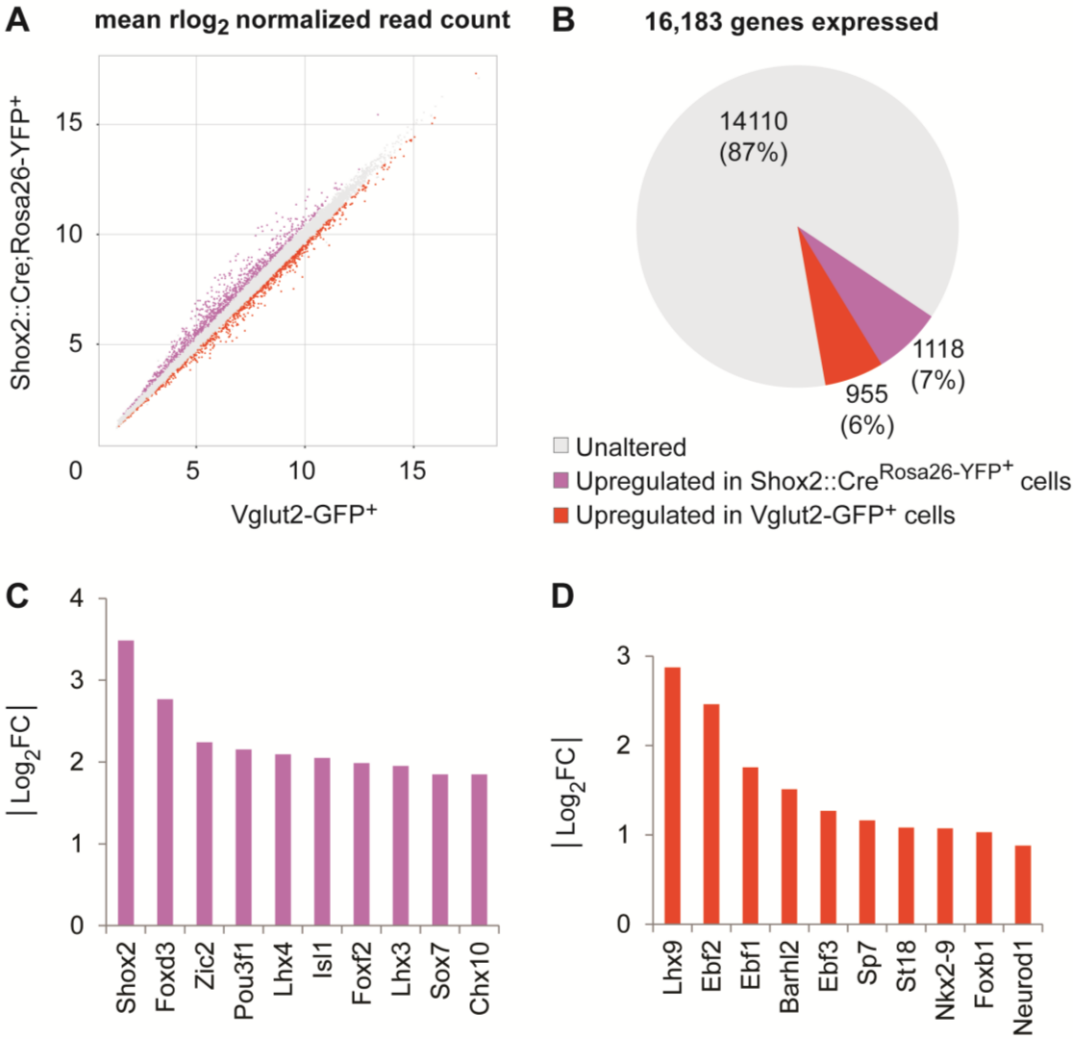


Figure 15. Distribution of differentially expressed genes between Vglut2-GFP$^+$ and Shox2::Cre; Rosa26-YFP$^+$ spinal neurons. (**A**) Scatterplot illustrating relative gene expression levels of differentially expressed transcripts up regulated in either YFP$^+$ (pink) or GFP$^+$ (red) cells from P3 spinal cords of *Shox2::Cre; Rosa26-YFP* and *Vglut2-GFP* mice, respectively. Transcripts with uniform expression between YFP$^+$ and GFP$^+$ cells are marked in grey. (**B**) Pie chart of DESeq2 analysis. DESeq2 comparison of YFP$^+$ and GFP$^+$ transcriptomes revealed a total of 14.183 genes expressed in YFP$^+$ and GFP$^+$ spinal cells, of which 1.118 were up regulated in YFP$^+$ cells (pink) and 955 were up regulated in GFP$^+$ cells (red). (**C** and **D**) Top 10 transcription factors enriched in either YFP$^+$ cells ($\geq$ 3.6-fold enrichment with padj $\leq$ 0.05) (**C**), or GFP$^+$ cells ($\geq$ 1.8-fold enrichment with padj $\leq$ 0.05) (**D**).

The main differentially expressed transcription factor identified as up regulated in YFP$^+$ cells was *Shox2*. Moreover, we also identified markers that are known to define subgroups of Shox2 interneurons embryonically amongst the top 10 transcription factors enriched in Shox2::Cre reporter cells, namely *Ils1*, *Lhx3* and *Chx10*

(Dougherty et al., 2013). Given that *Shox2* is down regulated postnatally, further investigation of the transcription factors enriched in Shox2::Cre reporter cells might reveal the postnatal identity of spinal Shox2 interneurons.

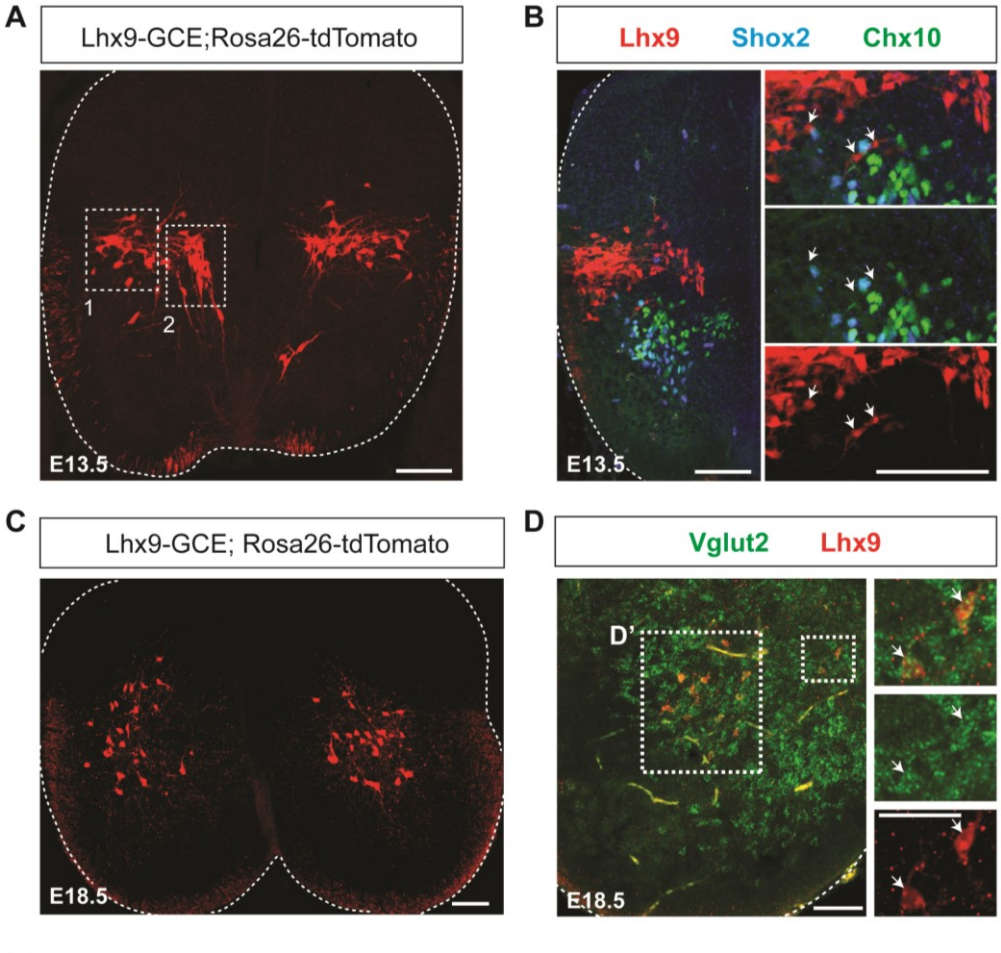
Similarly, further investigation of the transcription factors enriched in Vglut2-GFP$^+$ cells might reveal candidate markers for rhythm-generating neurons. Given rhythm-generating neurons are glutamatergic (Hagglund et al., 2010; Hagglund et al., 2013; Kiehn, 2006; Kiehn et al., 2008) and that ablation of the only glutamatergic group of neurons linked to rhythm generation, the Shox2 non-V2a interneurons does not completely abolish locomotor rhythm (Dougherty et al., 2013), there must be rhythm-generating neurons amongst the non-Shox2 glutamatergic population, some of which may belong to the excitatory Hb9::Cre-derived population.

4.3.3 *Lhx9*: top differentially expressed transcription factor enriched in Vglut2-GFP$^+$ spinal cord neurons

Lhx9 ranked as either the top two or top one differentially expressed transcription factor enriched in spinal Vglut2-GFP$^+$ cells (Figures 14C and 15C) in the first and second differential expression analyses carried out in this paper, respectively. Therefore, we set out to investigate if the transcription factor *Lhx9* defined a population of glutamatergic neurons in the postnatal ventral spinal cord that was distinct from the Shox2 population.

We verified that in *Lhx9-GCE; Rosa26-tdTomato* mice spinal cords (Balasubramanian et al., 2014) tamoxifen induced at E11.5, reporter expression was detected in both dI1i and dI1c interneurons groups (Wilson et al., 2008) (Figure 16A). We did not find any overlap between reporter-labeled cells and the Shox2 protein in the *Lhx9-GCE; Rosa26-tdTomato* mouse spinal cord (Figure 16B). Since many Shox2 interneurons also co-express *Chx10* (Dougherty et al., 2013), we investigated if any of the dI1 interneurons also expressed Chx10 protein but we did not observe any overlap between reporter-labeled and Chx10 interneurons (Figure 16B).

We also observed that at E18.5 reporter-labeled neurons settle in the ventral and intermediate spinal cord (Figure 16C) and that all of them are glutamatergic; *Vglut2* mRNA signal was detected extensively throughout the gray matter and in all Lhx9-GCE reporter cells (Figure 16D).



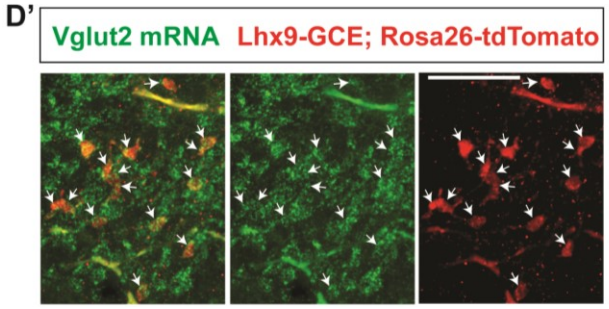


Figure 16. Lhx9 interneurons are glutamatergic and do not overlap with the Shox2 interneuron population. (**A**) Expression of tdTomato (red) in the spinal cord of *Lhx9-GCE;Rosa26-tdTomato* mice at E13.5. White boxes 1 and 2 indicate the positions of dI1i and dI1c interneurons, respectively. Scale bar: 100 µm. (**B**) Co-expression of tdTomato (red), Shox2 antibody (blue) and/or Chx10 antibody (green) in the *Lhx9-GCE;Rosa26-tdTomato* mouse spinal cord at E13.5. Rightmost pictures are magnifications of the white boxed area. Arrowheads indicate lack of overlap between reporter-labeled neurons (red) and Shox2/Chx10 (cyan), Shox2 (blue) or Chx10 (green). Scale bar: 100 µm. (**C**) Expression of tdTomato (red) in the spinal cord of *Lhx9-GCE;Rosa26-tdTomato* mice at E18.5. Scale bar: 100 µm. (**D**) Co-localization of *Vglut2* mRNA (green) and tdTomato (red) in the spinal cord of *Lhx9-GCE;Rosa26-tdTomato* mice at E18.5. Rightmost pictures are magnifications of the small white boxed area on the right. Arrowheads indicate *Vglut2* mRNA (green) detected in reporter-labeled neurons (red). Scale bar: 100 µm. (**D'**) Magnification of leftmost white boxed area in (**D**). Arrowheads indicate *Vglut2* mRNA (green) detected in reporter-labeled neurons (red). Scale bar: 100 µm.

These data further validate our experimental approach in that we have successfully isolated a transcription factor that both labels as well as fractionates the glutamatergic population in the mouse postnatal spinal cord. Moreover, this analysis brought to light a marker for glutamatergic interneurons in the ventral spinal cord described to be implicated in motor behavior (Bermingham et al., 2001; Wilson et al., 2008). Therefore, further investigation of *Lhx9*-expressing neurons might contribute to our understanding of the spinal locomotor network.

In the same way, further investigation of the expression pattern of other transcription factors enriched in Vglut2-GFP spinal neurons might lead to the identification of new neuronal subgroups and thereby help elucidate the identity of rhythm-generating neurons as well as contribute to a better understanding of the underpinnings of locomotor networks in the mouse spinal cord.

4.4 *PAPER IV*: DESCENDING COMMAND NEURONS IN THE BRAINSTEM THAT HALT LOCOMOTION

In this paper, we functionally evaluated the role of brainstem V2a neurons, characterized by the expression of the transcription factor *Chx10*, in motor output. We found that V2a neurons in the reticular formation constitute a major excitatory pathway to locomotor areas of the lumbar spinal cord, more specifically to the rhythm-generating layers of spinal locomotor networks. We also demonstrated that despite their excitatory nature, selective activation of V2a neurons in the rostral medulla or caudal pons either halts or negatively modulates ongoing locomotor-like activity in the isolated spinal cord and arrests locomotion in freely-moving mice. These data suggest that brainstem V2a neurons could be involved in the control of the episodic nature of locomotion.

4.4.1 Brainstem V2a neurons are excitatory

We found that the majority of V2a neurons in the brainstem are excitatory in that they express mRNA for the vesicular glutamate transporter 2 (Vglut2) regardless of their rostro-caudal or medio-lateral positioning (> 95%, n = 3 animals) (Figure 17F). We also verified that they do not express any inhibitory or monoamine neurotransmitters. These data is in agreement with previous investigations of the transmitter phenotype of V2a neurons (Al-Mosawie et al., 2007; Bretzner and Brownstone, 2013; Lundfald et al., 2007).

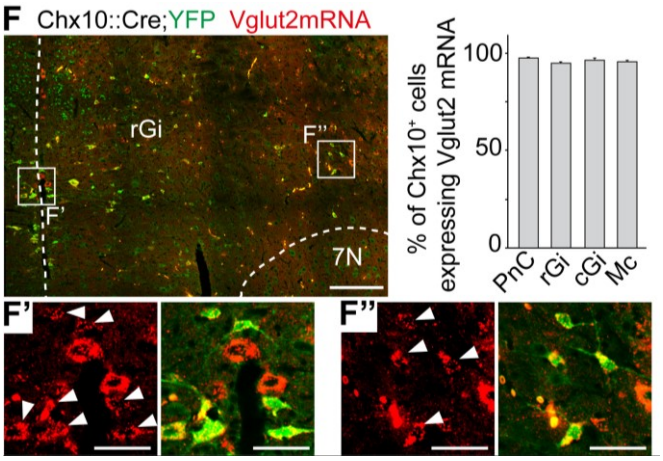


Figure 17. V2a brainstem neurons are excitatory. Transverse hemisection in the rGi indicating Vglut2^{+} (red) V2a neurons (YFP). Bar graphs show the average percentage of Vglut2^{+} V2a neurons (n = 3 animals). Insets in **F′** and **F″** are magnified views of *Vglut2* mRNA alone (left) and merged with YFP (right). White arrowheads indicate co-expression. Error bars are SEM. Scale bars: 200 µm and 50 µm.

4.4.2 Activation of rostral medulla/caudal pons V2a neurons stops ongoing locomotor-like activity

Our first objective was to investigate the effect of activation of V2a neurons on motor output and at the same time correlate this motor output to the specific region in the brainstem responsible for its generation. To that end, we performed four successive transverse cuts at different antero-posterior levels from the rostral to the caudal medulla in brainstems isolated from *Chx10::Cre; Rosa26-ChR2-YFP* mice, and targeted photo-illumination to the exposed plane (Figure 18A). We observed that light activation of the caudal pontine reticular nucleus (PnC) and rostral gigantocellular reticular nucleus (rGi) induced a complete arrest of ongoing locomotor-like activity (Figure 18B and C, respectively). Similarly, light activation of the caudal gigantocellular reticular nucleus (cGi) also led to an arrest of ongoing locomotor activity although a few low amplitude bursts were still observed (Figure 18D). In contrast, light activation of the caudal most descending V2a neurons from the magnocellular reticular nucleus (Mc), did not exert any effect in the frequency or amplitude of ongoing locomotor-like activity (Figure 18E).

Light activation at any of those segmental levels was unable to elicit locomotor-like or bursting activities in the absence of locomotor drugs.

Altogether, these data reveal that optogenetic activation of brainstem V2a neurons leads to an arrest of ongoing locomotor-like activity and suggest that this “stop” command either preferentially resides in the rostral Gi and PnC or that at least a critical number of cells is necessary for its functional manifestation. We therefore called these cells V2a stop neurons.

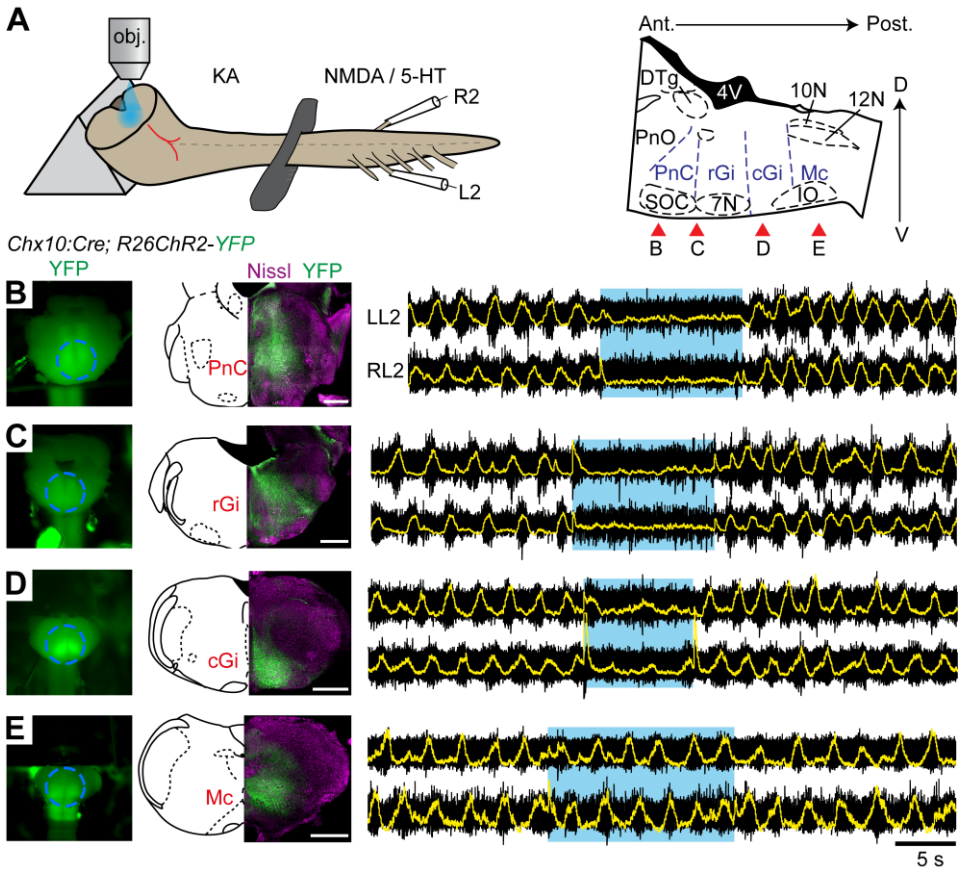


Figure 18. V2a stop neurons reside in the rGi and PnC. (**A**) Experimental set-up. The brainstem is sectioned transversally to expose a given transverse plane to the light. Red arrows on the right indicate approximate levels of the sections performed in (**B**)-(**E**). (**B**-**E**) Simultaneous electrophysiological recordings of L2 roots on both sides of the cord after a section exposing the PnC (**B**) and of the same preparation after having removed the PnC (**C**), and the rGi (**D**) or cGi (**E**). The ability of light stimulation to stop ongoing locomotion is lost when only the caudal-most medullary formation remains. The transient voltage deflections visible on the integrated traces at light onset and offset are light-mediated artifacts. Scale bar: 500 µm.

4.4.3 Optogenetic activation of brainstem V2a neurons halts locomotion *in vivo*

To determine the effect of activation of brainstem V2a neurons *in vivo*, we injected a Cre- dependent AAV-DIO-ChR2-mCherry virus bilaterally in the rGi of *Chx10::Cre* mice (Figure 19A) and investigated the effect of light activation on locomotor behavior. We observed that light activation of transfected V2a neurons promoted a complete locomotor arrest on average 140 ms after light onset, allowing the ongoing step to be completed and hence leading to a canonical stopping position; four feet on the ground and placed in front of the hips (Figure 19C).

Therefore, selective activation of rGi V2a neurons *in vivo* arrests quadrupedal locomotion and leads to a characteristic stopping position.

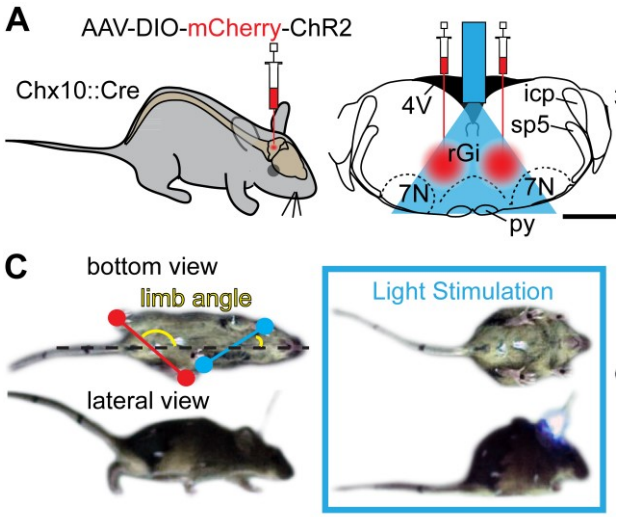


Figure 19. Optogenetic activation of brainstem V2a neurons halts in vivo locomotion. (A) Scaled reconstruction of the implantation and illumination range following bilateral viral injection in the rGi. (C) Snapshots of a freely-moving Chx10::Cre mouse 4 weeks post-injection before (left) and during (right) light stimulation using pulsed blue light. The limb angle is defined as the angle between a line joining the two hindpaws (red) or forepaws (blue), with respect to the midline of the animals.

4.4.4 V2a descending command is integrated in the spinal cord

We then set out to investigate if this arrest of locomotor activity observed upon activation of brainstem V2a neurons was due to recruitment of inhibitory descending pathways in the brainstem (Holstege, 1991) or whether it was integrated in the lumbar spinal cord. For this, we used a split-bath configuration that allowed blocking of glutamatergic transmission selectively in the brainstem with kynurenic acid (KYN, 4mM) (Figure 20). Under these conditions, light stimulation of V2a brainstem neurons still induced an arrest of locomotor-like activity, which was followed by a rebound of activity (Figure 20D and E). This rebound activity suggests that spinal locomotor neurons may have undergone synaptic inhibition during light stimulation.

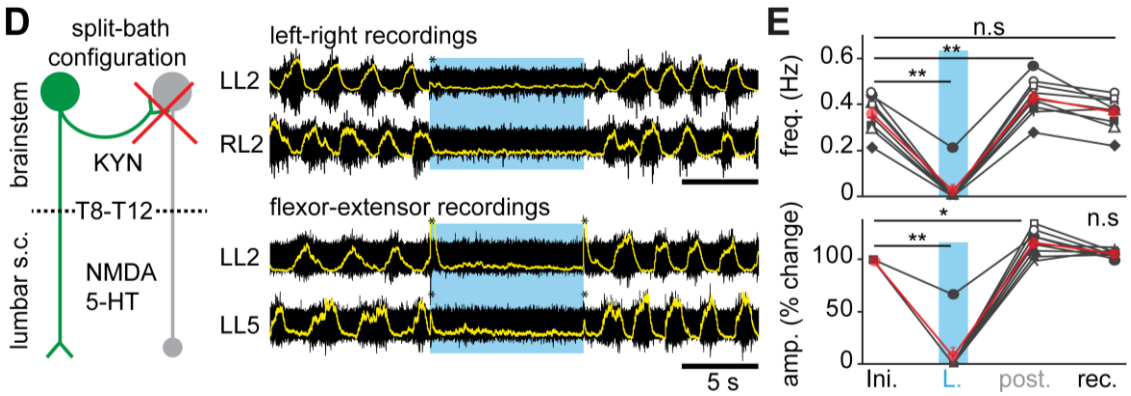


Figure 20. V2a-mediated arrest of locomotor-like activity is integrated in the spinal cord. (**D**) Left: blockage of recruitment of inhibitory descending neurons (gray) by V2a neurons (green) with KYN in a split-bath configuration. Right: recordings of L2 (flexor dominated) and L5 (extensor dominated) roots. Light activation (blue epoch) of brainstem V2a neurons stops ongoing locomotor-like activity. (**E**) Average per animal and grand average amongst animals (red) of the instantaneous frequency and of the percent change in amplitude of drug-evoked locomotor bursts before (Initial: ini.) and during light (L.), for 5 cycles following light offset (post), and for the following 20s (recovery: rec).

These findings indicate that the signal for arrest of locomotor activity is directly conveyed to the spinal locomotor networks.

4.4.5 V2a neurons in the rostral medulla terminate predominantly in lamina VII in the lumbar spinal cord

To determine the pattern of innervation of brainstem V2a neurons in the lumbar spinal cord, we performed anterograde labeling of brainstem V2a neurons with bilateral injections of AAV-DIO-ChR2-mCherry in the rostral Gi and investigated their axonal terminations in the lumbar spinal cord (Figure 21A and B). We verified that V2a neurons from the rostral Gi predominantly terminate in lamina VII (Figure 21C); lamina VII corresponds to the region where locomotor related neurons have been described (Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2006; McLean and Dougherty, 2015).

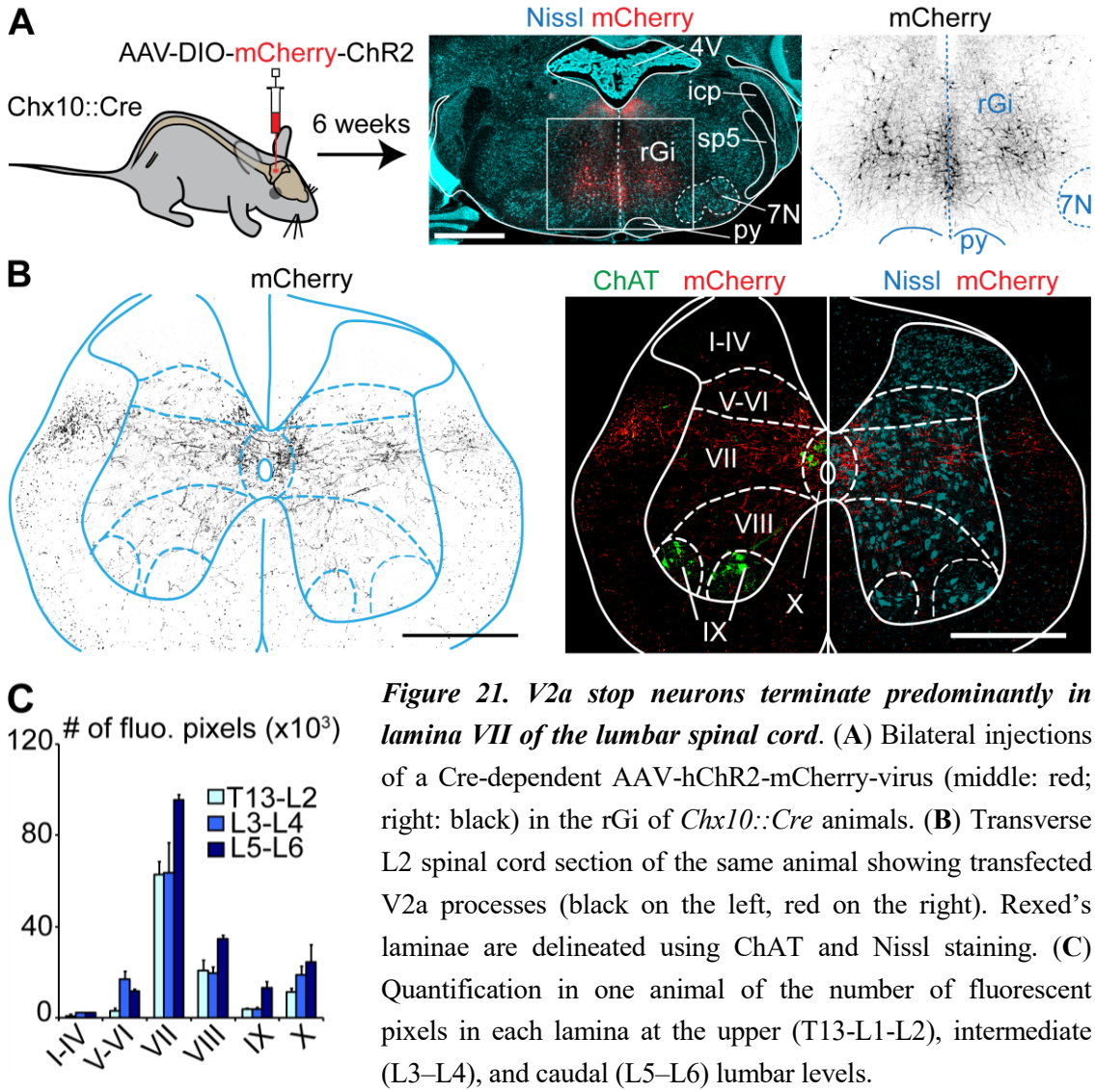


Figure 21. V2a stop neurons terminate predominantly in lamina VII of the lumbar spinal cord. (**A**) Bilateral injections of a Cre-dependent AAV-hChR2-mCherry-virus (middle: red; right: black) in the rGi of *Chx10::Cre* animals. (**B**) Transverse L2 spinal cord section of the same animal showing transfected V2a processes (black on the left, red on the right). Rexed's laminae are delineated using ChAT and Nissl staining. (**C**) Quantification in one animal of the number of fluorescent pixels in each lamina at the upper (T13-L1-L2), intermediate (L3–L4), and caudal (L5–L6) lumbar levels.

4.4.6 V2a-mediated locomotor arrest depresses rhythm-generating layers of the locomotor network

We then set out to investigate whether the locomotor arrest mediated by brainstem V2a neurons acted on the rhythm-generating layer or on the pattern formation layer in the spinal locomotor networks. To discriminate between an effect on the rhythm and pattern generating circuitries, we used a split-bath preparation and selectively challenged the spinal cord compartment to higher concentrations of locomotor drugs so that brainstem V2a activation did not completely arrest locomotor-like activity whereas excitatory synaptic transmission was simultaneously blocked in the brainstem.

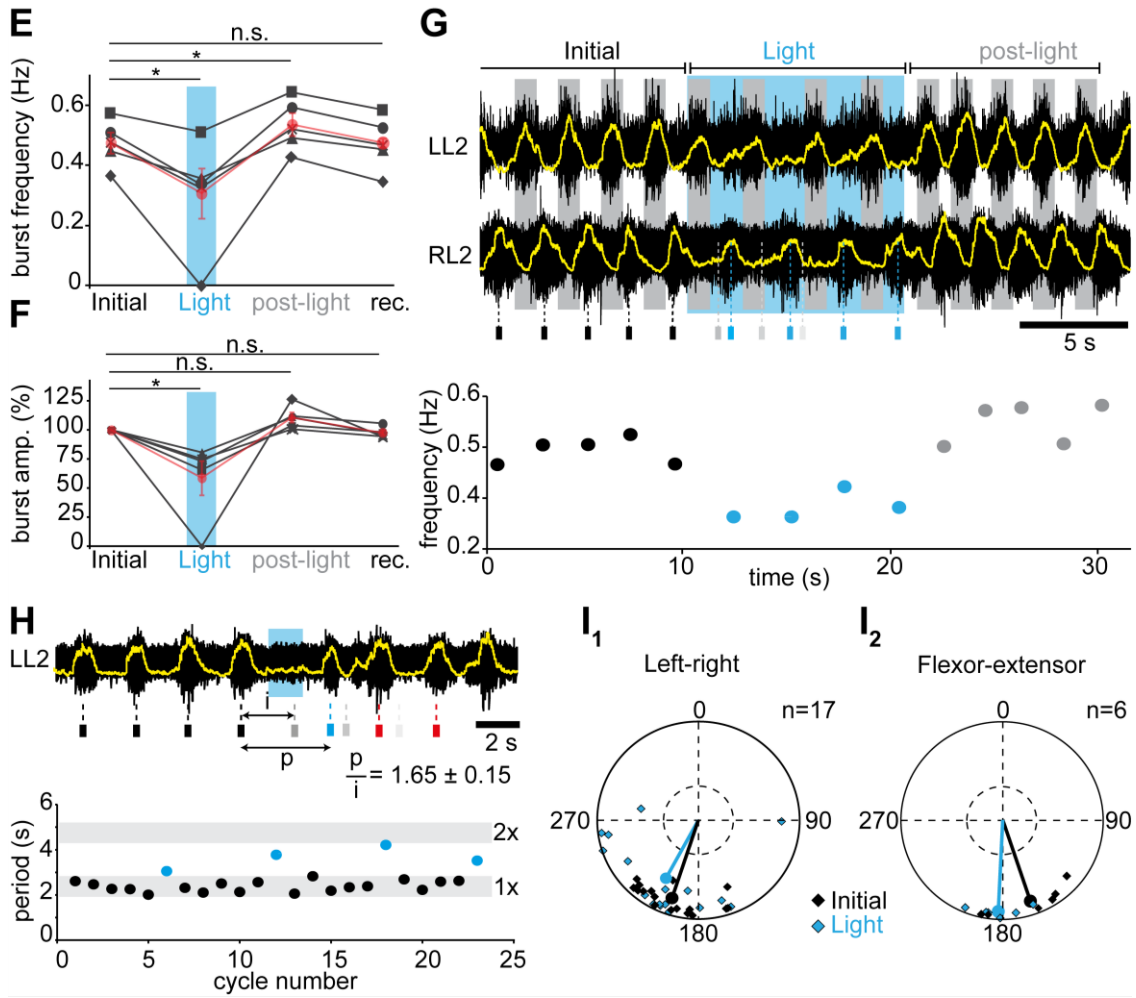


Figure 22. Light activation of brainstem V2a neurons depresses the rhythm-generating layers of spinal locomotor networks. (**E-F**) Average per animal (n = 5) and grand-average amongst animals (red) of the instantaneous frequency (**E**) and of the percent changes in amplitude (**F**) of L2 locomotor bursts on preparations facing high NMDA concentrations (> 8 µM). * indicates $p < 0.05$ (paired t test). (**G**) Typical L2 ventral root recording during high-frequency locomotor-like activity (~0.5 Hz). Small rectangles below indicate the time of peak of the control RL2 bursts (black), and their forecasted (gray) and actual occurrences (blue) during light activation of brainstem V2a neurons, showing a non-graded slowing of the rhythm. Below is plotted the corresponding instantaneous frequency of RL2 bursts. (**H**) L2 ventral root recording during drug-

evoked locomotor-like activity. The expected burst is silenced (gray bar below) and the rhythm reset by short light-pulse, as seen by the perturbed period (p) not falling in the range of twice the initial period (i). The graph below illustrates initial (black) and perturbed periods (blue) for four consecutive trials. (**I**) Circular plot showing the left-right (I1) or flexor-extensor (I2) phase-relationships for individual trials and for the mean preferred phase among all trials before (Initial, black) and during (Light, blue) light-activation. Phase values falling in the bottom-half of the outer circles indicate alternation. There is no significant difference between control and light conditions (Watson-William's test $p > 0.05$). Error bars in (E), and (F) are SEM.

At locomotor frequencies higher than 0.45 Hz, light activation of brainstem V2a neurons did not lead to a complete arrest of locomotor like activity but to a significant reduction in locomotor frequency and amplitude instead (Figure 22E-G). We also observed that shorter pulses of light given during a locomotor burst caused phase-resetting of locomotor rhythm (Figure 22H). Additionally, we did not observe any effects on left-right and flexor-extensor coordination upon light activation of brainstem V2a neurons (Figure 22I) which indicates that the pattern formation layer is not a target of brainstem V2a neurons. Moreover, we also observed that light activation of V2a stop neurons did not directly inhibit motor neurons.

Collectively, these data indicate that brainstem V2a neurons might arrest or modulate ongoing locomotor activity by acting on the rhythm-generating layer of the spinal locomotor networks.

4.5 OVERALL DISCUSSION

A lot of effort has been put into elucidating the wiring of spinal motor systems. To define the organizing principles of the spinal motor circuitry, we need to determine the diversity of the discrete interneuron subtypes that constitute it. Thus, the classification of spinal interneurons into 11 cardinal groups _ventral, V0-V3, and dorsal, dI1-dI6, domains_ (Goulding and Pfaff, 2005; Jessell, 2000) paved the way to our core understanding of the spinal locomotor circuitry.

In *Paper I* we demonstrated how axon guidance molecules can affect the wiring of the spinal circuitry and disrupt the pattern formation layer of the locomotor network. When the EphA4 signaling pathway is inactivated in glutamatergic neurons, there is an increase in the number of overcrossing glutamatergic neurons that would otherwise project ipsilaterally. This aberrant crossing of excitatory neurons leads to a hopping-like locomotor phenotype in which left-right alternation is partially maintained at low frequencies and completely lost at high locomotor frequencies.

While it is clear that the hopping phenotype can be explained by an increased excitatory overcrossing, an alternative explanation would be a reduction in the total number of crossing axons. For instance, a reduction in the number of crossing axons of commissural neurons involved in the direct or indirect inhibitory control of alternation (Talpalar et al., 2013) could in principle lead to a hopping phenotype. This would entail ipsilateral axon guidance defects which would lead to loss of connections, but, currently, there are no direct evidences to support this hypothesis.

So far we have not been able to pinpoint the exact molecular identity of the aberrantly crossing neurons in EphA4 signaling mutants although we were able to exclude spinal excitatory V2a neurons as candidates, because their axons were shown to not cross the midline in *EphA4 KO* mice (Lotta Borgius, unpublished data; Lundfald et al., 2007). Given the glutamatergic nature of these aberrantly crossing neurons, modeling studies have suggested it is plausible that rhythm-generating neurons themselves aberrantly cross the midline and synchronize the activity of the rhythm-generating cores on each side of the spinal cord in EphA4 signaling mutants (Rybak et al., 2013); this hypothesis definitely warrants further investigation.

The cardinal feature that should characterize rhythm-generating neurons is that their selective manipulation should have a direct impact on locomotor frequency. Their activation should be able to initiate locomotor rhythm and/or change the frequency of the ongoing rhythm whereas a selective reduction in their number should reduce the frequency of the ongoing locomotor rhythm (Dougherty et al., 2013; El Manira, 2014; Grillner and Jessell, 2009; Hagglund et al., 2013; Kiehn, 2016a; McLean and Dougherty, 2015; Roberts et al., 2010). In *Paper II* we pursued the functional removal of glutamatergic Hb9::Cre-derived INs from the network by selectively eliminating Vglut2 from the excitatory Hb9::Cre-derived population. By silencing synaptic transmission in glutamatergic Hb9::Cre-derived INs and investigating the effects on motor output in an isolated spinal cord preparation, we have shown that excitatory Hb9::Cre-derived INs are likely part of the locomotor rhythm generator, as demonstrated by a significant reduction in locomotor frequency. We acknowledge, however, that distinguishing between a role in tonic drive to the rhythm generator and a role in rhythm generation per se is difficult and nearly impossible experimentally, and therefore, we cannot exclude that this decrease in frequency might be partly due to a reduction in tonic drive to the rhythm-generating core.

It should also be emphasized that, similarly to what was concluded in studies on Shox2 neurons (Dougherty et al., 2013), we believe that it is unlikely that excitatory Hb9::Cre-derived INs are the sole rhythm-generating neurons in the mammalian locomotor network. In the absence of excitatory Hb9::Cre-derived INs, locomotor frequency is reduced but locomotor rhythm is not abolished. This is seen more clearly when compared to experiments where all excitatory neurons are silenced, which leads to a complete cessation of the rhythm (Hägglund et al. 2013). These observations suggest that several molecularly distinct groups of neurons may contribute to rhythm generation. It would, therefore, be interesting to eliminate both the excitatory Hb9::Cre-derived and the Shox2 populations from the spinal network in order to assess whether rhythm generation is cumulatively reduced or completely abolished.

In *Paper II* our classification of neurons as Hb9::Cre-derived INs does not sprout from the initial progenitor domain-defined classes of interneurons (Jessell, 2000). Instead, Hb9::Cre-derived INs in *Paper II* are those identified in the *Hb9::Cre* mouse line (Yang et al., 2001). They are a heterogeneous group of neurons that actually span several of the cardinal classes of interneurons. Similarly, studies on Shox2 INs have shown that, even within Shox2 non-V2a neurons, rhythm generation may be distributed amongst neurons derived from several progenitor domains (Dougherty et al., 2013). It has become apparent that the interneuron composition of the spinal cord is quite complex and that the cardinal classes of interneurons are actually comprised of a highly diverse set of transcriptionally distinct neuronal types (Bikoff et al., 2016; Dougherty et al., 2013; Enjin et al., 2010; Kiehn, 2016b; Zagoraiou et al., 2009).

The work in *Paper III* adds to this picture by showing that excitatory neurons in the mouse spinal cord express a broad range of transcription factors that are not necessarily captured by the canonical classes. In *Paper III* we carry out a fine-grained analysis of the transcriptome profile of spinal excitatory neurons and provide a comprehensive overview of the transcription factors, ion channels and metabotropic receptors expressed in glutamatergic neurons in the postnatal mouse spinal cord. We anticipate that analysis of gene expression databases in parallel with conjoint exposure to antibodies as well as *in situ* hybridizations for these transcription factors, ion channels and metabotropic receptors will determine their prevalence within the glutamatergic population and thereby delineate discrete subsets of glutamatergic interneurons in the mouse spinal cord, and hence pave the way for a comprehensive functional dissection of the neural circuits involved in locomotion. It is likely that this analysis will also identify groups of cells involved in functions other than rhythm

generation or even motor behavior. Additionally, since glutamatergic cells are found throughout the neuroaxis, our study will also be fundamental for defining new populations of cells in other areas of the nervous system as well.

One such example of glutamatergic cells that are found throughout the neuroaxis are the V2a neurons. V2a neurons in the mouse spinal cord are glutamatergic ipsilaterally projecting neurons described to be involved in the left-right coordination of locomotor activity (Al-Mosawie et al., 2007; Lundfald et al., 2007). In the brainstem, V2a neurons are thought to be glutamatergic and to send descending axons to the cervical spinal cord (Bretzner and Brownstone, 2013).

In *Paper IV* we investigated the role of brainstem V2a neurons in locomotion. We confirmed that brainstem neurons are exclusively glutamatergic and showed that they also send axonal projections to the lumbar spinal cord preferentially to the locomotor layer in lamina VII. We demonstrated that despite their excitatory nature, activation of V2a neurons leads to an arrest of ongoing locomotion and that this arrest is possibly mediated by depression of the rhythm-generating layers of the spinal locomotor networks. These findings indicate that brainstem V2a neurons might be involved in the important task of controlling the episodic nature of locomotion.

However, broad stimulation of all excitatory neurons in the caudal brainstem has been shown to lead to initiation of locomotor-like activity (Hagglund et al., 2010). Therefore, it is possible that glutamatergic brainstem neurons other than the V2a neurons might be involved in the initiation signal of locomotion. Alternatively, the large number of brainstem V2a neurons involved in locomotor arrest might hinder a selective manipulation of locomotor-initiating V2a neurons. A fine-grained molecular characterization of brainstem glutamatergic neurons in combination with genetic manipulation of projection-specific cell types might shed some light on this issue.

Since many molecularly defined groups of neurons are equally present in the spinal cord and in supraspinal regions, investigation of the expression pattern of markers enriched in spinal glutamatergic neurons might help define discrete subgroups of glutamatergic neurons not only in the spinal cord but also in supraspinal regions. This fractioning of the glutamatergic population into smaller discrete subgroups might help elucidate motor functions or mechanisms that still remain elusive or ambiguos.

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That’s all for now folks! THANKS!!!