Distinct limb and trunk premotor circuits establish laterality in the spinal cord

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I dedicate this work to Martina, my whole family and friends for always being there and supporting me. Thank you all so much!

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1. Summary

Motor behaviors such as swimming, walking or flying have evolved in various forms during evolution and with them dedicated neuronal circuits of the motor system within the central nervous system (CNS). Vertebrates ranging from lamprey to humans generate active movements by contraction of specific set of muscles, indicating that some basic motor elements are shared. One fundamental unit of the motor output system that is present in the most basal up to evolutionarily younger vertebrates are the motor neurons. This neuronal cell type establishes the direct connection from the networks of the CNS to the muscles, controlling contraction states of muscles and hence represents the last neuronal stage before the execution of a motor routine.

However, the motor neurons in all species need to be tightly controlled by neuronal circuits within the spinal cord and the brain, in order to ensure that motor neuron activity follows meaningful activation patterns.

The aim of this project is to study the premotor circuits – and hence the part of the network that ultimately decides if the contacted motor neuron pool will be active or not. The focus is put on muscle types with distinct biomechanical functions and their premotor circuits. The study investigates and compares the global structure, neurotransmitter phenotype contribution and progenitor domain origin of premotor networks controlling trunk- versus limb muscles.

The results demonstrate that fundamentally different premotor circuits control purely posture (trunk muscles) versus limb muscles. They significantly differ in their ipsi-contra distribution, commissural- and ipsilateral inhibitory input and contribution of distinct progenitor domains.

Furthermore, it turns out that motor neuron pool position and their dendritic structure are important parameters in establishing these distinct premotor networks in the spinal cord.

2. INTRODUCTION

Movements generated by the coordinated contraction of distinct types of muscles are found in countless varieties and facets throughout the animal kingdom. The basis of these motor behaviors is the interplay of neuronal circuits within the CNS and muscles in the periphery. The proper function of the motor system requires precise connectivity between neurons within the CNS and neurons from the CNS to the muscles.

In order to generate voluntary movement, an internal decision needs to be made within the brain. A structure implicated in this process of decision-making and action selection is the basal ganglia, which can promote a certain motor behavior. One of the structures lying downstream of the basal ganglia is the reticular formation, which in turn has direct access to the spinal networks controlling limb and axial muscles (Goulding, 2009). In addition to this higher-center information, local spinal networks further receive input from sensory modalities that allow the animal to monitor ongoing motor routines and adapt to changes in the environment. These neuronal computations within the spinal cord lead to an activation of motor neurons, which innervate a specific muscle target and control its activation. The precise temporal activation pattern of motor neurons leads to the sequential recruitment of muscle synergies, ideally generating the appropriate motor behavior.

2.1. Axial and limb muscles and the control of posture

With the water-to-land transition and the appearance of terrestrial locomotion, structures and systems of the body needed to evolve in order to counteract gravitational forces. Axial muscles are used by quadruped animals to stabilize the spine and trunk. In rat for example, axial muscles run along both sides of the vertebral column, stabilizing the back and tail, allowing to keep their trunk off the ground (Brink and Pfaff, 1980). Behavioral studies revealed that axial muscles on opposite sides of the body show an alternating activation

pattern during locomotion (Gramsbergen et al., 1999). For normal functional development of postural muscle activity, vestibular input is required. In rat, a lack of vestibular information from early postnatal stages, delays the appearance of adult-like postural motor behaviors (Geisler et al., 1998).

Besides regulation between axial muscles, it has been demonstrated in rat that the axial muscles on one side of the body are tightly coupled to the activity of flexor (FLEX) and extensor (EXT) muscles of the hind-limbs during walking. This coupling of axial and limb muscles is changing as the animal reaches more adult stages. At early stages (until P21) the axial muscles of one side are coactive with the EXT gastrocnemius (GS) muscle on the contralateral side. However, after this stage there is a shift in coupling resulting in the co-activation of axial muscles of one side and GS on the ipsilateral side of the body (Gramsbergen et al., 1999). These coordination of axial and limb muscles implies the existence of regions regulating both types of muscles. It has been proposed that one of these regions might be the reticular formation in the brainstem, which can be divided into distinct sub-regions, according to the type of motor neurons they control, ranging from axial- (neck and back) to fore- and hind-limb controlling motor neuron classes (Peterson et al., 1979). Recently, it was shown in decerebrate cats that brainstem and spinal circuits are sufficient to restore postural stability after disturbances to equilibrium such as a tilting platform (Musienko et al., 2014). Taken together, for motor behaviors such as walking, the interplay of axial- and limb muscles is crucial, making it necessary that neuronal circuits controlling them are closely communicating.

2.2. Limb muscles involved in walking and fine motor control

Terrestrial animals that locomote on the ground using limbs need to tightly regulate activity of different types of muscles in order to generate directed movements and counteract gravitational forces.

The muscles of the limbs control the opening and closing of different joints and referred to as EXT and FLEX, respectively. The temporal sequence of EXT/FLEX contractions leads to a specific movement of that extremity (Yakovenko et al., 2002). Considering a motor behavior such as walking, the limbs and their muscles need to be tightly controlled on the left and right of the body. Walking requires an alternation of EXT and FLEX muscles within one limb and furthermore the EXT muscles of one limb need to be out of phase with EXT muscles on the opposite body side (Kiehn 2006; Kiehn 2011).

In addition to the above-mentioned motor behaviors, there are motor routines, which require neuronal circuits controlling the precise activation of proximal muscles of the arm and smaller distal muscles of the hand in order to for example reach and grasp for an object (Esposito et al., 2014; Azim et al., 2014). However, irrespective of the type of motor behavior executed, the correct activation pattern of different muscle types requires dedicated neuronal circuits within the spinal cord.

2.3. Distinct motor neuron columns and -pools control trunk- and limb muscles

2.3.1. Motor neuron generation

A neuronal class within the spinal cord, which is critical to generate motor routines contains somatic motor neurons, which innervate and control skeletal muscles in the periphery. Motor neurons are the neuronal elements, which relay the information generated and computed within the CNS to the target muscle and hence are crucial to generate motor behaviors, since they directly link the CNS and the periphery. During embryonic development a generic set of motor neurons is generated from a specific progenitor domain (pMN) that is specified by the action of a secreted molecule – Sonic hedgehog (shh). Shh acts as a morphogen, establishing a concentration gradient (ventral^{high}, dorsal^{low}) inducing distinct ventral progenitor domains

exhibiting specific molecular programs. Shh has been shown to delineate the borders between ventral progenitor domains by inducing the restricted expression of homeodomain transcription factors, with Nkx6.1, Nkx2.2 and Irx3 defining the progenitor domain, which generates motor neurons (Jessell, 2000). A LIM homeodomain transcription factor called islet1 (Isl1) has been implicated to be essential in order to generate motor neurons in the spinal cord. In Isl1 mutant mice, there are no motor neuron specific genes expressed (e.g. Hb9 or Lhx3) during embryonic development, indicating that generation of motor neurons is abolished in this mutant (Pfaff et al., 1996).

Among the motor neurons generated in the wild-type spinal cord are somatic motor neurons, which subsequently are genetically further subdivided into motor neuron columns and –pools projecting to distinct muscle targets in the periphery (Figure 1).

2.3.2. Specification of distinct motor neuron columns

Transcriptionally distinct somatic motor neuron classes can be stratified in motor neuron columns, innervating specific types of muscles and settling at distinct locations within the spinal cord. A medially located motor neuron column, that is present at all segmental levels of the spinal cord, is called medial motor column (MMC) and harbors the motor neurons which innervate axial muscles. The MMC has been shown to maintain the expression of the LIM homeodomain transcription factor Lhx3 (whereas it is rapidly down regulated in other motor neurons), conferring its specific molecular profile. It has been demonstrated that ectopic expression of Lhx3 in all motor neurons induces MMC fate, breaking down the columnar subdivision within the spinal cord (Sharma et al., 2000). However, initial transient expression of Lhx3 by all motor neurons is important for proper ventral motor axon outgrowth from the spinal cord (Sharma et al., 1998). The mechanism, which induces the specification towards MMC-fate Lhx3-expressing motor neurons was shown to be a Wnt4/5 gradient (ventral^{high}, dorsal^{low}) in the ventral spinal cord. The suggested model predicts that motor neurons, which

are generated more ventrally (high Wnt4/5 concentration) within the pMN acquire a MMC fate whereas more dorsally-derived (low Wnt4/5 concentration) pMN motor neurons will give rise to segmentally restricted motor neurons of the HMC and LMC. This hypothesis is supported by an observed increase of MMC motor neurons in the *Nkx2.2* mutant mouse, in which the progenitor domain ventral to pMN is giving rise to motor neurons and not interneurons (Agalliu et al., 2009).

Both at cervical and lumbar levels is the lateral motor column (LMC) that contains all the motor neurons that control the muscles of the fore- and hind-limb, respectively. In the thoracic spinal cord are the autonomic motor neurons located in the preganglionic motor column (PGC) - called Column of Terni in the chick. In the chick is has been demonstrated that the positioning and correct genetic identity of LMC and CT is dependent on a Fibroblast Growth Factor (FGF) gradient, with caudal spinal cord exhibiting high levels of FGFs, where as the FGF concentration gradually decreases towards the rostral part of the spinal cord. It has been shown that members of the *Hox* gene family are expressed in specific types of motor neurons within confined rostro-caudal domains in the spinal cord, and interference with FGF gradient is leading to ectopic Hox gene expression (Dasen et al., 2003). Overexpression of FGF in the brachial spinal cord, leads to the induction of Hoxc9, which marks CT neurons normally only found at thoracic levels. Conversely, expression of Hoxc6 in the thoracic spinal cord, leads to the emergence of LMC markers, normally not found at these segmental levels (Dasen et al., 2003). Together these findings argue for a rostro-caudal FGF gradient in the spinal cord, inducing distinct *Hox* programs in motor neurons at different rostro-caudal levels, resulting in different motor neuron columns in the cervical, thoracic and lumbar spinal cord. This puts *Hox* transcription factors at center stage in the specification of different motor neuron columns (Dasen et al., 2003).

Interfering with *Hox* gene expression can lead to transformation of motor columns from one transcriptional motor column identity into another. It has been shown that deleting the *Hoxc9* gene in the mouse, leads to the expression of LMC marker genes in the motor neurons at thoracic levels. The reason for this finding is that *Hoxc9* normally represses *Hoxc6*, delineating the border between thoracic and cervical motor columns. With the loss of *Hoxc9*, *Hoxc6* is de-repressed and leads to motor neurons in the thoracic spinal cord expressing *Hoxc6* and LMC marker genes (Jung et al., 2010). At thoracic segments there are also motor neurons of the hypaxial motor column (HMC) that control muscles of the body wall and intercostal muscles. However, for the HMC less is currently known about its placing and specification.

Within these columns, the motor neurons further stratify into individual motor neuron pools, which are transcriptionally different from each other, and each pool innervating a specific muscle. In the genetic separation between motor neuron pool identity *Hox* genes have been proven to be again of critical importance. Individual *Hox* genes are expressed within the cervical LMC, positioning individual LMC motor neuron pools at specific rostro-caudal positions and assign distinct transcriptional profiles to different motor neurons pools (Dasen et al., 2005; Di Sanguinetto et al., 2008).

In the lumbar spinal cord of the mouse it was demonstrated that *Hoxc10* and *Hoxd10* are necessary to establish proper motor neuron columns and pools. In *Hoxc10* and *Hoxd10* double mutants, thoracic PGC motor column expands into lumbar spinal cord, at the expense of LMC motor neurons at these levels. Mutant mice show severe behavioral phenotype and aberrant motor axon projections to hind-limb muscles (Wu et al., 2008). For the chick, it has been shown that *Hoxd10* and *Hoxd11* are involved in the generation of the LMC and the delineation between thoracic PGC motor neurons and lumbar LMC motor neurons (Misra et al., 2009).

Hox genes are important patterning regulators of structures within the CNS of different species through out the animal kingdom. A recent publication revealed that lamprey, which represents the most basal vertebrates, shows a segmentally restricted *Hox* expression pattern in the hindbrain as it is seen in various modern vertebrate species, suggesting that the *Hox* code, patterning the hindbrain may be a feature already present in the evolutionary oldest vertebrates (Parker et al., 2014).

However, *Hox* genes are not only active in vertebrates, but for instance it was shown that in the leech, *Hox* gene activity is required for the generation of motor neurons controlling the male sex organ (Gharbaran and Aisemberg, 2013). In Drosophila, *Hox* genes have been shown to be involved in proper terminal arborization of motor axons and dendritic patterning of motor neurons innervating muscles of the legs (Baek et al., 2013).

Taken together, *Hox* gene activity is crucial in various animal species, in order to build a functional nervous system.

2.3.3. Motor axon guidance towards target structure

The acquisition of a certain genetic identity of distinct motor neuron pools leads to the expression of more downstream genes, encoding for example specific surface receptors, necessary for proper motor axon guidance to navigate towards its target structure (Tessier-Lavigne and Goodman, 1996). There are several studies describing the transcriptional profile of specific sets of LMC motor neuron pools and how they achieve precise wiring with their target limb muscle. One example of a receptor expressed in specific sets of motor neurons is EphA4, which is critical for correct LMC motor axon pathfinding at the dorsal/ventral limb bud junction (Eberhart et al., 2002). LMC motor axons, which are $EphA4^{+}$, are repelled from the ventral limb and innervate dorsal limb muscles, since ventral limb mesenchyme cells express the repulsive ligand ephrin-A (Kania and Jessell, 2003). It has been shown that

receptor-ligand interaction during motor axon guidance is more complex, with Eph-receptors and ephrins being expressed on the same growth cone leading to *cis*-attenuation of Eph-receptors (Kao and Kania, 2011). MMC motor neurons also express EphA4 and in addition – in contrast to LMC motor neurons – also EphA3. It has been demonstrated that EphA3/4 in MMC motor axons is crucial for proper axon guidance. MMC motor axons turn dorsally after leaving the ventral spinal cord in order to direct their growth towards their target axial muscles. This trajectory makes them passing the DRGs, which contain sensory neurons, expressing ephrin-A. The repulsive interaction of MMC motor axon EphA3/4 with sensory neuron ephrin-A, prevents motor axons from being miss-targeted into DRGs. Deletion of EphA3/4 leads to MMC motor axon guidance defects, with motor axons erroneously growing into DRGs (Gallarda et al., 2008).

However, many of these attractive and repulsive systems are acting in parallel during axon pathfinding, involving various receptor-ligand interactions, as for example Semaphorin-Neuropilin interactions, which are necessary as well for various steps of LMC motor axon ingrowth in dorsal- and ventral limb bud (Huber et al., 2005).

It has been demonstrated that during development axial muscles produce FGFs, which are acting as chemo-attractants for MMC motor axons. MMC motor neurons express FGF receptor 1 (FgfR1), which renders them sensitive for the signals from the axial muscles. In non-MMC motor neurons, maintained ectopic expression of Lhx3 leads to their conversion into MMC, accompanied by their expression of FgfR1, rendering them attracted towards axial muscles. (Sharma et al., 2000; Shirasaki, 2006; Bonanomi and Pfaff, 2010). These studies imply that Lhx3 is a key transcription factor in the separation between MMC and non-MMC spinal motor column identity and furthermore influences downstream programs such as motor axon pathfinding in the periphery.

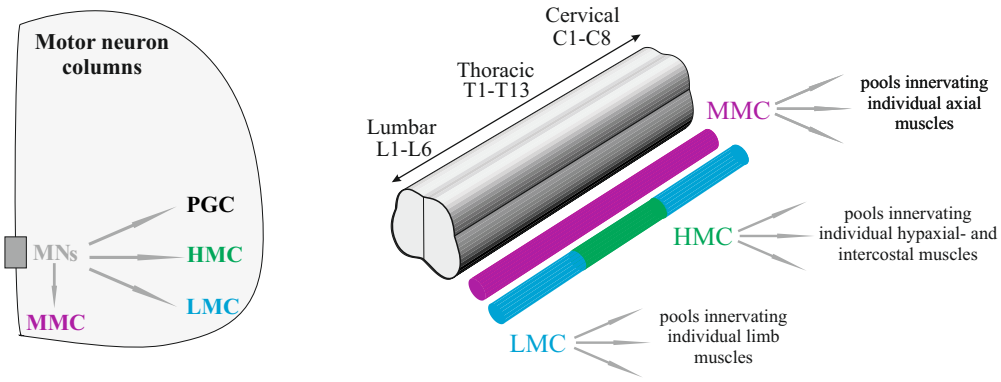

Figure 1. Motor neuron generation and transcriptional diversification in distinct motor neuron columns and –pools.
The spinal motor neuron progenitor domain gives rise to different motor neuron columns, which are located that specific sites within the spinal cord. The motor neuron columns then further stratify into distinct motor neuron pools each innervating a muscle in the periphery.

2.3.4. Spatial motor neuron pool positioning

Different motor neuron pools can be distinguished from one another by the expression of specific transcription factors. However, it also turned out that motor neuron pools within the LMC are topographically organized. Motor neuron innervating more proximal muscles of the extremity are located in a more ventral domain of the LMC, whereas motor neuron pools innervating progressively more distal muscles are located in the more dorsal LMC (Romanes, 1951). This spatial arrangement of LMC motor neuron pools was implicated to be of importance to ensure correct sensory input to a given LMC motor neuron pool (Jessell, 2011). Normally, LMC motor neuron pools form clusters that are confined to a particular area within the LMC and do not intermingle with motor neurons from other pools. This separation and clustering has been shown to be dependent on β- and γ-catenin signaling, since deletion of them leads to motor neuron pools, which are transcriptionally defined and innervate the correct muscle, but are not clustered anymore and scattered throughout the LMC (Demireva et al., 2011). The fact that these motor neurons still form transcriptionally defined pools which

project to the correct target, leads to the question, why a defined spatial matrix of motor neuron pools within the LMC is required. However, in order for a motor neuron pool to reliably control a muscle, it needs correct sensory input from homonymous and synergistic sensory fibers. In *FoxP1* conditional mutant mice (see below) in which the motor neuron pool position is scrambled and genetic identity altered, it turns out that sensory fibers from a given muscle still terminate in a specific domain within the spinal cord, irrespective of the type of motor neuron present (Sürmeli et al., 2011). This indicates that medio-lateral and dorso-ventral positioning of motor neuron pools has a profound influence on what type of (sensory) input they receive.

2.3.5. Motor neuron pools and their dendritic architecture

Motor neuron pools have been shown to be diverse in the structure of their dendrites, which has a profound influence on the type of input they receive. It has been demonstrated that dendritic organization of motor neuron pools within the spinal gray matter affects if they receive direct monosynaptic sensory input or not (Vrieseling and Arber, 2006). The study demonstrates that the Cutaneous Maximus (CM) muscle is controlled by motor neurons, which have their dendrites aligned with the grey-white matter border in the ventral spinal cord, basically avoiding central gray matter territory. In contrast, the motor neurons innervating Triceps (Tri) muscle have radially projecting dendrites, covering all the spinal gray matter territory around the soma. Electrophysiological recordings from motor neurons and stimulation of sensory neurons revealed that Tri motor neurons receive monosynaptic sensory input, whereas CM motor neurons do not (Vrieseling and Arber, 2006).

Upon elimination of the ETS transcription factor *Pea3*, motor neurons innervating the CM muscle show radially projecting dendrites, extensively covering a large field in the central grey matter. Analogous electrophysiological recordings demonstrated that CM motor neurons

in *Pea3* mutants receive direct monosynaptic sensory input (Vrieseling and Arber, 2006). This exemplifies the importance of the dendritic structure of motor neurons and how it influences the accessibility by sensory neurons. However, this of course applies in principle to any given presynaptic neuron in the spinal cord or the brainstem.

Taken together these studies demonstrate that proper wiring of motor neurons from different motor columns and -pools with their target muscles is crucial for the generation of adequate motor behaviors. In addition the spinal motor neurons have somata and dendritic fields at precise locations, which is important for proper function and integration into the neuronal network.

2.4. Spinal interneuron circuits control motor neuron activity

Distinct interneurons within the spinal cord are generated in different progenitor domains, express different neurotransmitters and project their axons either ipsi- or contralaterally with respect to their cell body (Arber, 2012).

During embryonic development there are ten progenitor domains in the spinal cord that generate different sets of interneurons, with 4 giving rise to ventral interneurons V0 – V3; and 6 generating dorsal interneurons dI1 – dI6. These distinct types of interneurons represent functionally different classes, generating and shaping specific parameters of motor behaviors or integrating various sensory modalities (Goulding, 2009; Alaynick et al., 2011). For the ventral interneurons, it has been shown that commissural V0 interneurons are shaping proper left-right alternation during various speeds. In mice lacking all V0 interneurons, left-right alternation is abolished (Lanuza et al., 2004). However, recently it has been demonstrated that the V0 interneurons can be subdivided according to function. Inhibitory V0 interneurons which are derived from the dorsal V0 domain are required at lower running speeds to generate proper left-right alternation, where as the excitatory ones generated in the ventral V0 domain

are needed at higher running speeds (Talpalar et al., 2013). Besides V0 interneurons influencing left-right alternation, there is a class of V0-derived cholinergic interneurons, which are located in the lamina X around the central canal. They are called partition cells, express the transcription factor *Pitx2* and form direct synaptic connections on motor neurons, called C-boutons (Zagoraiou et al., 2009; Stepien et al., 2010). Mice, in which the gene coding for the choline acetyl transferase (ChAT) has been deleted in these V0-derived interneurons, show a locomotor phenotype during swimming that is not observed in the wild-type situation (Zagoraiou et al., 2009).

The V1 domain was shown to generate ipsilaterally projecting interneurons that influence speed of motor output. The V1 domain is defined by the expression of *Engrailed-1* (*En1*) and ablation of these interneurons, using *En1-DTA* mice, results in a marked slowing of the locomotor cycle (Gosgnach et al., 2006). In addition it has been implicated that the V1 domain gives rise to Ia inhibitory interneurons (IaINs) mediating reciprocal inhibition, since V1-derived interneurons, which express Parvalbumin and receive input from Renshaw cells, have been found in a position, where IaINs have been found in the cat (Alvarez et al., 2005). Motor neurons receive direct excitatory homonymous sensory feedback from the muscle they innervate. However, during a motor behavior such as locomotion antagonistic muscles need to be active out-of-phase. IaIN get input form the sensory neurons of a muscle and make direct inhibitory contacts on motor neurons with opposite function. This mechanism ensures that sensory feedback directly activates homonymous or synergistic motor neurons, but inhibits the antagonistic motor neurons via the IaIN in between. Also Renshaw cells mediating recurrent inhibition, activated by motor neuron axon collaterals and shutting down the very same motor neuron by inhibitory recurrent connection, have been implicated to be generated by the V1 domain, based on immunohistochemistry and position, in agreement with electrophysiological data from the cat (Alvarez et al., 2005).

V2a-derived interneurons express *Chx10* and project their axons ipsilaterally and are necessary to coordinate left-right alternation, by connecting to commissural interneurons that synapse with neurons on the contralateral side of the spinal cord. *Chx10-DTA* mice, lacking V2a neurons, exhibit defects in left-right alternation (Crone et al., 2008).

V3-derived commissural interneurons express Sim1 and control burst robustness of motor neurons. Silencing Sim1 interneurons, using Sim1-TeNT mice results in a disruption of regularity of locomotion (Zhang et al., 2008). Furthermore, it has been recently shown that more than one class of interneurons can control a certain parameter of locomotion; flexor-extensor alternation has been demonstrated to be controlled by V1- and V2b derived interneurons acting in conjunction (Zhang, 2014). Taken together, the ventral spinal interneurons are controlling various parameters of locomotion, by distinct neurotransmitter phenotypes, axonal trajectories and postsynaptic partners (Stepien and Arber, 2008).

The functions of the dorsal interneuron classes are less well understood. The dI3-derived interneurons, expressing *Isl1*, have been shown to be involved in grip strength, allowing fine manipulation of hand and fingers. They make direct excitatory connections to motor neurons and receive monosynaptic input from sensory neurons. Genetic silencing of dI3-derived interneurons led to decreased capabilities in different motor task (Bui et al., 2013). The dI4 domain generates dorsal interneurons expressing the transcription factor *Ptf1a*. These interneurons form inhibitory synapses onto sensory terminals connecting to motor neurons, mediating presynaptic inhibition, in order to control proprioceptive input on motor neurons (Betley et al., 2009).

Lbx1-derived interneurons encompassing dI4 – dI6 have been described at the level of markers expressed, settling position of interneurons and their axonal projections. However, the lack of specific marker genes for only dI5- or dI6-derived interneurons leaves their functional roles currently unknown (Müller et al., 2002; Gross et al., 2002).

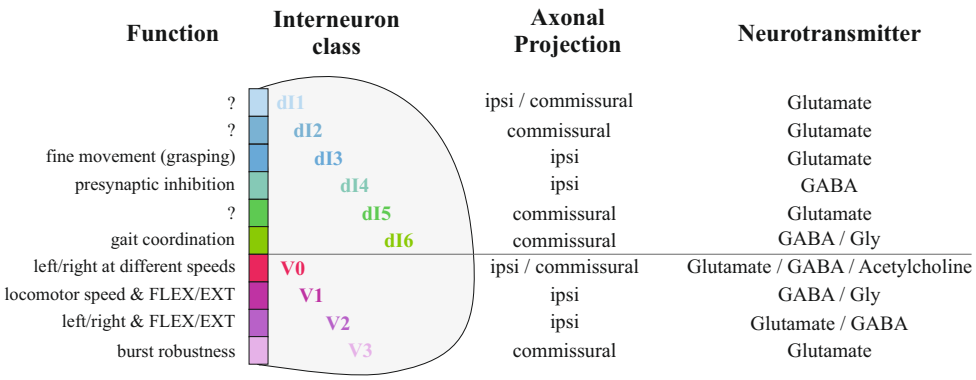

Figure 2. Function, axonal projection and neurotransmitter phenotype of spinal interneurons.
Different progenitor domains in the embryonic spinal cord give rise to distinct classes of spinal interneurons, which exhibit different axonal projection patterns, neurotransmitter phenotypes and functions in controlling locomotion.

This diverse array of spinal interneurons interconnect with each other, integrating commands from the brain, sensory information and ongoing computing within the spinal cord, generating specific motor outputs. The model that is proposed to underlie proper motor output patterns is called central pattern generator (CPG) (Grillner, 2003). CPG refers to interconnected interneurons in the spinal cord, which ultimately generate a specific pattern of motor neuron activity. The theory is that each side of the spinal cord contains CPG modules that control the activity of motor neurons on one side of the body. In addition the CPGs on each side of the spinal cord, interact with each other via commissural interneurons (CINs), which have midline-crossing axons, allowing the interconnection of interneurons on opposite sides, which is critical to have proper left-right coordination (Grillner, 2003). These circuit modules have been modeled in lamprey to demonstrate how they may generate undulating movements in the water and have also been adapted to explain the coordination of locomotion in limbed animals (Grillner 2003; Tresch et al., 2002). In order to understand better the circuits that directly control motor neurons, it is crucial to investigate the distribution and composition of neuronal circuits that synapse monosynaptically with them and hence directly shape their activity.

2.5. Monosynaptically restricted rabies tracing of premotor circuits

Recent development of monosynaptically restricted rabies tracing methodology that reveals premotor interneurons, allows to unravel the circuits that directly control specific motor neuron pools and thus the contraction state of the innervated muscle (Stepien et al., 2010). This technique has shown that premotor circuits of functionally distinct muscles of the limb are different in their overall distribution patterns (Tripodi et al., 2011). However, in order to generate a motor routine like walking, the interplay of limb- and trunk muscles is crucial. Therefore knowledge about the premotor circuits, which control trunk motor neurons, will shed further light on structure and composition of spinal premotor networks controlling distinct muscle types necessary to generate various forms of movements.

2.6. Transcriptionally reprogram motor neurons – *FoxP1* mutant

As described above each motor neuron column and –pool exhibits a specific transcriptional profile, which assigns molecular identity. The expression of *Hox* genes has a profound influence on establishing the spinal motor system and putting the correct sets of motor neurons at defined places within the spinal cord. *Hox* transcription factors act in conjunction with accessory factors, which are crucial for proper function. A *Hox* accessory factor that has been proven to be important in the generation of distinct spinal motor columns is *FoxP1*. In the full knock-out mouse mutant, it was shown that LMC and PGC motor neurons acquire the genetic identity of HMC motor neurons. In *FoxP1* mutants, the spinal cord basically harbors only two motor columns, the MMC (which is unaffected) and the ectopic HMC present at all segmental levels (Dasen et al., 2008). The proposed model for *FoxP1* function is, that it converts ground state HMC motor neurons into PGC and LMC motor neurons in a dose-dependent fashion. There is no *FoxP1* expressed in HMC, intermediate levels are detected in

the PGC, and LMC motor neurons express high levels of *FoxP1*. Recently it has been demonstrated that the regulation of *FoxP1* and motor column specification is at least partially mediated by a microRNA-dependent mechanism. It was shown that microRNA-9 controls the level of *FoxP1* transcript and hence its protein level, in the different spinal motor neuron columns, leading to the already described difference in concentration of *FoxP1* in HMC, PGC and LMC (Otaegi et al., 2011). The study, performed in chick, revealed that overexpression of microRNA-9 led the emergence of more motor neurons with MMC identity, whereas knockdown of microRNA-9 led to the generation of more LMC-like motor neurons. This study supports a model already proposed by *Dasen et al.* in 2008, in which *FoxP1* acts in a dose-dependent fashion. MicroRNA-9 overexpression reduces the amount of *FoxP1*, thereby "pushing" motor neurons away from LMC and PGC fate, in contrast knock-down leads to increased *FoxP1* in motor neurons, followed by increased number of motor neurons with LMC transcriptional program (Otaegi et al., 2011).

Interestingly, in *FoxP1* mutants, ectopic HMC motor neurons at limb levels still project out of the spinal cord, invading the limb, establishing an almost normal innervation pattern of the muscles (Dasen et al., 2008).

The recent generation of a conditional mutant mouse allows removing *FoxP1* only in motor neurons, resulting in mice, which survive after birth but show severe motor deficits. Motor neurons innervating a specific muscle are no longer forming clusters, as seen in the wild-type, but are scattered throughout the ectopic HMC motor column (Sürmeli et al., 2011). However in this mutant the sensory neurons still enter the spinal cord and entering domains, where LMC motor neurons in the wild-type situation are located. In this mutant, the sensory-motor connection specificity is no longer intact, with motor neurons innervating certain muscles and not receiving matched sensory input anymore.

2.7. Beyond the mouse – premotor circuits in the zebrafish

The zebrafish has become an important model system in neuroscience to study networks controlling motor behaviors. The fact that the fish is transparent at early larval stages and more and more neuronal subtypes become genetically accessible makes this system very promising to reveal circuits, manipulate them and study functional consequences. Zebrafish contains interneurons, which are possible homologue counterparts of the ones in the mouse spinal cord (Goulding, 2009). This makes it interesting to study the function of defined interneurons in both species, revealing if some functional properties have been maintained during evolution.

A recent study showed that V2a interneurons in the zebrafish spinal cord form microcircuits driving slow-, intermediate- or fast motor neurons, implying that the recruitment of gradually faster muscle fibers requires distinct circuits within the spinal cord (Ampatzis et al., 2014). It has also been demonstrated that optogenetic activation of V2a interneurons in the zebrafish spinal cord is enough for the induction and maintenance of locomotion (Ljunggren et al., 2014).

In evolutionary terms, the mouse and its motor system represents a rather modern vertebrate system with highly evolved appendages and fine control of extremity limb muscles, allowing grasping or reaching behaviors. However, the vertebrates appeared about 500 million years ago and were aquatic organism without appendages, today represented by for example the lamprey (Jessell and Grillner, 2009). The zebrafish is an interesting model to study the motor system, since it represents an evolutionary older state compared to the mouse. Interestingly, the zebrafish and the mouse, both have axial muscles, which are innervated by MMC motor neurons. However, in the zebrafish, the axial muscle represent the main source of force generation in order to move within the water, where as in the mouse they are mainly involved

in postural control. From this evolutionary and functional perspective it would be of great interest to compare the circuits controlling axial muscles in the zebrafish versus the mouse.

3. Distinct limb and trunk premotor circuits establish laterality in the spinal cord

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3.1. Summary

Movement coordination between opposite body sides relies on neuronal circuits capable of controlling muscle contractions according to motor commands. Trunk and limb muscles engage in distinctly lateralized behaviors, yet how regulatory spinal circuitry differs is less clear. Here we intersect virus technology and mouse genetics to unravel striking distribution differences of interneurons connected to functionally distinct motor neurons. We find that premotor interneurons conveying information to axial motor neurons reside in symmetrically-balanced locations while mostly ipsilateral premotor interneurons synapse with limb-innervating motor neurons, especially those innervating more distal muscles. We show that observed axial and limb distribution differences reflect specific premotor interneuron subpopulations defined by genetic and neurotransmitter identity. Synaptic input across the midline reaches axial motor neurons preferentially through commissural axon arborization and to a lesser extent through midline-crossing dendrites capturing contralateral synaptic input. Together, our findings provide insight into principles of circuit organization underlying weighted lateralization of movement.

3.2. Introduction

Motor behavior reflects the sequential contraction of many muscles, moving the body according to the commands of the nervous system. An important aspect in the control of movement is the coordination of motor programs between opposite body halves. The degree of lateralization of a movement and as a consequence the need for motor output pathway interaction regulating ipsi- and contralateral muscle contractions differ depending on the type of movement executed. Whereas basic locomotion and posture require careful bilateral coordination of muscle contractions to biomechanically stabilize the animal, lateralized movements to independently control muscle groups on opposite sides of the body are essential for uncoupled manipulative activities with extremities. While such behavioral observations are straightforward, the organization of neuronal circuitry mediating these distinct programs is still under investigation.

Execution of motor programs relies on the temporally precise activation of motor neurons in the spinal cord regulating the contraction of skeletal muscles as elementary units of movement. Motor neurons in the mammalian spinal cord exhibit several layers of organization reflecting their functionally distinct roles in the control of movement. Whereas motor neurons innervating limb muscles reside in the lateral motor column (LMC) at both cervical and lumbar spinal levels, the more proximal axial and body wall muscles are targeted by motor neurons resident in medial (MMC; all spinal levels) and hypaxial (HMC; thoracic levels) motor columns (Brink et al., 1979; Dasen and Jessell, 2009; Gutman et al., 1993; Vanderhorst and Holstege, 1997). Motor columns can be further subdivided into pools each innervating a separate muscle. Motor neuron pools innervating limb muscles are topographically organized, and cell body positions in the spinal cord correlate with proximo-distal axis of the limb muscle innervated (McHanwell and Biscoe, 1981; Romanes, 1951; Vanderhorst and Holstege, 1997). This organization results in a grid in which the more

ventrally positioned LMC motor neuron pools innervate proximal limb muscles and progressively more dorsal motor neurons project to more distal limb muscles. Developmental studies revealed the involvement of transcription factors and regulated cell surface molecules in the establishment of motor column- and pool-specific axonal trajectories, thereby providing detailed mechanistic insight into this process (Bonanomi and Pfaff, 2010; Dasen et al., 2005; De Marco Garcia and Jessell, 2008; Kania et al., 2000; Philippidou and Dasen, 2013). In contrast, the development of central connectivity patterns to distinct motor neuron pools in order to ensure differential motor output profiles according to these functional subdivisions remains surprisingly unexplored.

Commissural interneurons are essential to connect circuits on opposite sides of the spinal cord. Work in aquatic vertebrates such as lamprey proposes a circuit model in which inhibitory commissural interneurons connect to excitatory interneuron modules and motor neurons across the midline resulting in reciprocal inhibition of left and right body sides (Buchanan, 1982, 1999; Grillner, 2003; Kiehn, 2011). Commissural communication in the mammalian spinal cord is significantly more complex, but the general need for carefully balanced excitation/inhibition (E/I) ratios by midline-crossing axons is conserved (Jankowska, 2008; Kiehn, 2011). Several transgenic mouse models with specific genetic mutations affecting commissural neurotransmitter balance exhibit severe perturbation in left-right motor coordination (Arber, 2012; Goulding and Pfaff, 2005; Kiehn, 2011; Kullander et al., 2003; Lanuza et al., 2004; Talpalar et al., 2013), and pharmacological blockade of inhibition leads to complete loss of alternation in sided motor output (Cohen and Harris-Warrick, 1984; Cowley and Schmidt, 1995; Kullander et al., 2003). Together, these findings suggest that connectivity and neurotransmitter phenotype of commissural circuit modules fulfill an important role in ensuring appropriately weighted laterality of motor output.

Different spinal interneuron populations derive from separate progenitor domains during development and can be marked genetically by the expression of transcriptional programs subdividing interneurons into 4 ventrally-derived (V0-V3) and 6 dorsally-derived (dI1-dI6) cardinal classes (Alaynick et al., 2011; Arber, 2012; Goulding and Pfaff, 2005; Kiehn, 2011). A common theme emerging from these studies is that genetically defined spinal interneuron populations often exhibit laterality in their projection trajectories, arborizing predominantly ipsi- or contralaterally in the spinal cord. Electrophysiological and anatomical studies demonstrate that motor neurons receive direct synaptic input from many different functional classes of spinal interneurons including ipsi- and contralateral subpopulations (Hultborn et al., 1971; Jankowska, 2008; Jankowska et al., 2009; McCrea and Rybak, 2008; Renshaw, 1941) and recent work begins to align functional subtypes to genetic identity (Alaynick et al., 2011; Arber, 2012; Kiehn, 2011). Moreover, overall distributions of premotor interneurons exhibiting direct connections to motor neurons have been assessed by virtue of transsynaptic rabies virus approaches, revealing biased ipsilateral residence for interneurons connected to several LMC motor neuron pools (Stepien et al., 2010; Tripodi et al., 2011). It remains to be explored how motor neuron function and biomechanical properties of innervated muscle targets are matched. This question is particularly pertinent for how connectivity to functionally distinct motor neurons by spinal premotor interneuron subtypes diverges and the mechanisms by which such distinctions emerge.

Using virus technology intersectionally with mouse genetics, here we reveal different weights in laterality of spinal premotor interneuron distributions and sources of excitation-inhibition stratified by motor columnar and pool identity. MMC motor neurons receive significantly more direct input from contralateral interneurons than LMC motor neuron pools, themselves exhibiting a gradual decrease in the degree of direct contralateral synaptic input in correlation with more dorsal cell body position. While total E/I balance for premotor input is matched

across columns, sources of inhibition are opposite with dominant inhibitory input to MMC by contralateral and to LMC by ipsilateral spinal interneurons. We find that commissural axon trajectories favor direct synaptic access to MMC over LMC motor neurons, and that MMC dendrites elaborate midline-crossing branches to capture synaptic input derived from unilaterally projecting contralateral interneurons. Together, our findings demonstrate that spinal interneurons communicate with contralateral motor neurons at distinct stringencies and are established by different mechanisms. These communication channels provide a higher degree of direct input to motor neurons innervating muscle groups closer to the body axis with increased demand on bilateral motor coordination than to motor neurons innervating distal limb muscles with more functional independence, providing insight into the principles of circuit organization underlying lateralization of movement.

3.3. Results

3.3.1. Distinct distribution of premotor interneurons connected to axial and limb motor pools

To compare the distribution of spinal interneurons with direct connections to motor neurons innervating axial or limb muscles, we used transsynaptic virus-based technology with monosynaptically-restricted labeling (Stepien et al., 2010; Tripodi et al., 2011). Making use of their differential columnar organization and associated peripheral trajectories (Figure 3A, B), we infected MMC or LMC motor neurons retrogradely through axial or hindlimb intramuscular co-injection of glycoprotein-deficient Rabies virus encoding fluorescent marker protein (Rab-FP) and adeno-associated virus expressing glycoprotein (AAV-G) (Figure 3C). As a representative MMC motor neuron pool, we used the lumbar extensors of the spine (Brink et al., 1979; Brink and Pfaff, 1980), and as lumbar LMC motor neuron pool the thigh

muscle Quadriceps (Q), unless otherwise stated. We found that many spinal interneurons were labeled upon initiation of transsynaptic spread from either the LMC or MMC motor neuron pool (Figure 3D, G).

To assess and compare distribution patterns for LMC and MMC spinal premotor interneurons quantitatively, we assigned x-y-z coordinates to each Rab-FP marked neuron in spinal segments from mid-thoracic (T8) to sacral (S1) levels. Transversal projection analysis revealed that MMC premotor interneuron distribution is highly distinct from the one observed for LMC premotor neurons (Figure 3E, H). Both LMC and MMC cohorts were broadly distributed in the spinal cord ipsilateral to muscle injection (Figure 3E, H). In contrast, while LMC premotor interneurons located contralateral to injection were largely restricted to a ventro-medial domain in Rexed's lamina VIII (Figure 3E), contralateral MMC-premotor neurons distributed much more broadly (Figure 3H). Moreover, in an overall quantification of ipsi- versus contralateral spinal residence, we found that 75±3% of all LMC premotor neurons were located ipsilateral to injection (Figure 3F), in agreement with previous results (Stepien et al., 2010). In sharp contrast, MMC premotor interneurons exhibited a nearly symmetrically balanced distribution with a slight prevalence for neurons residing contralaterally to muscle injection (59±1%) (Figure 3I). These differences were also obvious in an overall medio-lateral interneuron density analysis, for which the highest peak of LMC premotor interneuron density was found ipsilaterally, whereas MMC premotor interneurons displayed the highest neuronal density contralateral to injection (Figure 3J). Analysis of overall distribution patterns across different mice demonstrated that intra-columnar (MMC::MMC or LMC::LMC) values were highly correlated, whereas inter-columnar comparison between MMC and LMC premotor patterns segregated into distinct clusters (Figure 3K).

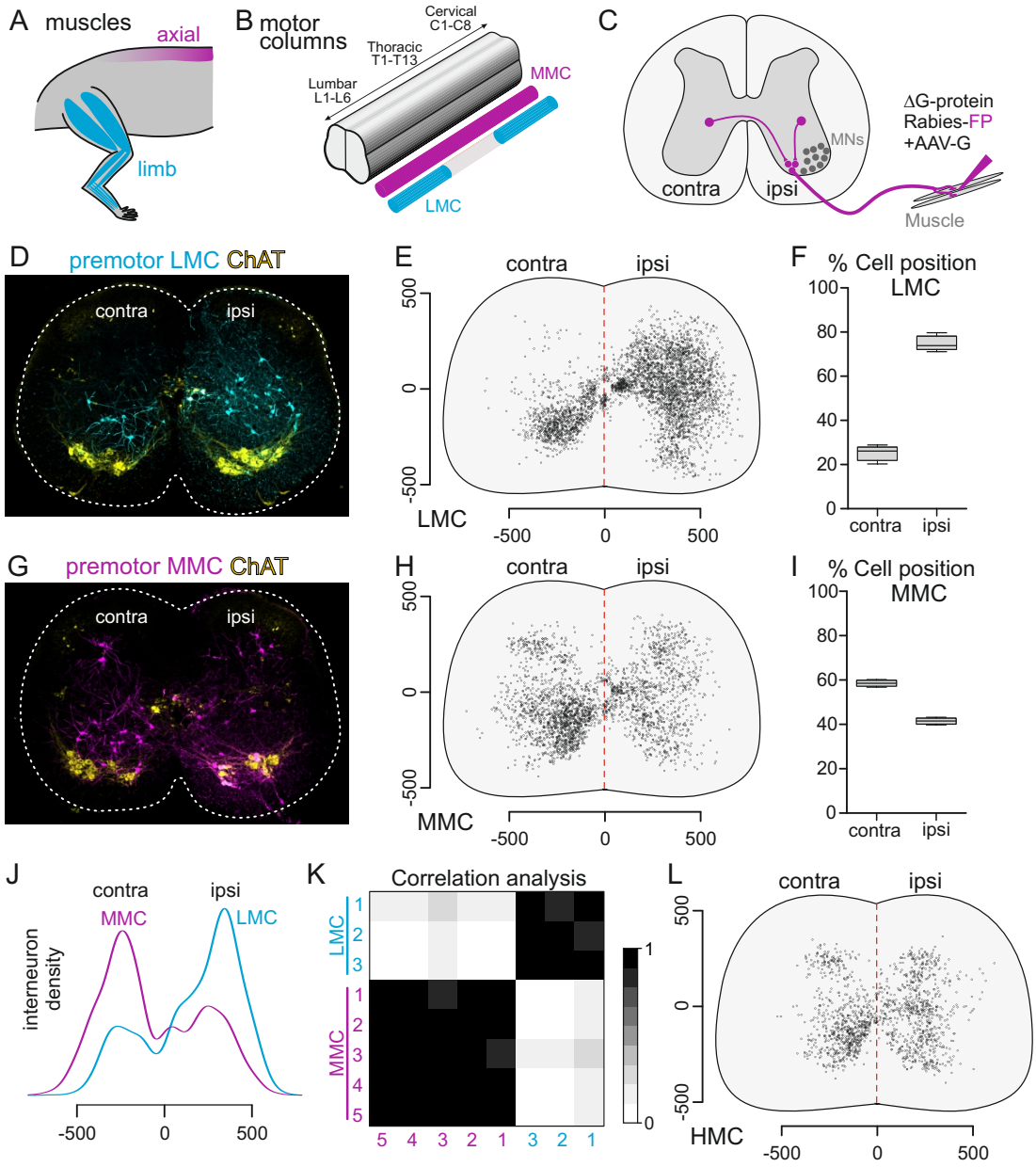

Figure 3. Symmetrical distribution of axial premotor network

(A) Scheme depicting the location of axial (magenta) and limb muscles (blue). (B) Axial muscles are innervated by motor neurons of the medial motor column (MMC) present at all segmental levels of the spinal cord. In contrast, motor neurons controlling limb muscles reside in the segmentally restricted lateral motor columns (LMC). (C) Diagram illustrating the employed monosynaptic rabies-tracing strategy. The target muscle is co-injected with ΔG-protein Rabies-FP and AAV-G, leading to infection and fluorescent labeling of the innervating motor neuron pool as well as connected premotor interneurons (see also: (Stepien et al., 2010)). (D-F) Transverse spinal cord section at L1, showing LMC (Q) premotor interneurons (turquoise) and ChAT^{ON} motor neurons (yellow). Scatter plot shows digitally reconstructed distribution of premotor interneurons (each dot represents soma position) from T8 to S1 (E). Boxplot displays dominant ipsilateral LMC (Q) premotor interneuron distribution (n=5) (F). (G-I) Transverse spinal cord section at L1, showing axial premotor

interneurons (magenta) and ChAT^{ON} motor neurons (yellow). Scatter plot shows digitally reconstructed distribution of premotor interneurons (each dot represents soma position) from T8 to S1 (H). Boxplot displays symmetrical MMC premotor interneuron distribution (n=5) (I). (J) Medio-lateral premotor interneuron density differences between MMC and LMC (Q) premotor circuits. MMC premotor density peak is contralateral to injection, whereas highest premotor density for LMC (Q) premotor is ipsilateral to injection (MMC n=5; LMC n=3). (K) Correlation analysis shows significant differences between MMC and LMC (Q) premotor circuits. Moreover, premotor interneuron distribution patterns in different mice are highly reproducible.
(L) Digitally reconstructed HMC premotor network, exhibiting symmetrical distribution of premotor interneurons similar to MMC (each dot represents soma of premotor interneuron).

To determine whether the observed distribution for MMC premotor interneurons is a more general feature of muscles spanning along the body axis, we next set out to map the distribution of premotor interneurons connected to motor neurons of the hypaxial motor column (HMC), innervating intercostal and abdominal body wall muscles. We found that the HMC premotor network distribution is strikingly similar to the one observed for MMC. Quantitatively, ~50% of HMC premotor interneurons were located in the spinal cord contralateral to muscle injection (Figure 3L). Together, these data demonstrate that both MMC and HMC motor columns innervating proximal muscles including trunk and body wall muscles receive major direct synaptic input from contralateral spinal interneurons.

3.3.2. Proximo-distal limb axis scales with decreasing contralateral premotor input

The observation that MMC and HMC are both motor columns innervating muscles close to the body axis prompted us to determine the laterality values of premotor inputs responsible for the control of muscles at different proximo-distal positions along the limb axis and innervated by LMC motor neuron pools with progressively more dorsal cell body position in the spinal cord (Figure 4A). To directly address this question, we chose to compare lumbar motor neuron pools innervating three muscle groups with progressively more distal location

along the mouse hindlimb axis. We analyzed the distribution of premotor interneurons connected to motor neurons innervating the thigh muscle Q, the more distally located calf muscle tibialis anterior (TA) and the most distally positioned foot muscles (Figure 4A).

We observed the highest value in the percentage of contralaterally-positioned LMC premotor interneurons for cohorts connected to the Q motor neuron pool innervating the most proximally studied limb muscle (25±3%), with decreasing values for the progressively more distally positioned TA and foot muscles (Figure 4A-E).

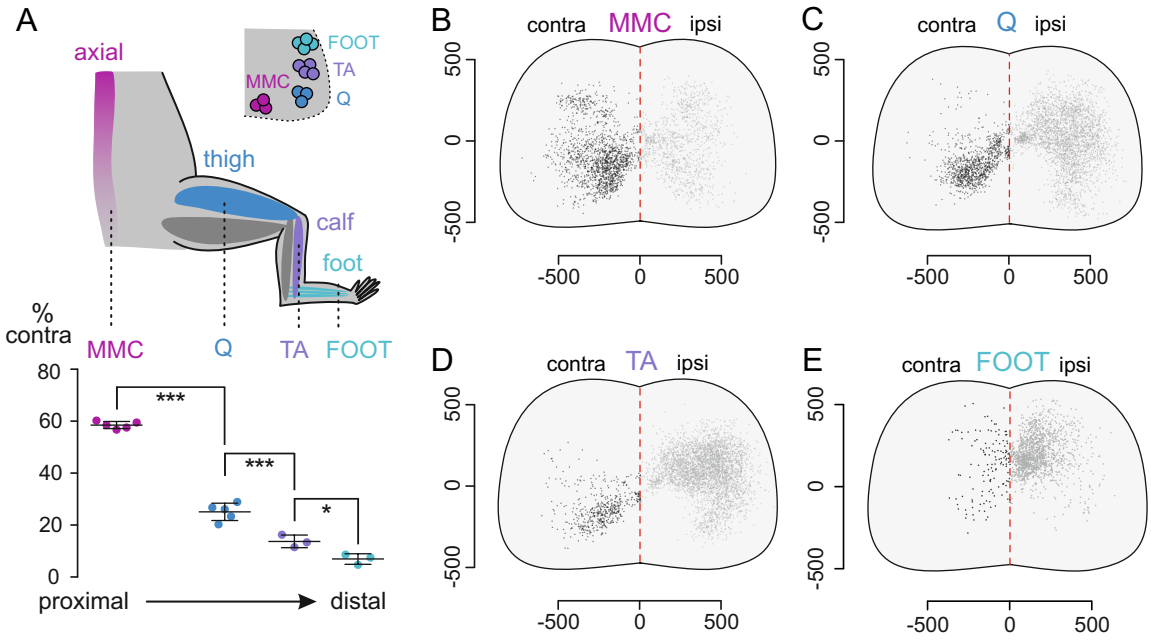

Figure 4. Differential control of LMC motor pools by contralateral premotor network

(A) Scheme illustrating correlation between muscle position along the proximo-distal body axis and the fraction of contralateral premotor interneurons of the motor neuron pool innervating the respective muscle. Motor neurons controlling proximal muscles exhibit higher contralateral premotor fractions than distal muscle counterparts. Top right: approximate position of analyzed motor neuron pools in ventral spinal quadrant is shown. (B-E) Digital reconstructions of premotor networks of different motor neuron pools analyzed. Motor neurons innervating axial muscles exhibit ~60% of contralateral premotor interneurons. Moving along the proximo-distal axis of the hindlimb, the access to contralateral premotor interneurons gradually decreases from thigh to foot motor neurons (ANOVA $p<0.0001$, MMC n=5; Q n=5; TA n=3; Foot n=3).

This observation was confirmed using an alternative method with centrally targeted motor neuron infection to initiate transsynaptic spread (Figure 5).

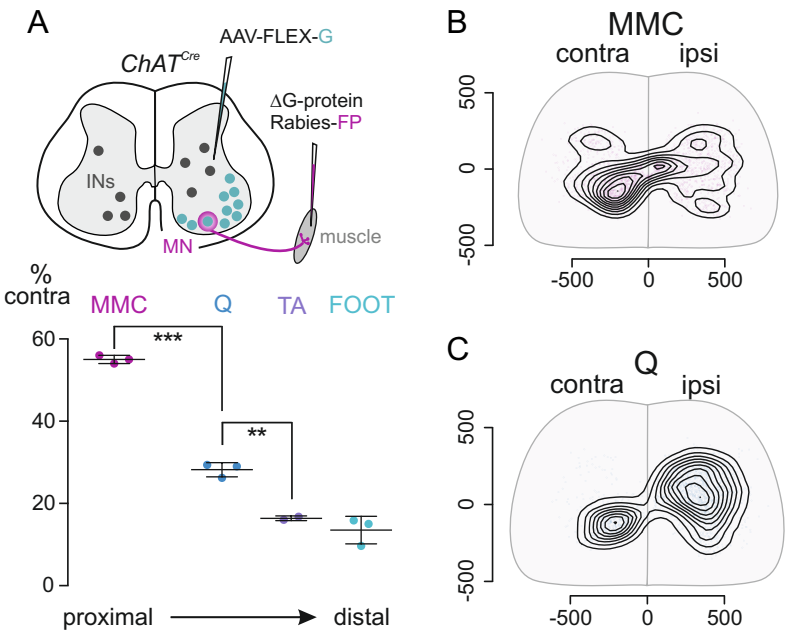

Figure 5. Premotor mapping by use of central motor neuron infection to express G

(A) Diagram for alternative monosynaptic rabies-tracing strategy to map premotor interneuron distribution. Intraspinal injection of AAV-CAG-FLEX-G in *ChAT*^{Cre} mice was sequentially followed by rabies-FP injection into axial or limb muscles (Q, TA, foot). This method reveals similarly decreasing contralateral access from MMC to dorsal LMC motor neuron pools as conventional tracing methods (ANOVA $p<0.0001$, MMC n=3; Q n=3; TA n=2; Foot n=3). (B-C) Scatter and overlaid density plots show distribution pattern of MMC and Q premotor interneurons (every dot represent soma position).

Together, these findings provide evidence that motor neuron pools innervating limb muscles receive progressively less direct input from contralateral spinal interneurons the more distal the innervated limb muscle is located along the limb axis and the more dorsally the corresponding motor neuron pool resides in the spinal cord. These findings raise the question of the cellular origin(s) responsible for achieving such different ratios of contra- versus ipsilateral contribution to the premotor network of distinct motor columns.

3.3.3. Interneuron subtypes coopted by both MMC and LMC motor neurons

We first set out to determine whether some spinal interneuron subtypes are recruited by both MMC and LMC motor neurons. Two well-studied interneuron populations, which are thought to represent unique subtypes based on functional criteria and for which also molecular markers exist, are cholinergic partition cells and Renshaw cells.

Cholinergic partition cells provide neuromodulatory input to motor neurons through C-boutons and are located in Rexed's lamina X around the central canal (Conradi and Skoglund, 1969; Hellstrom et al., 2003; Miles et al., 2007). To map the distribution of partition cells connected to MMC or LMC motor neurons, we gated the analysis specifically to cholinergic premotor neurons upon application of monosynaptic rabies injections to corresponding muscles (Figure 6A). We found that for both the LMC and MMC premotor network, the majority of connected partition cells were positioned ipsilateral to muscle injection, and a smaller fraction was found contralateral to injection (Figure 6B-D). These findings demonstrate that the contralateral dominance of the MMC premotor network is not a general feature of all interneuron subtypes, and that certain defined subpopulations such as cholinergic partition cells exhibit similar distribution patterns and ipsi-contra ratios for MMC and LMC.

To determine whether cholinergic partition cells can represent truly shared interneuron populations between MMC and LMC or whether these are separate populations, we made use of the observation that a fraction of partition cells establish bifurcating axonal arborizations to contact motor neurons contralateral to injection (Stepien et al., 2010). In experiments marking MMC premotor neurons by unilateral monosynaptic rabies virus injections into axial muscles, we analyzed whether vAChT^{ON} C-boutons labeled by rabies-expressed fluorescent protein

contact LMC motor neurons in the contralateral spinal cord (Figure 6E). We found that vAChT^{ON} MMC-premotor terminals indeed make close contact with LMC motor neurons, suggesting that at least a fraction of cholinergic partition cells establish divergent synaptic connections to both MMC and LMC motor neurons and are hence truly shared interneurons.

We next assessed the distribution of Calbindin^{ON} Renshaw cells connected to MMC motor neurons (Figure 6F) (Alvarez et al., 2005; Renshaw, 1941). We found that MMC-premotor virus marked Renshaw cells resided in proximity to motor neurons close to initiation of transsynaptic spread and exclusively on the side ipsilateral to virus injection, connectivity similar to the one described in cat and assessing recurrent inhibition to axial motor neurons electrophysiologically (Jankowska and Odutola, 1980). The observed pattern was highly reminiscent to the one previously observed for LMC motor neurons (Stepien et al., 2010), providing evidence that Renshaw cells represent a functional interneuron subtype commonly recruited by many motor neuron subtypes.

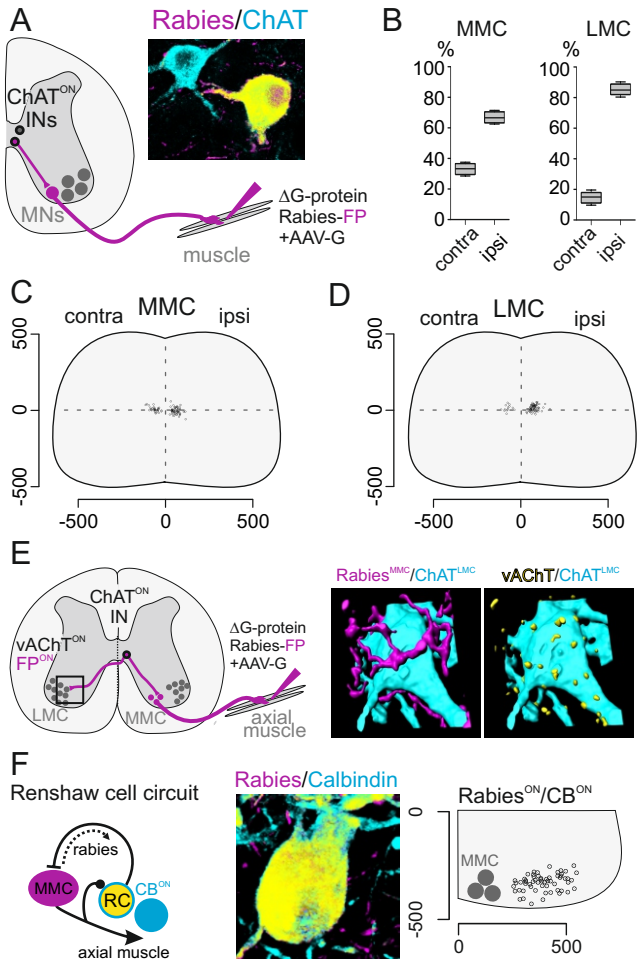

Figure 6. Interneuron subtypes coopted by both MMC and LMC motor neurons

(A) Use of monosynaptic rabies tracing to reveal partition cells (ChAT^{ON} premotor interneurons in Rexed's lamina X) directly connected to motor neurons. (B-D) Partition cells are part of the LMC (Q) - as well as MMC

premotor network, with dominant ipsilateral contribution for both premotor populations (MMC n=5; LMC n=5). (E) Monosynaptic rabies tracing from MMC labels $ChAT^{ON}$ partition cells. High resolution imaging of contralateral LMC area reveals rabies labeled axons forming $vAChT^{ON}$ C-bouton contacts with $ChAT^{ON}$ LMC motor neurons. This indicates that at least a fraction of partition cells, which are part of the MMC premotor network, have an axon collateral directly connecting to LMC on the opposite side of the spinal cord. (F) Renshaw cells ($Calbindin^{ON}$ premotor interneurons in the most ventral part of the grey matter, mediating recurrent inhibition) are part of the MMC premotor circuit. They are located in a ventro-lateral domain with respect to the MMC, coherent with previous findings on limb-muscle innervating motor neuron pools (Stepien et al., 2010).

Together, these findings demonstrate that premotor synaptic input to MMC and LMC motor neuron pools examined originates from common subsets of spinal interneurons distributed in similar overall patterns. At the same time, they put further emphasis on the important question of how the overall distinct distribution patterns between MMC and LMC premotor interneurons can be explained, and which interneuron subtypes contribute to these patterns.

3.3.4. Lbx1-derived interneurons connected differentially to MMC and LMC motor neurons

MMC premotor interneurons exhibit a much more prominent contribution to the contralateral premotor network than their LMC counterparts, prompting us to begin to dissect their identity and connectivity profiles. We noted that contralateral MMC premotor interneurons can largely be divided into two main categories: (1) a ventral population overlapping in occupied territory approximately with Rexed's lamina VIII; and (2) a population in the intermediate spinal cord dorsal to the central canal, which is essentially devoid of LMC premotor interneurons. Spinal neurons developmentally derived from progenitor domains dI4-6 express the transcription factor Lbx1 (Gross et al., 2002; Muller et al., 2002) and can be visualized at mature stages by intersectional mouse genetics crossing $Lbx1^{Cre}$ and reporter mice ($Tau^{lox\text{-}STOP\text{-}lox\text{-}mGFP\text{-}INLA}$ or $Tau^{lox\text{-}STOP\text{-}lox\text{-}Flp\text{-}INLA}$ mice; Figure 7A) (Hippenmeyer et al., 2005; Pivetta et al., 2014; Tripodi et al., 2011). The neuronal cohort derived from dI4-6 progenitors

comprises populations settling in the intermediate and dorsal spinal cord (dI4; dI5) as well as the ventrally migrating dI6 commissural neuron population settling in Rexed's lamina VIII (Alaynick et al., 2011; Gross et al., 2002; Muller et al., 2002), thus representing a possible genetic identity tag for at least a fraction of contralateral MMC premotor interneurons.

We therefore mapped the spinal distribution of $Lbx1^{LacZON}$ MMC and LMC (Q) premotor interneurons using monosynaptic rabies injections into axial and Q muscles in mice with genetically marked Lbx1-derived neurons (Figure 7A-C). We subdivided the spinal cord into four quadrants according to neuronal residence ventral or dorsal to the central canal, and ipsi- or contralateral to muscle injection (Figure 7D). We found that the large majority of $Lbx1^{LacZON}$ LMC premotor interneurons were located in the ipsilateral dorsal quadrant, whereas the other three quadrants each only contributed minor synaptic input to LMC motor neurons (Figure 7B, D, E). In contrast, a very different picture emerged for MMC premotor interneurons for which >50% of all $Lbx1^{LacZON}$ neurons resided in the contralateral ventral quadrant (Figure 7C-E). In addition, the contribution of interneurons to the contralateral dorsal quadrant was ~2.5 fold higher than for the corresponding LMC population, whereas MMC premotor neurons in the ipsilateral dorsal quadrant were ~3.5 fold less numerous than LMC premotor interneurons (Figure 7B-E).

Together, these findings reveal major differences in the contributions of $Lbx1^{LacZON}$ neurons to the premotor network of MMC and LMC motor neurons respectively (Figure 7A-E). Most strikingly, Lbx1-derived MMC premotor interneurons residing in Rexed's lamina VIII and hence most likely representing inhibitory dI6 commissural neurons made up the dominant population in the cohort (Figure 7D, E). Much in contrast, Lbx1-derived LMC premotor interneurons provide the most pronounced contribution from the ipsilateral intermediate spinal cord to motor neurons (Figure 7D, E). These observations suggest that functionally

distinct motor columns recruit direct synaptic input to highly varying degrees from different spinal interneuron cohorts and that these can be identified by a combination of spinal location and genetic marking by progenitor domain origin during development.

3.3.5. Isl1-derived interneurons connect preferentially to LMC motor neurons

Lbx1-premotor interneuron analysis demonstrated that differential connectivity profiles of premotor interneurons to MMC and LMC motor neurons ipsilateral to injection can be pronounced despite the fact that no obvious gaps in spinal occupancy between the two cohorts are evident at the overall premotor level. These findings prompted us to further dissect the ipsilateral premotor network assessing the status of premotor interneurons derived from the single progenitor domain dI3. These neurons are marked by the transcription factor Isl1, connect to LMC motor neurons, and were described to contribute to circuitry regulating grasping behavior (Bui et al., 2013; Stepien et al., 2010).

To analyze the connectivity profiles between dI3 spinal interneurons and MMC or LMC motor neurons, we applied a recently developed strategy intersectionally using mouse genetics and intraspinal viral injections (Pivetta et al., 2014). Interbreeding of $Isl1^{Cre}$ mice with $Tau^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ mice leads to permanent expression of FLP recombinase in dI3-derived spinal interneurons. Local intraspinal injection of FRT-flanked AAV viruses conditionally expressing a fusion protein between Synaptophysin and GFP (AAV-FRT-SynGFP) in these mice can be used to track synaptic output of marked neurons (Pivetta et al., 2014). DI3 neurons labeled using this approach at L1 projected exclusively ipsilaterally in the spinal cord (data not shown), in agreement with previous results (Bui et al., 2013; Stepien et al., 2010). We found that targeting of spinal motor neurons was highly distinct for motor neurons of different columnar identity. Whereas LMC motor neurons analyzed at L2/L3

spinal levels were readily contacted by dI3 marked interneurons, MMC neurons at the same segmental level were largely devoid of such synaptic input (Figure 7F).

Together, these findings lend further support to the notion that LMC and MMC motor neurons receive differential input from selected spinal interneuron subpopulations, likely contributing to their distinct functional roles and recruitment during motor behaviors.

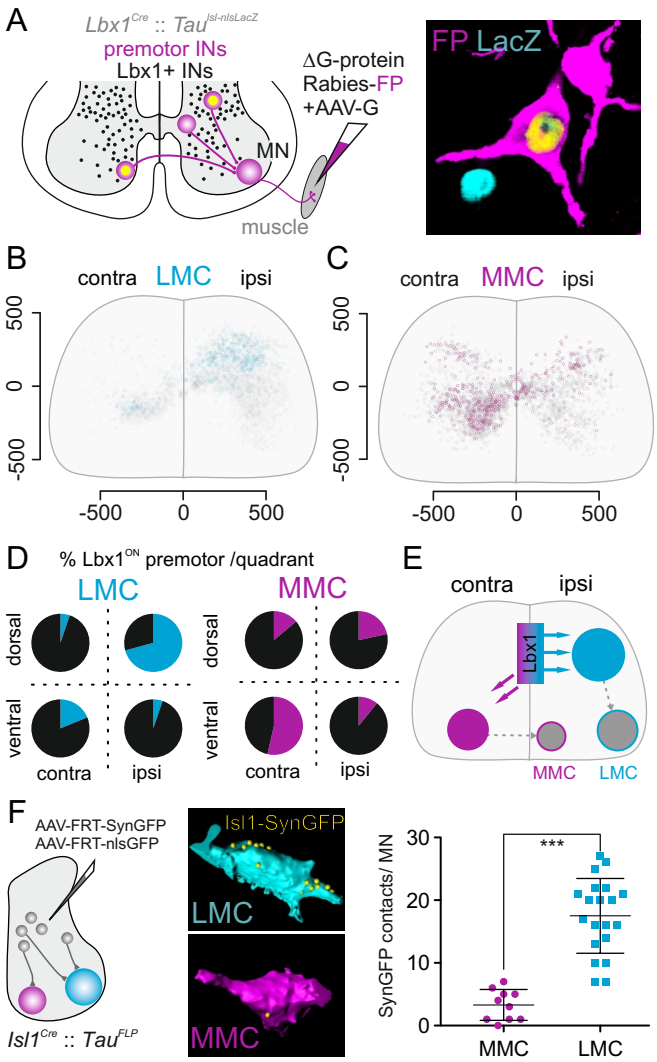

Figure 7. Premotor populations with motor column preferences

(A) Monosynaptic rabies tracing strategy from either LMC (Q) or axial muscles in an Lbx1^{LacZON} background reveals premotor interneurons derived from the Lbx1 progenitor domain. Lbx1-derived premotor interneurons are FP^{ON}/Lbx1^{LacZON} (right). (B-C) Digital reconstruction of premotor interneurons (grey) and Rabies^{ON}/Lbx1^{LacZON} Q (B) or MMC (C) premotor interneurons displayed in color. (D) Spinal quadrant analysis of Lbx1-derived premotor interneurons reveals differential contribution for the different premotor circuits. The majority of Lbx1-derived MMC premotor interneurons resides in the contralateral ventral spinal cord, whereas the ipsilateral dorsal spinal cord provides the main source of Lbx1-derived interneurons within the LMC premotor cohort (MMC n=2; LMC n=2). (E) Summary diagram illustrating observed differential contribution of the Lbx1-domain to MMC- versus LMC premotor networks. (F) Anterograde synaptic-tagging strategy to reveal input to ChAT^{ON} MMC- and LMC motor neurons from dI3 derived Isl1^{ON} spinal interneurons. Isl1-SynGFP input to ChAT^{ON} MMC (magenta) and LMC (turquoise) motor

neurons was reconstructed (middle). Quantification of Isl1-SynGFP contacts per ChAT^{ON} MMC- or LMC motor neuron at same segmental level reveals significantly more Isl1-SynGFP contacts on LMC- than MMC motor neurons (MMC MNs n=10; LMC MNs n=20).

3.3.6. Distinct origin of spinal inhibition to MMC and LMC motor neurons

To elucidate the functional implications of differential distribution of MMC and LMC premotor interneurons, insight in neurotransmitter identity and in particular E/I balance across the premotor network provides important information. Two major and functionally antagonistic sources of spinal interneurons connecting to motor neurons are vGAT^{ON} inhibitory neurons (GABAergic and/or glycinergic) and vGlut2^{ON} glutamatergic neurons. To map the distribution pattern of vGAT^{ON} neurons within the MMC and LMC premotor cohort, we used mice with transgenically marked vGAT^{ON} neurons (nlsLacZ), derived from intersectional breeding of *vGAT^{Cre}* and *Tau$^{lsl\text{-}INLA}$* mice (Hippenmeyer et al., 2005; Vong et al., 2011). Upon injection of monosynaptic rabies virus into axial or LMC (Q) muscles, we determined the position of vGAT^{ON} neurons marked by rabies-expressed FP (Figure 8A, B), a strategy targeting both GABAergic and glycinergic interneuron populations (Wojcik et al., 2006).

We first assessed the overall inhibitory component within the premotor network, including ipsi- and contralateral populations. We found that ~40% of all marked neurons were vGAT^{ON} for both MMC and LMC premotor populations (Figure 8C), demonstrating that E/I balance at the overall premotor level is comparable between these two motor columns. Moreover, we analyzed overall distribution profiles of all marked premotor and vGAT^{ON}/premotor interneurons of each cohort separately, using contour density analysis. We found that MMC premotor neurons as a whole population exhibited a very similar distribution profile to vGAT^{ON} MMC premotor neurons, and the same feature was also observed for LMC premotor

neurons (Figure 8D-G). These findings support the notion that within the overall premotor population, vGAT^{ON} neurons are distributed in a seemingly random pattern.

Ipsi- and contralateral spinal interneurons convey distinct information to motor neurons. We therefore determined the proportion of vGAT^{ON} MMC or LMC premotor interneurons resident ipsi- or contralaterally to muscle injection (Figure 8H, I). We found that of all inhibitory MMC premotor neurons, ~68% were located in the contralateral spinal cord (Figure 8H, I). In contrast, ~83% of inhibitory LMC premotor interneurons were located ipsilaterally (Figure 8H, I).

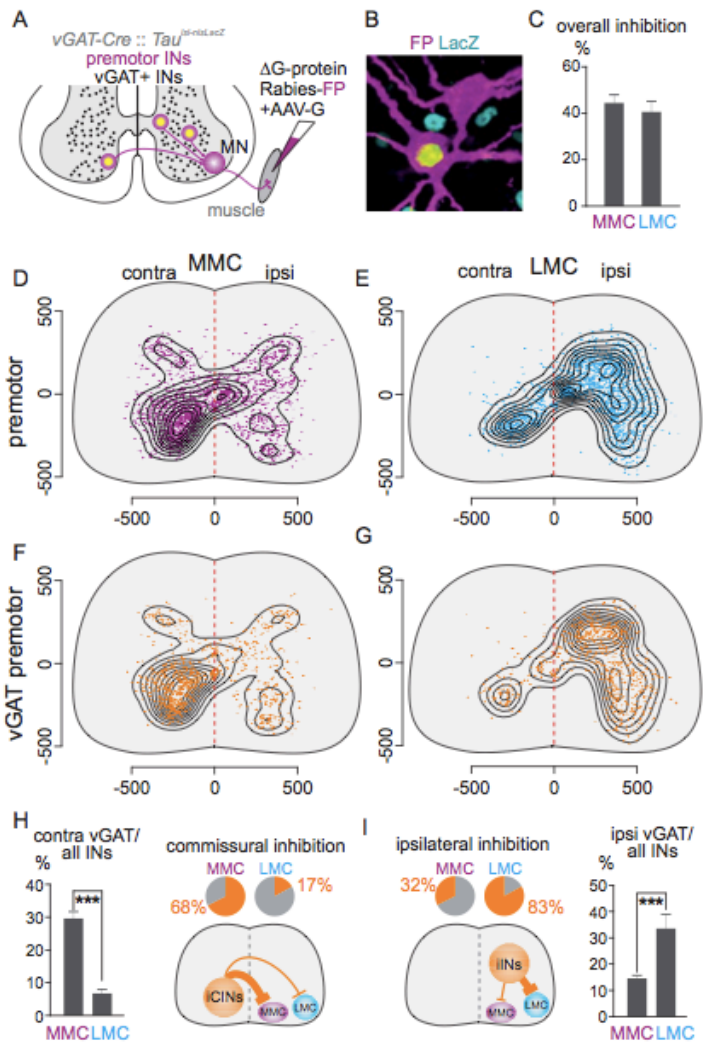

Figure 8. MMC and LMC controlled by opposing inhibitory premotor networks

(A, B) Monosynaptic rabies tracing strategy in vGAT^{LacZON} mice reveals FP^{ON}/vGAT^{LacZON} inhibitory premotor interneurons (yellow). (C) MMC and LMC (Q) receive comparable amount of overall spinal premotor inhibition (MMC n=5; LMC n=4). (D-G) MMC and LMC (Q) show uniform distribution of inhibitory premotor interneurons (orange) within the entire premotor cohort (MMC: magenta; LMC: turquoise). (H-I) Comparison of contralateral and ipsilateral contribution of inhibitory premotor interneurons displays a dominance of inhibition from the contralateral side to MMC motor neurons compared to LMC. Conversely, dominant inhibition on LMC compared to MMC is observed on the ipsilateral

side of the spinal cord. Bar plots show the fraction of contralateral or ipsilateral vGAT^{ON} premotor interneurons normalized to all premotor interneurons. Pie charts illustrate the fraction of commissural or ipsilateral vGAT^{ON} premotor interneurons normalized the all vGAT^{ON} premotor interneurons (MMC n=5; LMC n=4).

Thus, despite comparable overall fractions of inhibitory interneurons in the premotor network, strikingly distinct and essentially opposite contributions are derived from the ipsi- or contralateral spinal side to muscle injection for the LMC and MMC premotor network respectively. Conversely, comparative analysis of putative excitatory premotor interneuron distributions by digital subtraction revealed that these are less strongly biased than inhibitory counterparts (Figure 9).

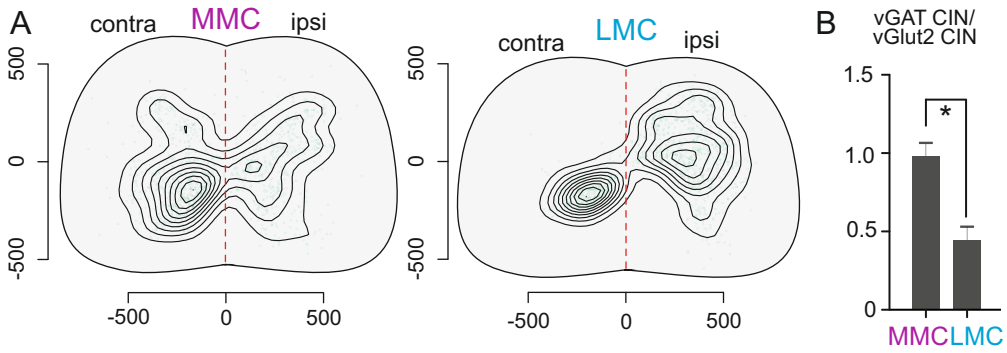

Figure 9. Control of MMC and LMC by putative excitatory interneurons

(A) Digital subtractions of all mapped premotor interneurons minus vGAT^{ON} neurons (see Figure 5) minus ChAT^{ON} partition cells to determine ipsi- and contralateral contribution of putative excitatory interneurons to premotor interneuron network for MMC and LMC (Q) injections. Note that due to transient developmental expression of vGluT2 in more neurons than mature vGluT2^{ON} neurons, it is not possible to use a genetic lineage tracing approach to determine these values. (B) Bar plot displays the ratio of the percentage of commissural inhibitory premotor cells (number of contralateral inhibitory premotor neurons normalized to total number of premotor neurons) divided by the percentage of commissural putative excitatory premotor cells (number of contralateral putative excitatory premotor neurons normalized to total number of premotor neurons). Note that MMC premotor circuits have a significantly higher commissural inhibition relative to commissural putative excitation compared to LMC (Q) premotor circuits (Mann-Whitney test, MMC n=5; LMC n=4).

Our findings uncover that MMC motor neurons receive the major part of their inhibitory spinal input from contralateral interneurons whereas LMC motor neurons recruit mostly ipsilateral inhibitory interneurons (Figure 8H, I).

3.3.7. Commissural interneuron trajectories explain differences in inhibitory premotor input

The striking finding on distinct sources of inhibitory input to MMC and LMC motor neurons revealed by our retrograde rabies tracing experiments prompted us to determine the mechanism by which inhibitory commissural axons preferentially target MMC over LMC motor neurons. For this purpose, we used unilateral intraspinal injection of conditional AAVs expressing SynGFP upon Cre recombination (AAV-FLEX-SynGFP) in *vGAT*^{Cre} mice (Pivetta et al., 2014; Vong et al., 2011), allowing us to assess overall synaptic termination domains of inhibitory commissural interneurons in the spinal cord and to quantify their synaptic output to motor neurons residing in different spinal positions (Figure 10A).

We found that unilateral injection of AAV-FLEX-SynGFP into the lumbar spinal cord of *vGAT*^{Cre} mice resulted in a high contralateral density of SynGFP^{ON} synapses in Rexed's lamina VIII and in close vicinity of MMC motor neurons, whereas LMC motor neurons were outside this domain of strong synaptic termination of inhibitory commissural interneurons (Figure 10A). To get a quantitative view of inhibitory commissural input to different motor neurons in relation to identity and spinal position, we next acquired high-resolution confocal images of SynGFP input to ChAT^{ON} motor neurons. For this purpose, we kept track of MMC/LMC motor neuron identity and cell body position, in parallel with the quantification of synaptic input to each analyzed motor neuron (Figure 10B). We found that the highest synaptic input derived from vGAT^{ON} commissural interneurons was targeted towards MMC motor neurons (Figure 10B, C). Synaptic input to LMC motor neurons was significantly

lower than to MMC, and in addition, motor neurons positioned ventrally within the LMC were targeted by more vGAT^{ON} synapses from commissural interneurons than motor neurons located more dorsally in the same column (Figure 10B, C). These data reveal the existence of a gradient in inhibitory commissural synaptic input to motor neurons in the following order MMC > LMCv > LMCd (Figure 10C).

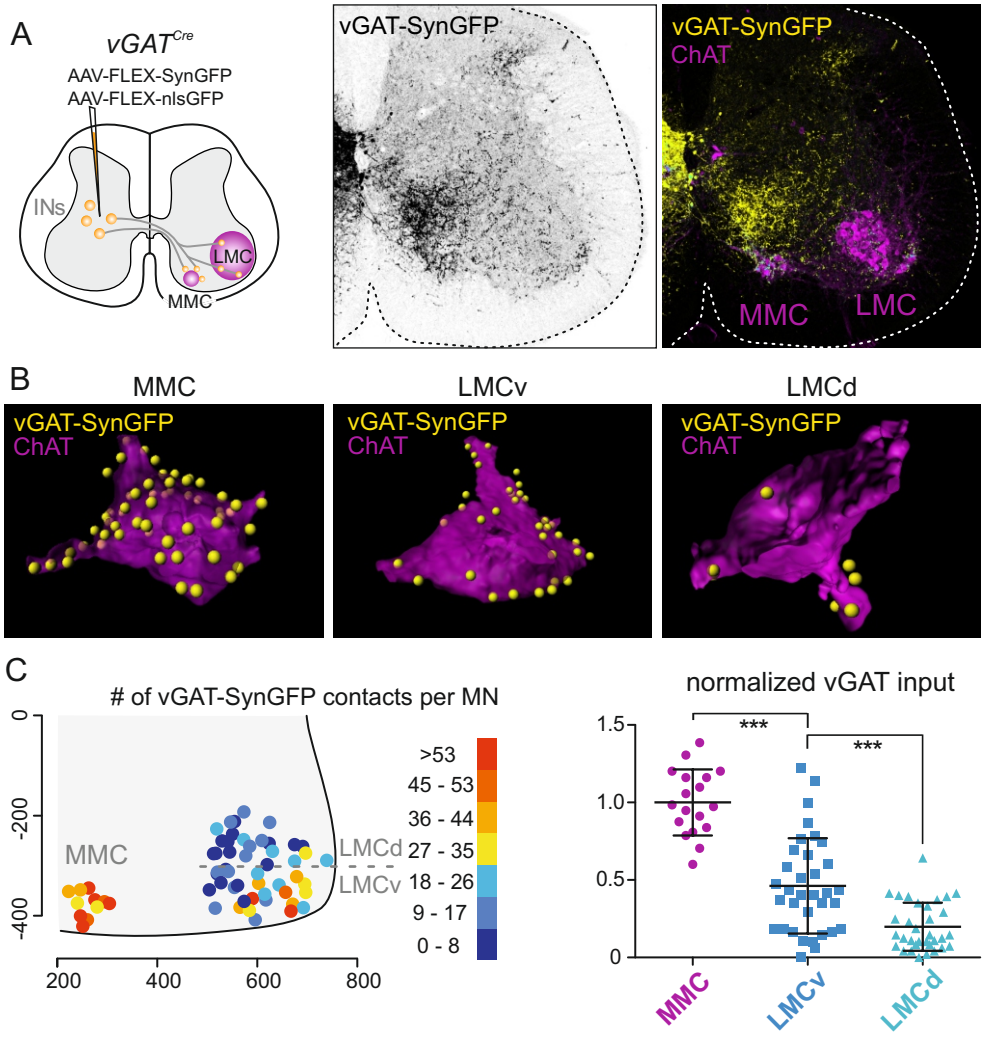

Figure 10. Motor neuron cell body position influences access to contralateral premotor interneurons

(A) Injection scheme for anterograde fluorescent-tagging of inhibitory synaptic terminals on the side contralateral to injection. Images to the right show contralateral vGAT-SynGFP terminals at low resolution in relation to MMC and LMC ChAT^{ON} motor neurons. (B, C) Representative examples of reconstructed motor neuron surfaces of MMC, ventral LMC (LMCv), dorsal LMC (LMCd) motor neurons and their commissural inhibitory input (vGAT-SynGFP: yellow). Analysis of motor neuron cell body position and inhibitory input per

motor neuron at L2 reveals that MMC motor neurons receive significantly more vGAT-SynGFP input than LMCv and LMCd. Within the LMC, LMCv receives higher input than LMCd (left: MMC MNs n=11; LMC MNs n=49 – right: 2 pooled animals, ANOVA p<0.0001, MMC MNs n=18; LMC MNs n=67).

Together, our findings provide an explanation for the dominant inhibitory synaptic input to MMC motor neurons and the lower accessibility of LMC motor neurons through this route (Figure 11).

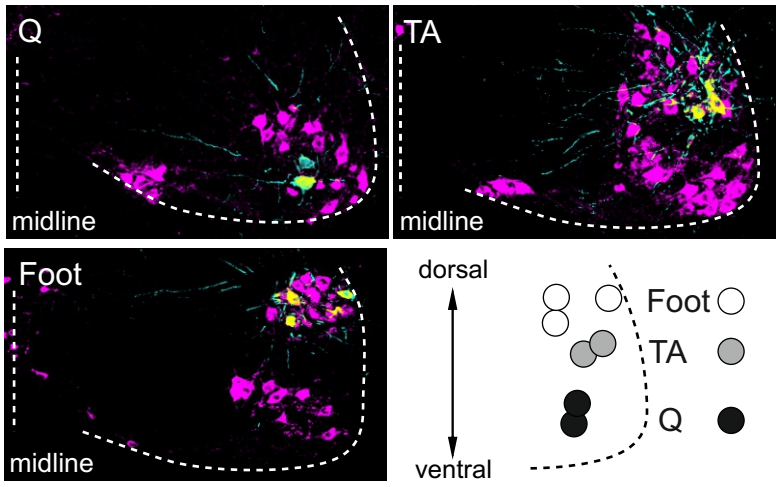

Figure 11. Distinct dorso-ventral positions of LMC motor neuron pools

Rabies tracing of motor neurons innervating Q, TA and foot muscles (Rabies-FP: turquoise; ChAT: magenta). Note different dorso-ventral positions of the individual motor neuron pools, with more proximal muscles innervated by more ventrally located pools, and distal muscles controlled by motor neurons residing more dorsally within the LMC.

3.3.8. Ipsilaterally projecting interneurons connect to MMC midline-crossing dendrites

Motor neurons elaborate dendrites that represent an important anatomical substrate for synaptic input. In order to determine the spinal domains in which MMC neurons can receive presynaptic input, we analyzed dendritic arborization of MMC motor neurons by several different approaches. First, we used intramuscular injection of Rabies-FP to retrogradely label MMC motor neurons. We found that MMC motor neuron dendrites are mostly directed in two antipodal orientations, one extending towards the more laterally positioned LMC motor

neurons and into Rexed's lamina VII, and the second one projecting medially towards the midline (Figure 13A). We noted that these medially projecting MMC dendrites do not stop at the midline but frequently cross the midline and grow into contralateral spinal territory around and below the central canal (Figure 13A). This feature is a distinctive property of MMC motor neurons at these segmental levels, since comparative injections of Rabies-FP into Q or foot muscles resulted in visualization of elaborate dendritic trees of marked motor neurons but neither of them crossed the midline (Figure 12A).

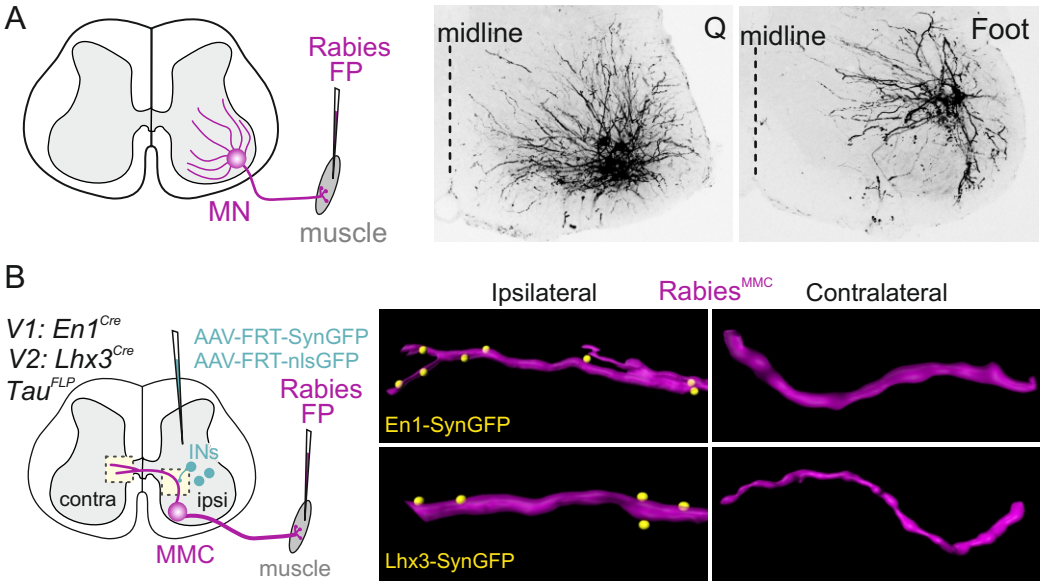

Figure 12. MMC but not LMC motor neurons exhibit midline-crossing dendrites

(A) Injection of Rabies-FP in Q or foot muscles reveals retrogradely marked motor neurons including dendrites. Note that both Q and foot motor neurons elaborate dendrites which do not cross the midline.

(B) Synaptic output tracing of V1 (En1) and V2 (Lhx3) interneurons with AAV-FRT-SynGFP. Rabies-FP is injected intramuscularly to reveal MMC motor neurons ipsilateral to intraspinal injection. Dendritic compartments ipsilateral to intraspinal injection receive direct input from interneurons derived from both V1 and V2 domains, whereas the ones located contralaterally do not.

To substantiate the observation that MMC motor neuron dendrites extend across the midline and to reveal their trajectory in more detail, we carried out unilateral intraspinal injections of AAV-FRT-FP into *Isl1*^{Cre}::*Tau*^{FLP} mice, leading to labeling of motor neurons (Figure 13B).

Also using this independent approach, we found that MMC motor neuron dendrites coarse towards the midline in bundles and frequently cross the midline barrier. Together, these findings demonstrate that medially projecting MMC dendrites cross the midline to invade contralateral territory. These results raise the question of whether exclusively ipsilaterally-projecting spinal interneurons target MMC motor neurons with cell bodies residing on the opposite spinal side but with dendrites extending across the midline. Through this mechanism, spinal interneurons with axons restricted to ipsilateral spinal territory may be granted synaptic access to contralateral motor neurons by establishing contacts to midline-crossing dendrites.

To directly address this question, we marked the synaptic output of V1 interneurons, identified by the expression of the transcription factor Engrailed-1 (En1) and a known major ipsilaterally projecting inhibitory neuronal cohort in the spinal cord (Alaynick et al., 2011; Alvarez et al., 2005). Unilateral intraspinal injection of AAV-FRT-SynGFP into $En1^{Cre}$::Tau^{FLP} mice led to almost exclusively ipsilateral SynGFP output, allowing us to ask whether these synapses contact MMC dendrites emerging from the opposite spinal side. We targeted contralateral MMC motor neurons by retrograde injection of Rabies-FP into axial muscles on the side opposite to intraspinal injection and analyzed synaptic input of SynGFP terminals on crossing MMC dendrites (Figure 13C). We found that indeed contralaterally located MMC motor neurons receive synaptic input from V1 interneurons on the crossing part of their dendrites, but are devoid of such input on the dendrite stretch prior to midline crossing (Figure 13C, data not shown). In contrast, in experiments injecting Rabies-FP and intraspinal AAV-FRT-SynGFP on the same side, MMC dendrites received V1 input on the side of injection but contralateral stretches were devoid of input (Figure 12B). We next carried out similar experiments with the V2 population of spinal interneurons, marked by the transcription factor Lhx3 and known to project predominantly ipsilaterally (Alaynick et al., 2011). We found that midline crossing MMC dendrites also represent a synaptic substrate for

ipsilaterally projecting V2 interneurons on the opposite side to muscle injection (Figure 13C, Figure 12B). Together, these findings demonstrate that medially extending MMC dendrites receive synaptic input from two different sources of V1 and V2 interneurons. Whereas dendritic stretches located ipsilaterally to cell bodies receive input from ipsilateral V1 and V2 interneurons, midline-crossed dendrites capture V1- and V2-input from the contralateral spinal cord.

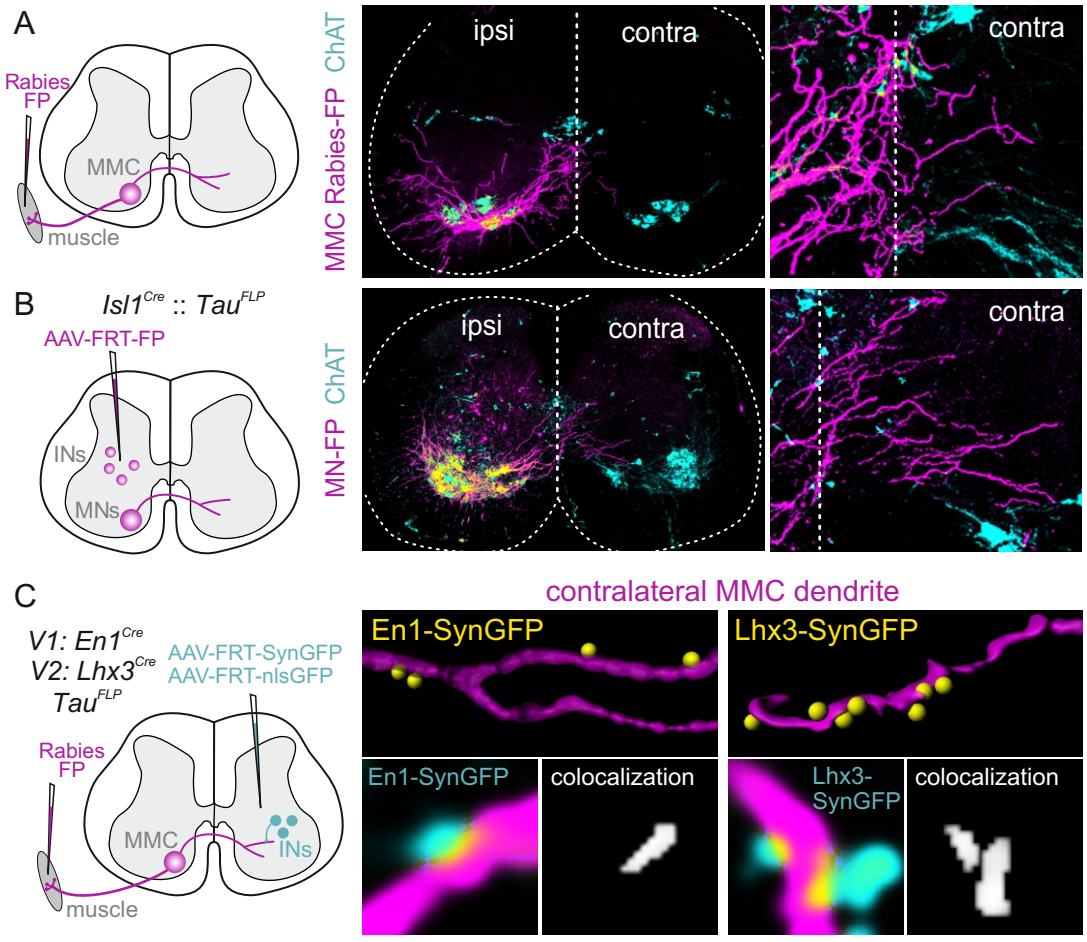

Figure 13. Motor neuron dendrites influence accessibility to contralateral interneurons

(A) Injection of Rabies-FP into lumbar axial muscles reveals MMC motor neurons and their dendrites. MMC dendrites orient in a bipolar fashion running along the ventral grey matter laterally and medially. Dendrites directed towards the midline cross it allowing access of contralateral grey matter territory. (B) Intraspinal injection of AAV-FRT-FP in *Isl1*^{Cre}*::Tau*^{FLP} mice reveals motor neurons and midline-crossing dendrites. (C) Injection strategy to test whether contralateral MMC dendrites receive input from contralateral ipsilaterally-projecting V1 (En1) or V2 (Lhx3) interneurons. Intraspinal coinjection of AAV-FRT-SynGFP/AAV-FRT-nlsGFP in either *En1*^{Cre}*::Tau*^{FLP} or *Lhx3*^{Cre}*::Tau*^{FLP} mice, combined with Rabies-FP into lumbar axial muscles

contralateral to intraspinal injection. Fluorescently labeled contralateral MMC dendrites receive synaptic input from contralateral V1 (En1-SynGFP) and V2 (Lhx3-SynGFP) interneurons.

Taken together, these experiments provide evidence that midline-crossing MMC dendrites receive synaptic input from the contralateral spinal cord derived from interneurons with unilaterally-confined synaptic output patterns. Thus, one additional mechanism contributing to distinct MMC- and LMC premotor distribution patterns is the elaboration of midline-crossing dendrites by MMC motor neurons.

3.4. Discussion

We found that motor neurons innervating trunk or limb muscles receive synaptic input from partly shared and partly distinct spinal interneuron subpopulations. We elucidate the cellular origins of distinct premotor network connectivity across the spinal midline associated with the two most widespread mammalian motor columns MMC and LMC. Here we discuss our findings in the context of previous work on spinal circuitry and motor control to present an integrative view on (1) the mechanisms involved in the establishment of synaptic input to functionally distinct motor neurons, (2) our understanding of the organizational logic and function of circuits implicated in bilateral coordination of motor behavior and (3) motor circuit evolution in the spinal cord.

3.4.1. Cellular mechanisms regulating synaptic input specificity to motor columns and pools

Motor neuron activity is regulated in a profound manner by input from premotor interneurons in the spinal cord, yet only scant information is available on how functionally distinct motor

neurons recruit distinct interneuron subpopulations to serve their synaptic regulation. Previous work using intraspinal tracer injections at segmental levels L1 versus L4 as proxy for the functionally distinct motor columns MMC or LMC to retrogradely reveal neurons with axonal projections to these segments provided preliminary evidence for differential input from premotor interneurons to these two columns (Puskar and Antal, 1997). Our experiments using monosynaptic rabies methodology now directly demonstrate that LMC and MMC premotor networks exhibit striking differences in overall organization and provide insight into their cellular composition as well as the mechanisms involved in achieving these differences.

Division of premotor interneurons into subpopulations by neurotransmitter identity and developmental ontogeny was instrumental to highlight differences in synaptic input specificity between LMC and MMC. While we found that some premotor interneuron subtypes including Renshaw cells and cholinergic partition cells exhibit similar distribution irrespective of their connectivity to analyzed LMC or MMC motor neurons, other interneuron subtypes show highly preferential connectivity profiles in favor of one or the other motor column. These column-skewed distributions together sum up to lead to a connectivity profile in which MMC motor neurons receive direct spinal inputs from interneurons with symmetrically-balanced overall distribution, whereas a strongly ipsilaterally-biased connectivity profile emerged for LMC motor neuron pools analyzed (Figure 14A). In addition, we found that the more dorsal an LMC motor neuron pool was located in the spinal cord, the less input from contralateral interneurons it receives (Figure 14A). These differences cannot be explained by traits related to extensor-flexor function of the innervated muscle since previous work demonstrated that motor neurons innervating ankle flexor (TA) or extensor (GS) muscles receive input from ipsilateral interneurons at comparable rate (Tripodi et al., 2011). Together, these findings raise the important question of the underlying reasons for these observed differential connectivity matrices.

We found that the mechanisms explaining these differences are at least twofold, both relating to the organizational logic of spinal motor neurons and ultimately regulating information transfer across the midline. First, many contralateral interneurons establish midline-crossing axonal trajectories to reach the opposite spinal side in close proximity to MMC motor neurons (Figure 14B), thereby granting them higher accessibility to MMC than LMC motor neurons. Second, MMC motor neurons establish midline-crossing dendrites, allowing them to capture synaptic input from ipsilaterally-projecting interneurons on the opposite spinal side that would otherwise be off-limits for these motor neurons (Figure 14C).

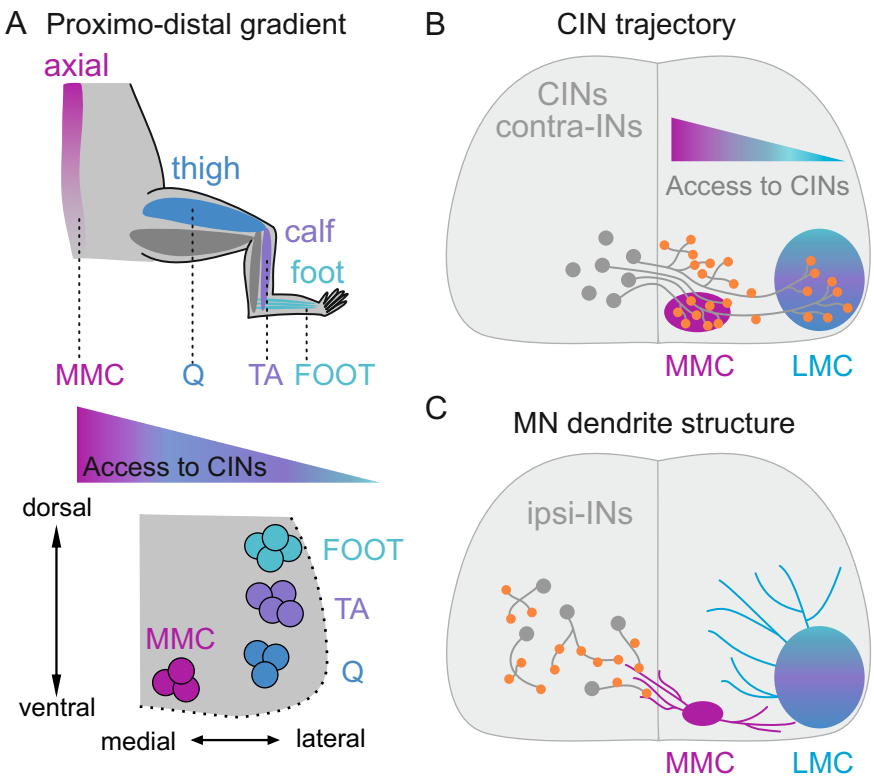

Figure 14. Motor neurons exhibit distinct premotor connectivity profiles

Summary diagram illustrating main findings presented in this study. (A) Proximo-distal gradient along mouse hindlimb muscles correlates with decreased synaptic access of motor neuron pools (Q, TA, foot) by contralateral spinal interneurons (CINs). MMC motor neurons innervating axial muscles receive the highest CIN input. (B, C) CIN trajectory and MMC dendrite structure both contribute to the observed differences in premotor circuit organization (synapses depicted in orange) between MMC and LMC motor neurons.

Together, our findings demonstrate that connectivity between premotor interneurons and distinct contralateral motor columns and pools relies on a combination of motor neuron positional information and dendritic structure. Irrespective of the nature of the cellular mechanisms involved in establishing this connection matrix however, both lead to higher information transfer from contralateral spinal interneurons to MMC- than LMC motor neurons on the opposite side of the spinal cord.

Motor neuron pool-specific synaptic input was also recently observed between V1 and V2b spinal interneuron populations and ipsilateral LMC motor neurons (Zhang et al., 2014). Since both interneuron subtypes establish ipsilateral trajectories and reside in close proximity to LMC motor neurons, a mechanism related to motor neuron position and/or dendrite elaboration seems less likely, making a connection strategy based on molecular identity more plausible in this case. Other input to motor neuron pools with known synaptic specificity is derived from group Ia proprioceptive sensory neurons, providing monosynaptic feedback from muscle spindles to motor neurons innervating the same and functionally related muscles (Eccles et al., 1957). For these synaptic inputs, a combination of motor neuron positional cues and molecular mechanisms likely explain the emergence of the observed connectivity matrices (Arber, 2012; Fukuhara et al., 2013; Pecho-Vrieseling et al., 2009; Surmeli et al., 2011). Taken together, emerging evidence supports a model in which spinal motor neuron position is an important parameter in the regulation of synaptic input specificity to functionally distinct motor neuron classes.

3.4.2. Organizational logic of circuits implicated in bilateral coordination of motor behavior

Execution of most motor behaviors requires close interplay between the two sides of the spinal cord. The circuit interface mediating left-right communication is the commissural interneuron system, which establishes connections to contralateral interneurons and motor neurons (Grillner, 2003; Jankowska, 2008; Kiehn, 2011). The differences in weighted laterality for premotor networks to functionally distinct motor neurons revealed here raise the question of the functional implications of these organizational patterns. The observed lower direct contralateral interneuron connectivity to LMC motor neurons innervating distal limb muscles compared to motor neurons innervating more proximally located muscles is particularly interesting in this context. Namely, distal limb muscles can be used for movements carried out in independence from the opposite body side, in particular in tasks such as gripping during climbing or food retrieval. The regulation by predominantly ipsilateral premotor input is consistent with such behavioral usage.

Previous work has implicated E/I balance across the midline as an important parameter in the motor coordination on opposite sides of the spinal cord (Jankowska, 2008), and genetic perturbation of these ratios interferes with motor output (Arber, 2012; Goulding and Pfaff, 2005; Kiehn, 2011; Kullander et al., 2003; Lanuza et al., 2004; Talpalar et al., 2013). However, E/I balance has previously not been assessed at the premotor level and stratified by motor columnar identity. It can be argued that a high degree of inhibition across the midline likely leads to suppression of motor output on the opposite side, in particular if these inputs are delivered directly to motor neurons. In agreement, general pharmacological blockade of inhibition results in bilaterally synchronous motor bursting in a fictive locomotor preparation (Cohen and Harris-Warrick, 1984; Cowley and Schmidt, 1995; Kullander et al., 2003). Here we show that LMC and MMC motor neurons receive input from very similar percentages of

inhibitory interneurons but MMC motor neurons receive most direct inhibitory input from contralateral interneurons whereas inhibitory regulation to LMC motor neurons has predominantly ipsilateral origin.

Postural stabilization during walking is one of the most important functions mediated by axial musculature. The strong crossed premotor interneuron network revealed here regulating MMC motor neurons is a likely contributor to this function. Moreover, previous work on descending pathways regulating posture provides evidence for access of these same motor neurons through crossed networks (Galea et al., 2010). In particular, stimulation of either contra- or ipsilateral pyramidal neurons in the cortex evokes similar effects in motor neurons of the back through crossed indirect circuits, and consistent with this model, unilateral cortical lesions affect trunk muscle control to a much lesser extent than limb movement (Galea et al., 2010). Taken together, the organization of premotor interneuron networks connected to functionally distinct motor neurons appears to correlate well with the functional needs of the regulated muscles. Since our anatomical reconstructions do not provide information about activity patterns of premotor interneurons, future work will address how these mapped interneuron populations contribute to differential motor function.

3.4.3. Evolutionary aspects of spinal motor control

Our findings on different motor columns can also be reviewed from an evolutionary angle. Vertebrates emerged about 500 million years ago as limbless aquatic organisms moving by contraction of MMC-regulated axial musculature to generate undulation. Subsequently, when vertebrates transitioned from water to land, limbs evolved to promote efficient over-ground locomotion, and these changes were accompanied by adjustments in the central nervous system to control the newly acquired appendages (Fetcho, 1992; Grillner and Jessell, 2009; Murakami and Tanaka, 2011). Lamprey is an ancient aquatic vertebrate still alive today, in

which a dominantly inhibitory commissural system is essential to control MMC motor neurons regulating undulation (Buchanan, 1982; Grillner and Jessell, 2009). MMC, HMC and LMC motor columns coexist in evolutionarily younger and limbed animals, making it difficult to disentangle behavioral roles of these columns and connected circuitry. It should be noted however that limbed reptiles have extremities with rather limited degrees of freedom to support motility and these animals still use undulation of the spine to locomote. In contrast, undulation is essentially absent in walking rodents, which points to a less pronounced usage of these circuits for this behavior. Since we found premotor networks in mice to span over multiple spinal segments, it is feasible that in the course of evolution, undulatory circuits may at least in part have been co-opted for use in HMC premotor circuits to coordinate bilateral control and contraction of body wall muscles during breathing. Our study in mice raises the intriguing possibility that aspects of the striking synaptic organization of ancient MMC motor neurons were maintained throughout evolution, but that they may also have developed further to support other or additional functions aligned with new mechanical demands of the evolving body.

3.5. Acknowledgements

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3.6. Experimental Procedures

Mouse Genetics

Mouse strains used in the present study have been previously described: *vGAT*^{Cre} (Vong et al., 2011), *En1*^{Cre} (Sapir et al., 2004), *Lhx3*^{Cre} (Sharma et al., 1998), *Lbx1*^{Cre} (Sieber et al., 2007), *Isl1*^{Cre} (Srinivas et al., 2001), *ChAT*^{Cre} (Jackson Laboratory stock number 006410), *Tau*$^{lox\text{-}STOP\text{-}lox\text{-}mGFP\text{-}IRES\text{-}nlsLacZ}$ (Hippenmeyer et al., 2005), *Tau*$^{lox\text{-}STOP\text{-}lox\text{-}Flp\text{-}IRES\text{-}nlsLacZ}$ (Pivetta et al., 2014). Mice used for intercrosses were maintained on a mixed genetic background (129/C57BL6) and Local Swiss Veterinary Offices approved all the procedures.

Monosynaptic rabies tracing and retrograde motor neuron infections

Monosynaptic rabies tracing from individual muscles was performed as previously described, using rabies-GFP and rabies-mCherry (Stepien et al., 2010; Tripodi et al., 2011). Injections were performed at postnatal day 5 (P5) and animals perfused at P13, using ice-cold PBS followed by 4% Paraformaldehyde (PFA). To confirm premotor interneuron distributions, we also used an alternative tracing strategy. We targeted glycoprotein expression to motor neurons by injecting AAV-CAG-FLEX-G (Pivetta et al., 2014) intraspinally at lumbar levels in *ChAT*^{Cre} mice at P1. Rabies-FP was injected into muscles at P5, and animals were perfused 6-7 days after rabies-FP injection. Spinal cords were dissected by ventral laminectomy and post-fixed for 6 hours in 4% PFA, followed by 1-2 days of cryoprotection in 30% Sucrose/PBS. We based our assignment of muscle identity on previous nomenclature (Greene, 1935). Specifically, to mark MMC motor neurons, we injected the lumbar extensors of the spine (Brink et al., 1979; Brink and Pfaff, 1980). These injections targeted motor neurons at lumbar (L) level L1 in a medial and ventral position, consistent with previous observations (Smith and Hollyday, 1983). For HMC motor neurons, abdominal body wall muscles including oblique and rectus abdominis muscles were injected. As a representative

motor neuron pool of the lumbar LMC, we used Quadriceps (Q) throughout the study unless otherwise stated.

Anterograde viral tracing

For intraspinal anterograde synaptic tracing, we used AAV-CAG-FLEX-nlsGFP, AAV-CAG-FLEX-SynGFP, AAV-CAG-FRT-nlsGFP, or AAV-CAG-FRT-SynGFP produced using standard procedures and serotype 2.9 (Pivetta et al., 2014). Unilateral intraspinal injections were performed at P12 and animals perfused at P21. In experiments, in which also MMC motor neurons were traced, G-protein-coated rabies was injected intramuscularly at P19. Spinal cords of P21 animals were post-fixed in 4% PFA at 4°C over night, followed by 2-3 days in 30% Sucrose/PBS. Spinal cords were embedded in Tissue-Tek using dry ice and transverse sections at 40mm were cut using a cryostat.

Immunohistochemistry and Imaging

The following primary antibodies were used: Chicken anti-GFP (1:1000; Invitrogen), Chicken anti-LacZ (1:1000; Abcam), Goat anti-ChAT (1:1000; Chemicon), Guinea pig anti-vAChT (1:1000; Chemicon), Rabbit anti-Calbindin (1:5000; Swant), Rabbit anti-RFP (1:5000; Rockland). Fluorescently coupled secondary antibodies from Jackson Laboratories were used at 1:1000. For image acquisition, a custom-made dual spinning disc microscope (Life Imaging Services GmbH, Basel, Switzerland) (Tripodi et al., 2011) and Olympus confocal microscopes (FV500 and FV1000) were used. LMCv and LMCd identity (Figure 10) was defined based on equidistance to the most ventral- and most dorsal LMC motor neuron for which input was quantified within all LMC motor neurons at the analyzed segmental level. The scatter graph (Figure 10C, right) displays pooled data from two $vGAT^{Cre}$ mice with unilateral injection at L2, in which vGAT-SynGFP input to contralateral motor neurons was quantified. Individual data sets were normalized to the value of the mean of inputs on MMC

motor neurons. These showed the same decreasing trend allowing pooling of data within one graph.

Statistical Analysis

We used GraphPad PRISM Version 6.0 to analyze data, perform statistical tests, and create box-, scatter- and barplots. For all boxplots shown, the horizontal line in the box represents the median value, bottom, and top limits if the box display 25^{th} and 75^{th} percentile, and whiskers indicate smallest (min) and largest (max) values. All scatter- and barplots show mean value and whiskers indicate SD. We reconstructed interneuron positions within the spinal cord using `*Qu*` in MATLAB and we used R (R Foundation for Statistical Computing, http://www.r-project.org) to generate scatter- and density plots (for detailed description see: (Tripodi et al., 2011)). To calculate significances, one-way ANOVA followed by post-hoc Tukey`s HSD test was performed in Figure 4A, 5A and 10C; a two-sided unpaired *t*-test was performed in Figure 7F, 8H and 8I; a Mann-Whitney test was performed in Figure 9B. To indicate significance levels, *p<0.05, **p<0.01 and ***p<0.001 were used in all graphs.

4. Additional Results

4.1. Anterograde tracing of Lbx1-derived interneurons in the spinal cord

Monosynaptically-restricted rabies tracing technique revealed that interneurons, which are derived from the Lbx1 domain, connect differentially to different motor neuron columns (see Figure 7). According to these results, Lbx1-derived interneurons in the ipsilateral dorsal spinal cord connect preferentially to LMC compared to MMC motor neurons. Here, we used an intersectional strategy using mouse genetics and viral tracing to reveal the synaptic output pattern of ipsilateral dorsal Lbx1-derived interneurons. We crossed $Lbx1^{Cre}$ mice with reporter mice expressing Flip-recombinase and nlsLacZ after Cre mediated STOP cassette excision, resulting in $Lbx1^{Cre}$ :: Tau^{FLP} mice. These mice were injected with AAV-FRT-nlsGFP/AAV-FRT-SynGFP viruses, leading to nuclear labeling of infected Lbx1-derived interneurons and their synaptic terminals with GFP (Figure 15A). Reconstruction of infected Lbx1-derived interneurons marked with nlsGFP revealed that in the injected spinal cord, the majority of infected interneurons reside within the dorsal spinal cord, ipsilateral to injection (Figure 15B). The center of injection was at spinal lumbar level 3 (L3) and we gated the analysis on MMC and LMC motor neurons at this segmental level. High resolution imaging of MMC and LMC area, defined by $ChAT^{ON}$ motor neurons, revealed Lbx1-SynGFP terminals in close apposition to motor neurons (Figure 15C).

The synaptic input to MMC and LMC motor neurons was quantified on the same sections, allowing direct comparison of absolute number of inputs. It turned out that MMC motor neurons only receive minor Lbx1-SynGFP input from the ipsilateral dorsal spinal cord; in contrast the LMC was heavily contacted by fluorescently labeled SynGFP-terminals (Figure 15D). The amount of ipsilaterally dorsal derived Lbx1 input was significantly higher on LMC motor neurons than MMC motor neurons (Figure 15E).

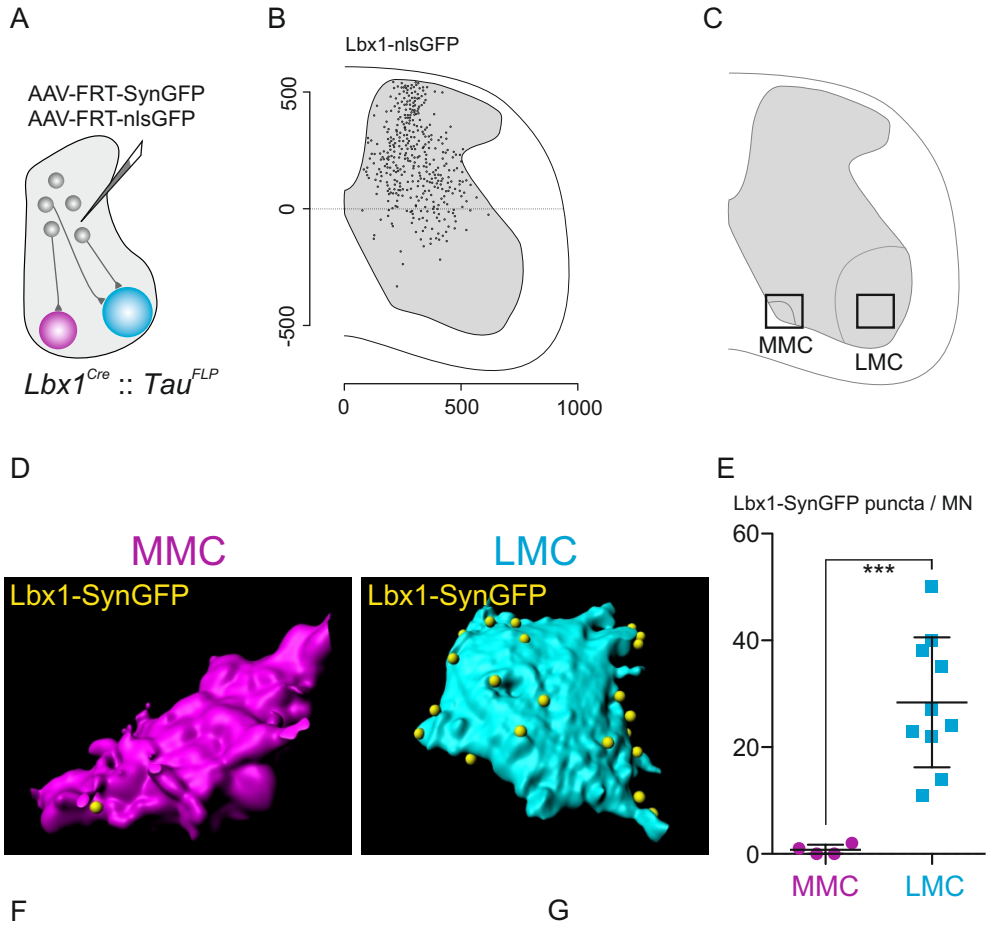
A
AAV-FRT-SynGFP
AAV-FRT-nlsGFP
Lbx1Cre :: TauFLP
B
Lbx1-nlsGFP
500
0
-500
0
500
1000
C
MMC
LMC
D
MMC
LMC
Lbx1-SynGFP
Lbx1-SynGFP
E
Lbx1-SynGFP puncta / MN
60
40
20
0
***
MMC
LMC
F
ipsi MMC
Lbx1-synGFP
ChAT
G
ipsi LMC

Figure 15. Anterograde synaptic tracing of ipsilateral dorsal Lbx1-derived interneurons

(A) Scheme illustrating the experimental design. A mix of AAV-FRT-SynGFP and AAV-FRT-nlsGFP is injected intraspinally at L3 in $Lbx1^{Cre}$ :: Tau^{FLP} mouse. (B) Reconstruction of the position of nlsGFP expressing Lbx1-derived interneurons (each dot represents GFP^{+} nucleus). (C) High resolution confocal imaging of MMC and LMC region. Motor neurons visualized by staining against ChAT. (D) Lbx1-SynGFP input on MMC (magenta) and LMC (cyan) motor neurons was quantified (each yellow dot represents SynGFP terminal). (E) Quantification of absolute number of Lbx1-SynGFP terminals on MMC and LMC motor neurons of the same section of the spinal cord. LMC motor neurons receive a significantly higher number of Lbx1-SynGFP inputs compared to MMC motor neurons (unpaired t-test p=0.0008, plot shows mean with SD, MMC n=4; LMC n=10). (F) High resolution images of ipsilateral MMC motor neurons expressing ChAT (bottom) and the Lbx1-SynGFP terminals in this domain. (G) High resolution image of ipsilateral LMC motor neurons expressing ChAT (bottom) and the Lbx1-SynGFP terminals in this domain.

High resolution imaging of ipsilateral MMC and LMC motor neurons at the level of injection shows high density of Lbx1-SynGFP puncta in the LMC area but very few in the MMC domain (Figure 15F, G). Taken together, these results nicely confirm the pattern obtained with the retrograde rabies approach, in which the ipsilateral dorsal Lbx1-derived interneurons exhibit a strong bias towards LMC compared to MMC.

4.2. Anterograde synaptic tracing of commissural vGluT2 interneurons

In order to assess and quantify the amount of commissural excitation reaching MMC and LMC motor neurons, we injected $vGluT2^{Cre}$ mice intraspinally at lumbar level 2 (L2) with AAV-FLEX-SynGFP and AAV-FLEX-nlsGFP. Injection was unilaterally confined, infecting excitatory interneurons on one side of the spinal cord. On the contralateral side, vGluT2-SynGFP terminals, which make direct connections to $ChAT^{ON}$ MMC and LMC motor neurons, can be detected (Figure 16A). High resolution imaging of different motor neurons and their excitatory commissural input reveals that MMC, ventral LMC (LMCv) and dorsal LMC (LMCd) receive comparable amount of excitatory inputs on their soma and proximal dendrites (Figure 16B).

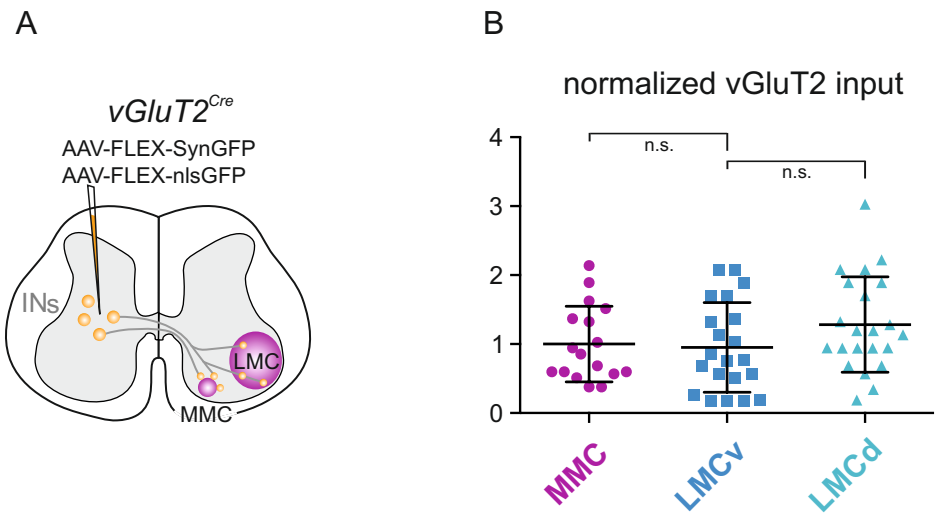

Figure 16. Anterograde synaptic tracing of excitatory commissural interneurons

(A) The scheme displays the strategy. Intraspinal injection at L2 in mice in which Cre-recombinase is expressed in excitatory neurons (vGluT2^{Cre}) with AAV-FLEX-SynGFP and AAV-FLEX-nlsGFP fluorescently marks the synaptic terminals and nuclei of the infected excitatory interneurons. Analysis was gated to ChAT^{ON} MMC- and LMC motor neurons contralateral to injection side. High resolution imaging of motor neurons allowed the quantification of vGluT2-SynGFP puncta contacting motor neurons. (B) Graph shows pooled data from two independent injections as described, data sets were normalized to the mean of inputs to the MMC. MMC, LMCv and LMCd do not receive significantly different amount of excitatory commissural input (ANOVA and Tukey's HSD test $p>0.05$, plot shows mean with SD, MMC n=17; LMCv n=21; LMCd n=22).

These data suggest, that MMC and LMC motor neurons do not receive differential excitatory commissural input on their soma and proximal dendrites. However, our rabies data suggests a higher fraction of commissural excitation than inhibition on LMC than MMC, implying that the more distal dendrites of LMC motor neurons receive a high amount of commissural excitation.

4.3. Monosynaptic rabies tracing of V1-derived premotor interneurons

We traced the premotor network of MMC and LMC in mice in which ipsilaterally-projecting inhibitory V1-derived interneurons are labeled ($En1^{Cre}$ :: $Tau^{nlsLacZ}$), since we wanted to investigate if there is a differential contribution of this domain to MMC versus LMC premotor circuits. In addition, this experiment will give further insight on whether the midline-crossing MMC dendrites receive monosynaptic input from contralaterally located ipsilaterally projecting interneurons.

The tracing revealed premotor interneurons connecting to MMC and LMC and in addition the nuclei of V1-derived interneurons were marked with LacZ (Figure 17A). When the premotor network of the MMC is revealed, V1-derived premotor cells are found mainly within the ipsilateral ventral quadrant (Figure 17B). However, in addition there are V1-derived premotor cells in the contralateral spinal cord, located at different locations within the ventral quadrant (Figure 17B). The premotor circuit of the LMC shows the vast majority of V1-derived interneurons ipsilateral to injection and only one V1-derived premotor cell was found on the contralateral side of the spinal cord out of three injections (Figure 17C).

We then wanted to directly compare the V1-domain contribution to the ipsilateral or contralateral premotor network of MMC and LMC. To compare them, we calculated the percentage of ipsilateral V1-derived premotor cells normalized to all premotor cells. This allows direct comparison between MMC and LMC, revealing that there is a significantly higher fraction of ipsilateral En1-derived LMC premotor cells (14%) than found in the ipsilateral MMC premotor cohort (8%) (Figure 17D). However, if the analogous analysis is applied to the contralateral side, a significant higher fraction of V1-derived interneurons is found in the MMC premotor network (0.5%) than in the LMC premotor circuit (0.1%) (Figure 17E). In summary, these data demonstrate that the V1 domain exhibits a differential connectivity preference towards MMC and LMC motor neurons. The LMC is preferentially contacted over the MMC by ipsilateral V1-derived interneurons, whereas MMC receives

more V1-derived input from the contralateral side compared to LMC. Furthermore, the fact that the MMC premotor network shows several contralateral V1-derived interneurons, implies that these ipsilaterally projecting V1 interneurons make a monosynaptic connection to midline-crossing MMC dendrites.

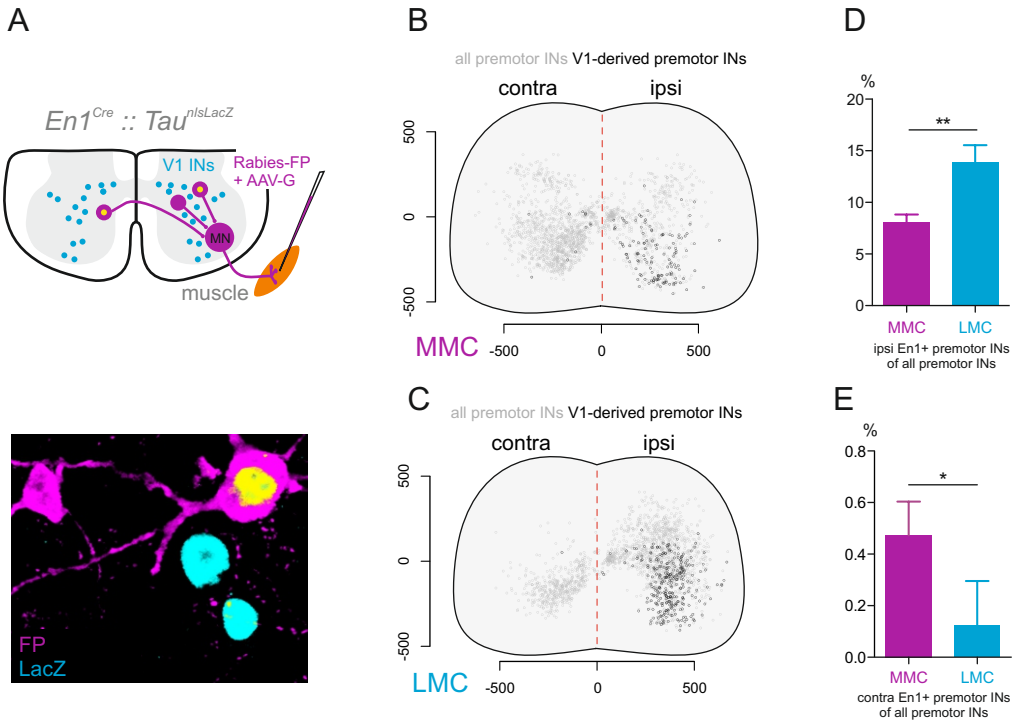

Figure 17. Contribution of V1-derived interneurons to MMC and LMC premotor circuits

(A) We used $En1^{Cre}$ :: $Tau^{nlsLacZ}$ mice in order to reveal the interneurons which are derived from the V1-domain. This was combined with monosynaptic rabies tracing from either axial or limb – Q – muscles to fluorescently label the premotor interneurons (FP) connecting to MMC versus LMC and at the same time reveal if they are derived form V1-domain (LacZ) or not. (B and C) Rabies tracing reveals MMC and LMC – Q – premotor interneurons (grey dots) and in addition the contribution of V1-derived premotor interneurons can be assessed (black dots). (D) Fraction of V1-derived ipsilateral premotor interneurons normalized to all premotor interneurons, displays a significantly higher bias towards LMC compared to MMC (unpaired t-test p=0.005, plot shows mean with SD, MMC n=3; LMC n=3). (E) Conversely, the fraction of V1-derived contralateral premotor interneurons normalized to all premotor interneurons, displays a significantly higher number for MMC than LMC (unpaired t-test p=0.04, plot shows mean with SD, MMC n=3; LMC n=3).

4.4. Contribution of V2-domain to MMC premotor network

The V2-domain, defined by the expression of Lhx3, gives rise to ipsilaterally projecting interneurons with excitatory- or inhibitory neurotransmitter phenotype. For the Q premotor circuit, it has already been demonstrated that V2-derived premotor interneurons reside on the spinal side ipsilateral to muscle injection and encompass about 3% of the total number of Q premotor interneurons (Stepien et al., 2010). We then used the monosynaptically-restricted rabies tracing method in order to assess the contribution of the V2-domain to the MMC premotor circuit. We injected *Lhx3*^{Cre} :: *Tau*^{nlsLacZ} mice with glycoprotein-deficient rabies and AAV-G virus into lumbar axial muscles. This revealed the MMC premotor interneurons and in addition the V2-derived ones amongst them (Figure 18A). As seen for the Q premotor network, we find ipsilateral V2-derived interneurons, which are premotor to MMC. They are located in the ventral- and intermediate spinal cord and close to the central canal (Figure 18B). However, in addition we also found V2-derived MMC premotor interneurons contralateral to injection in the area close to the central canal (Figure 18B). This implies that the contralateral premotor cohort may in fact contain ipsilaterally projecting axons, which make monosynaptic connections on midline-crossing MMC dendrites. The fact that no contralateral V2-derived interneurons have been detected in the Q premotor, further supports this notion, since Q motor neurons do not have midline-crossing dendrites.

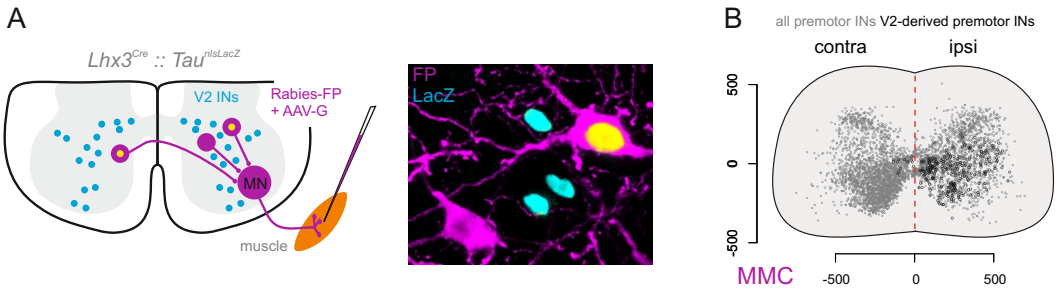

Figure 18. Contribution of V2-derived interneurons to MMC premotor network

(A) We intramuscularly injected a mix of glycoprotein-deficient rabies virus and AAV-G in axial muscles in order to initiate trans-synaptic spread from the MMC. This was done in $Lhx3^{Cre}$ :: $Tau^{nlsLacZ}$ mice, allowing to determine the contribution of the V2-domain to MMC premotor circuit, by assessing the fraction of fluorescent-protein (FP) expressing rabies infected premotor interneurons positive for the nuclear marker of V2-derived interneurons (LacZ). (B) MMC premotor interneurons are displayed as grey dots, whereas the black dots represent the premotor interneurons, which are V2-derived (each dot represents soma position).

4.5. Premotor circuit tracing in *FoxP1* conditional mutants

We wanted to investigate the premotor circuits in a situation, in which motor neurons innervating a specific muscle have an altered transcriptional program. This would allow studying how premotor networks are organized, when their target neuron has been changed at molecular level. In addition, in this mutant the spatial confinement of motor neuron pools does no longer exist, with motor neurons being scattered and not forming pools at defined locations. This feature allowed us to address, if the fraction of contralateral premotor interneurons is changing with the dorso-ventral position of the contacted motor neurons no longer being given. To address these questions, we used *FoxP1* conditional mutant mice (*FoxP1* cKO) by crossing $Olig2^{Cre}$ with $FoxP1^{flox/flox}$ mice, resulting in the deletion of *FoxP1* in motor neurons (Figure 19A). The lack of *FoxP1* in the motor neurons leads to a spinal cord, with MMC and HMC motor columns but lacking a LMC and a PGC (Figure 19B) (Sürmeli et al., 2011). The ectopic HMC motor neurons at lumbar levels, still project to limb muscles, innervating them, yet motor neurons innervating a particular muscle are no longer grouped in a spatially confined cluster.

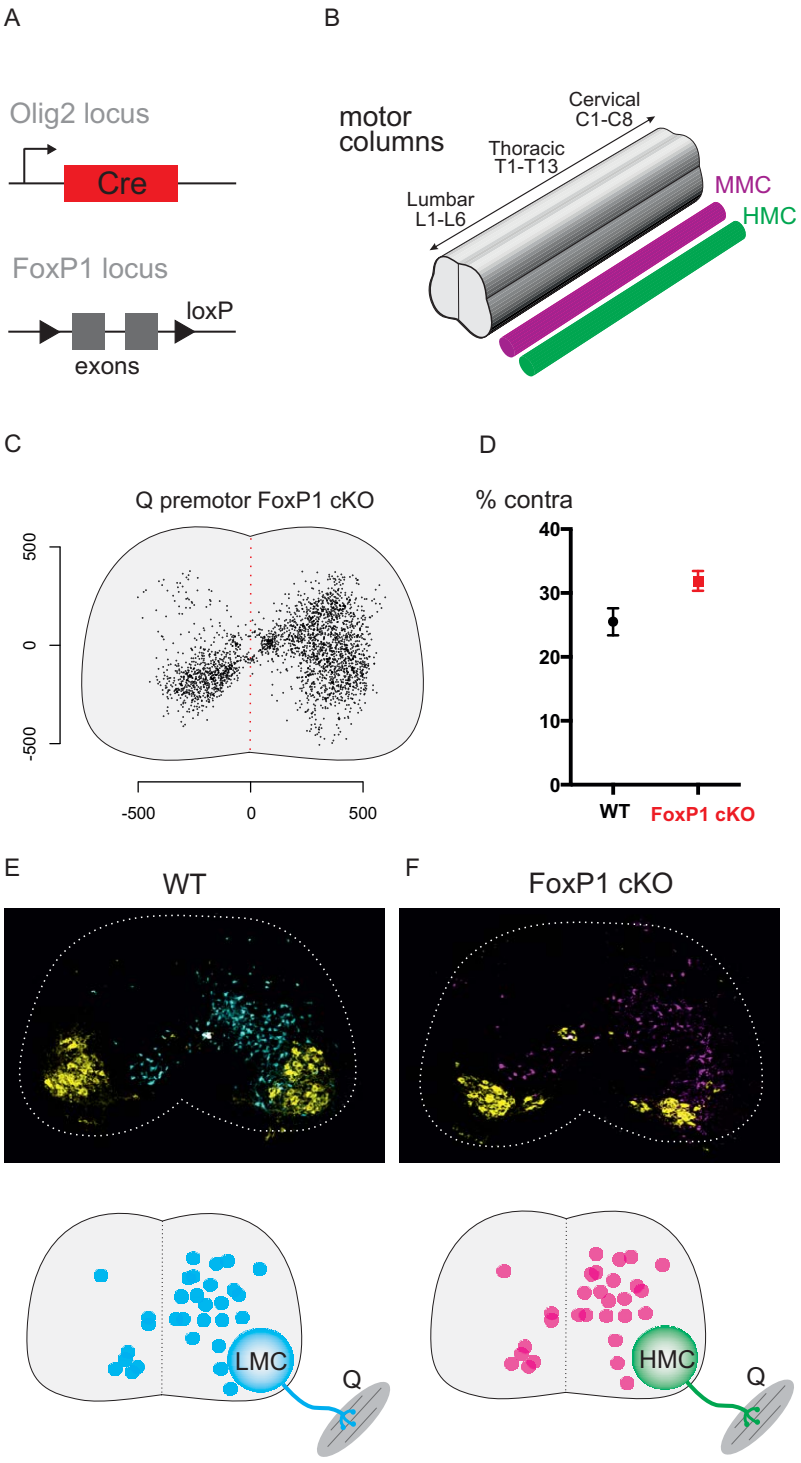
A
Olig2 locus
Cre
FoxP1 locus
loxP
exons
B
motor
columns
Cervical
C1-C8
Thoracic
T1-T13
Lumbar
L1-L6
MMC
HMC
C
Q premotor FoxP1 cKO
500
0
-500
-500
0
500
D
% contra
40
30
20
10
0
WT
FoxP1 cKO
E
WT
F
FoxP1 cKO
LMC
Q
HMC
Q

Figure 19. Premotor circuit tracing in the *FoxP1* cKO mouse

(A) *FoxP1* condictional knock-out (cKO) were generated by deletion of two critical exons in the *FoxP1* gene, using Cre-lox system, with the Cre being expressed in motor neurons ($Olig2^{Cre}$). (B) This resulted in the transcriptional conversion of LMC and PGC motor neurons into HMC motor neurons. The spinal cord of the *FoxP1* cKO contains only the MMC and HMC motor column, both present at all segmental levels of the spinal cord. (C) Monosynaptically-restricted rabies tracing was used in order to reveal the premotor network controlling the ectopic HMC motor neurons innervating Q muscle. Each dot represent soma position of a premotor interneuron (D) Comparison of ipsi-contra ration of Q premotor circuits in the wild-type and the *FoxP1* cKO, indicates, that this ratio is comparable in both situations, with contralateral fraction being slightly higher in the *FoxP1* cKO. (E) Spinal cord transverse section showing Q premotor interneurons (cyan) and $ChAT^{ON}$ motor neurons (yellow) in the wild-type situation. (F) Spinal cord transverse section showing Q premotor interneurons (magenta) and $ChAT^{ON}$ motor neurons (yellow) in the *FoxP1* cKO situation.

This led us to investigate how the premotor circuit of such a molecularly reprogrammed motor column is organized. For this, we injected complemented rabies virus in the Q muscle and revealed the premotor interneurons in the *FoxP1* mutant. Surprisingly, the overall pattern of the premotor circuit controlling the Q is very similar between the mutant and the wild type. In *FoxP1* mutant mice, ipsilateral Q premotor interneurons are found in lamina VII, lamina X and the intermediate spinal cord. Contralaterally the majority of premotor is interneurons is confined to lamina VIII, as observed in the wild-type situation (Figure 19C, E, F). A slight increase in the fraction of contralateral premotor interneurons was observed in the mutant compared to wild type, with *FoxP1* mutants displaying about 32% of contralateral premotor interneurons for the Q muscle (Figure 19D).

4.6. Monosynaptic rabies tracing from axial muscles in the zebrafish

Since zebrafish has become an important model system to study motor behaviors, we set out in collaboration with Abdel El-Manira and Konstantinos Ampatzis from the Karolinska Institute, to try to apply the monosynaptically restricted rabies tracing system to the zebrafish. To reveal first spinal motor neurons innervating axial muscles, we injected larvas at early stages with Dextran-Rhodamine intramuscularly using a picospritzer (Figure 20A). After one night of incubation, motor neurons were labeled brightly seen in the whole mount of the larva (Figure 20B, C). Subsequently, the same experiment was then performed injecting glycoprotein-deficient rabies virus together with HSV-EF1a-G in order to complement the glycoprotein. The injection in axial muscles was performed in zebrafish larva which where 6 days post-fertilization. Daily investigation of larva under fluorescent lamp revealed rabies expressing premotor interneurons 7 days after injection. Premotor neurons were found in the spinal cord rather broadly distributed along the rostro-caudal axis and several rabies labeled neurons were found in the brainstem of the fish (Figure 20D – F). These findings demonstrate that the monosynaptically restricted rabies tracing method can be applied to trace premotor circuits controlling axial muscles of the zebrafish, even labeling long-projecting neurons residing in the brainstem.

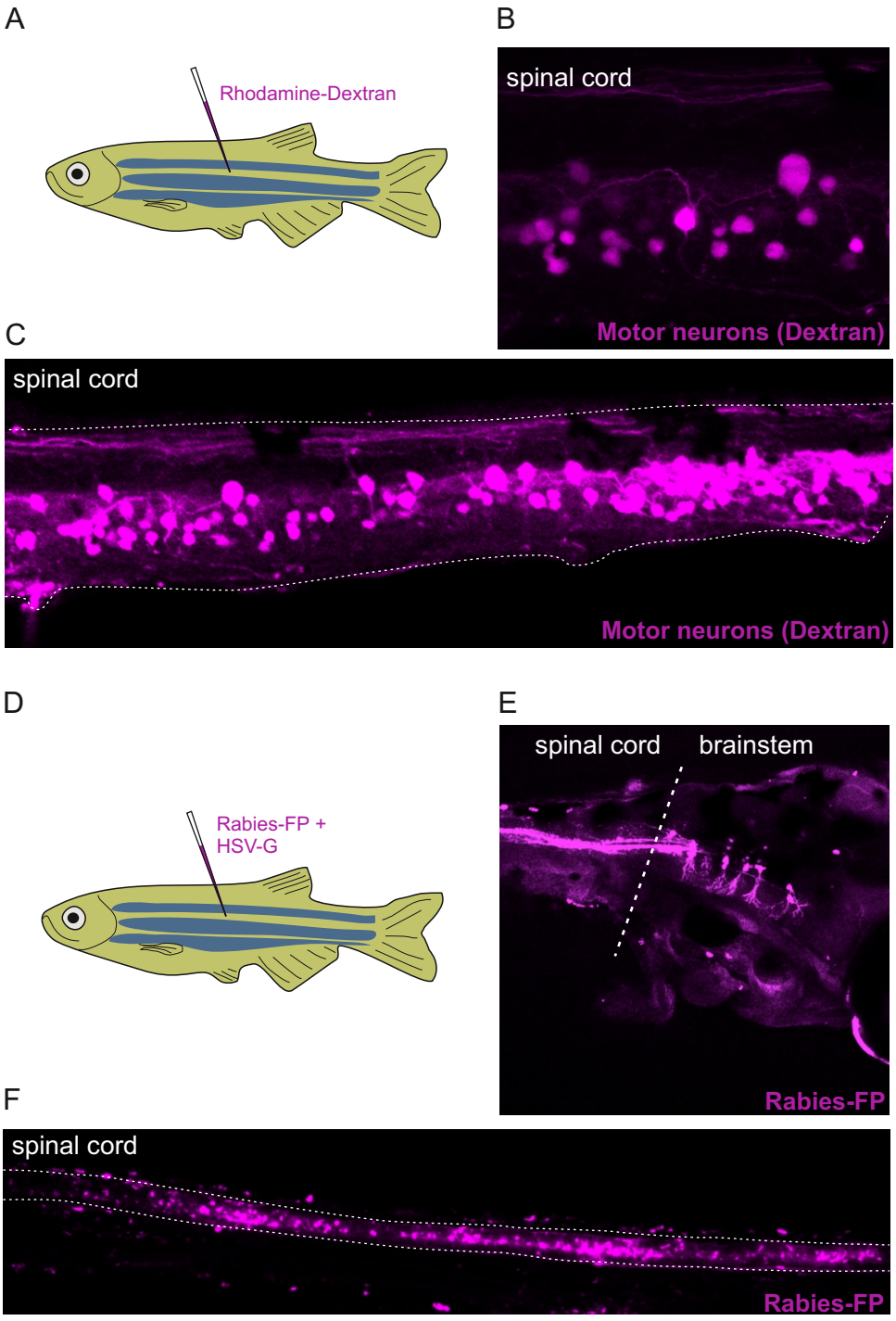

Figure 20. Premotor circuit tracing of axial muscles in the zebrafish

(A-C) Dextran-Rhodamine injection with a picospritzer into axial muscles at early larval stages fluorescently labels motor neurons in the spinal cord. (D) Injection of glycoprotein-deficient rabies virus and Herpes Simplex Virus expressing G (HSV-EF1a-G) in axial muscles using a picospritzer on order to infect spinal motor neurons and subsequently premotor interneurons. (E and F) Rabies labeled premotor interneurons are found within the brainstem and the spinal cord.

5. Discussion

5.1. Neuronal circuits controlling distinct motor behaviors

A vast literature is available on interneuron circuits within the spinal cord and how they shape motor neuron activity. There is a rather profound knowledge about individual spinal interneurons classes and their functional roles (Goulding, 2009; Alaynick et al., 2011; Arber 2012). Nevertheless, these populations are part of an entire network of interneurons, acting in conjunction to control the activity of motor neurons. For this it is important to investigate the circuits in a more complete fashion, in order to better understand how they are organized and how they function. The development of viral circuit tracing has proved to be a powerful tool to investigate connectivity of the motor system. Monosynaptically-restricted rabies tracing methodology allows to directly visualize the interneurons connecting to a specific motor neuron pool (Stepien et al., 2010; Tripodi et al., 2011). In our study we used this technique to compare premotor networks controlling different spinal motor columns. We found that these premotor circuits contain interneuron classes, which have been shown to be involved in shaping distinct parameters of locomotion. Despite the fact that some of these interneurons classes were part of both MMC and LMC premotor networks, some of them exhibited differential connectivity preferences between different motor neuron types. It has already been proposed for some interneuron classes, that they have motor neuron pool preferences. One example is the cohort of V1- and V2b-derived interneurons, which shows differential connectivity towards different LMC motor neurons innervating proximal hind-limb muscles (Zhang et al., 2014).

We found that the most striking difference, however, at the level of premotor network differences between axial- and limb muscles is their laterality, with axial muscles being controlled by a significantly higher fraction of contralateral premotor interneurons than limb muscles. Furthermore, a significantly higher inhibitory commissural input is observed to

MMC motor neurons than LMC motor neurons, indicating that axial muscles are controlled by strong crossed-inhibitory networks. Interestingly, this crossed inhibition is proposed for the lamprey spinal cord, a limbless aquatic organism only using its axial musculature to propagate within water. In a computational model underlying undulation of the lamprey, excitatory interneurons interconnect on one side of the spinal cord, forming a network, controlling axial muscle contraction on one side of the body. However, in this model both sides of the spinal cord are interconnected by inhibitory commissural interneurons, mediating alternating excitation-inhibition of motor neurons in one segment of the lamprey spinal cord (Buchanan, 1982; Grillner 2003). This alternation at a specific segment combined with a rostro-caudal propagation of the excitatory wave, creates an undulating movement (Grillner, 2003).

Interestingly, it was demonstrated that in early postnatal rats, the axial muscles of the trunk create a bending movement of the spine during locomotion, propagating along the rostro-caudal axis of the body (Falgairolle and Cazalets, 2007). This trunk-bending motor behavior appears reminiscent of the undulation of the lamprey generated by axial muscles. One could speculate, that during early postnatal periods, undulating movements of the spine represent the evolutionary basal locomotor state, later transitioning in the adult-like locomotion that is limb-based, with axial muscles stabilizing the trunk. In the light of this hypothesis, one could also explain the motor behavior of *FoxP1* conditional mutants, which display an undulating movement of their body at adult stages, using their axial muscles. Given the fact that the spinal cord in this mutant only contains MMC and HMC – the two most ancient motor columns – could further support the notion, that in a situation in which limbs are not functional, the locomotion acquires basal features seen only in evolutionary older animals.

Functionally, this feature of high contralateral premotor fraction and strong commissural inhibition seen in the mouse, may serve as a substrate to tightly control axial muscles on both sides of the body. The high number of contralateral premotor interneurons may be sampling

sensory inputs or receive direct information from the CPG of the side contralateral to the motor neurons contacted. This strong commissural premotor channel therefore may feed a lot of information about the status of the contralateral spinal network activity directly to motor neurons influencing the integration process of the motor neuron pool contacted and ultimately the decision whether an axial muscle will contract or not.

For limb muscles, we find a bias of the premotor interneurons towards the ipsilateral side, implying a more sided output of the extremities, allowing a more independent usage of one side over the other. Previous work in the mouse has shown that premotor circuits controlling EXT or FLEX muscles of a joint, segregate spatially within the ipsilateral spinal cord, however they overlap on the contralateral side (Tripodi et al., 2011). On the ipsilateral side, their positioning influences the amount of direct sensory input premotor interneurons in the intermediate spinal cord receive, suggesting that ipsilateral positioning of limb premotor circuits is important for proper function (Tripodi et al., 2011). Our findings also emphasize the correlation of more independent sided motor output of limb muscles and the ipsilateral premotor bias, since we found that there is an increasing ipsilateral premotor fraction, as one moves to more distal limb muscles. On the level of circuits this could imply that less direct information of the contralateral side is needed, since motor behaviors can be executed which do not need strong bilateral control.

However, motor routines such as walking need a close co-regulation of axial- and limb muscles. This implies that at some level – and this does not necessarily need to be at the premotor level – the networks need to have some kind of co-regulation. One interneuron type, which we found at the premotor level, that turned out to directly co-connect MMC and LMC motor neurons on opposite sides of the spinal cord are the cholinergic partition cells (Zagoraiou et al., 2009). These interneurons have been shown to increase the excitability of motor neurons, and hence have strong influence if a motor neuron is activated or not (Miles et

al., 2007). However, technical constraints of the monosynaptically-restricted rabies tracing method do not allow to assess quantitatively the premotor interneurons double-connecting to two different motor neuron pools or –columns. With the rabies data we generated for MMC and LMC, we can only infer that part of their premotor circuits overlap spatially within the spinal cord, at least making it plausible that some of the interneurons are double connecting. MMC and LMC (Q) premotor networks for example show strong overlap in the ipsilateral spinal cord and contralateral lamina VIII. Nevertheless, we revealed that the MMC premotor circuit contains a contralateral dorsal population, which is basically absent in the LMC (Q) premotor network.

Importantly, in order to address the question of coordination between different motor neuron pools and –columns, one should not be solely focusing on the spinal cord, since likely candidates for the co-regulation of the axial and limb networks include descending brainstem nuclei. It has been demonstrated that electrical stimulation of the medullary reticular formation (MRF) is able to induce calcium transients in lumbar MMC and LMC motor neurons in a brainstem–spinal cord preparation (Szokol et al., 2008). Furthermore, stimulation of the vestibular nucleus evokes activation of axial nerves in the lumbar spinal cord (Brink and Pfaff, 1981). Another study also pointed in the direction that the vestibular nucleus is involved in the activation of axial and limb controlling motor neurons (Kasumacic et al., 2010). From this perspective, our spinal premotor tracing reveals an important part of the entire circuit controlling distinct muscle classes, but further studies will hopefully shed more light on whether the differences in premotor structures also applies to the brainstem.

5.2. Development of postural control

After birth, terrestrial mammals are exposed to gravitational forces acting on their bodies. However, in order to execute motor behaviors such as walking, rearing or grooming, animals need to have a functional postural system, allowing them bear their own weight and stabilize their body during a motor routine. Muscles, which are crucial to achieve this stabilization, are the postural muscles. They encompass axial muscles, running along the vertebral column and stabilizing the spine. Furthermore, extensor muscles of the limb are required as well to adequately counteract gravity. The interplay of axial- and limb muscles is essential to create coordinated motor behaviors, involving the whole body of the animal.

It has been shown that in rat, postural control involves transitions in the use of specific muscles to achieve an adult type of locomotion pattern (Geisler et al., 1993). It was demonstrated that within the first week after birth, the only type of postural control that developed was the horizontal movement and lifting of the head. However, about 10 days after birth, the rats are standing on their four feet, with their ventral side not touching the ground anymore, demonstrating postural control and weight bearing of the trunk. Two weeks after birth, rats exhibit an adult-like locomotion pattern, with axial muscles stabilizing the trunk and head, and limbs exhibiting flexor-extensor alternation (Geisler et al., 1993).

The appearance of these postural support systems of course needs to be paralleled by the correct functioning of circuits within the CNS, which control postural muscle contractions. Interestingly, alternating leg movements can be observed in rats before birth, indicating that circuits in the CNS are present to control these movements (Bekoff and Lau, 1980). In fact, it has been shown in the mouse that critical elements of the spinal motor circuitry are already established during embryonic development (Ladle et al., 2007).

Due to current technical limitations, our rabies premotor data shows the premotor connectivity at postnatal day thirteen, basically exactly coinciding with the appearance of fully developed postural motor behavior. In this respect it would be interesting to see

connectivity at a time point, where the animals has acquired its complete postural repertoire. Anterograde synaptic tracing strategy, using AAVs allowed us to reveal connections at more mature stages of the spinal cord circuitry. The results we obtained at these later stages are in line with what we find with the rabies tracing at earlier stages. This argues that the premotor network we reveal with the rabies tracing is a connectivity status that remains fairly constant, although there is a dramatic transition in postural motor behavior. Despite these findings it is important to note that in our study, anterograde tracing results were analyzed three weeks after birth, hence it would be interesting to investigate these connectivity profiles in animals, which are even older giving insight into possible rearrangements as the motor repertoire greatly expands due to newly acquired skills.

5.3. Dendritic structure of motor neurons controlling posture

Motor neurons control the activity of muscles and hence their input greatly influences whether a muscle contracts or not. Motor neuron dendrites are critical compartments of motor neurons to receive various inputs. However, besides the inputs received, these motor neuron dendrites have specific spatial arrangements, which can create proximity between different motor neurons. In rat, it has been demonstrated that motor neurons innervating postural muscles exhibit dendrite bundles, intermingling with each other (Scheibel and Scheibel, 1970). Furthermore, these dendrite bundles have been shown to express gap-junctions, allowing electrical coupling between these motor neurons (van der Want et al., 1998). Interestingly, this dendritic feature seems to be only established as postural control reaches its adult-like status. In early neonate rats, the soleus muscle, which is a limb muscle involved in posture, displays dendrites oriented in a seemingly random fashion. However, about two weeks after birth these motor neuron dendrites started to form bundles, exactly coinciding with the appearance of adult-like locomotion pattern (Westerga and Gramsbergen, 1993).

Also in humans, these dendritic bundles have been observed for spinal motor neurons in the domains where axial- and extensor limb innervating motor neurons are located (Schoenen, 1982). This suggests that this feature of dendritic clustering is conserved during evolution of the vertebrate motor system.

These findings demonstrate that motor neurons controlling postural muscles on the same side of the body form dendrite bundles. In addition, it was also proposed that axial motor neuron pools form dendritic bundles with the correspondent contralateral motor neuron dendrites in the medial spinal cord (Scheibel and Scheibel, 1973; Gramsbergen et al., 1996). In our study, we also found indications that axial motor neurons might in fact be coupled by this mechanism across the midline. In our MMC neuron dendrite tracing we found midline-crossing dendrites, running closely to contralateral MMC dendrites, intermingling with them (data not shown). Hence this mechanism of electrical coupling of dendrites could also be used to synchronize axial motor neuron activity on opposite sides of the spinal cord.

5.4. Influence of motor neuron molecular identity and position on premotor connectivity

Motor neuron pools and –columns are found at stereotypic positions within the spinal cord (Jessell, 2000). These positional cues have been implicated to be of importance for correct premotor connectivity between sensory neurons and motor neurons. It has been suggested that sensory neurons of the limb terminate within precisely defined domains of the LMC and misplacing motor neurons leads to sensory contacts to motor neurons which would not be found in the wild-type (Sürmeli et al., 2011). The authors hypothesized that sensory neurons invade a specific domain of the LMC, irrespective of which muscle the local motor neurons innervate, establishing erratic sensory – motor connections (Sürmeli et al., 2011). We used the same mouse mutant, called *FoxP1* conditional mutant in order to study commissural premotor

connectivity. In the wild-type tracing we found that motor neuron positioning correlates with the amount of commissural input a motor neuron pool receives. The misplacement of motor neurons in the LMC region of the *FoxP1* mutant allowed us to investigate the premotor connectivity to motor neurons at different dorso-ventral positions. We chose the Q muscle to reveal the premotor distribution of this muscle in the mutant. However, we did not find a dramatic change in the overall distribution pattern. We only found a slight increase of contralateral premotor interneurons. Regarding our hypothesis of motor neuron positioning, it could be that some of the motor neurons of which spread was initiated in the mutant, were located in the very ventral domain (in the wild-type, Q motor neurons are not residing within the most ventral domain) of the limb-innervating motor column, encountering more commissural synapses. In fact it has been shown that motor neurons innervating the limb in the *FoxP1* mutant are less numerous and the whole ectopic motor column is less expanded in the dorso-ventral dimension, which consequently leaves more limb controlling motor neurons in the ventral domain, theoretically contacted by high number of commissural synapses (Sürmeli et al., 2011).

Besides this position-centric view, the molecular component of motor neuron–premotor connectivity needs to be taken into account. It has been demonstrated that the sensory–motor neuron connection specificity involves genetically encoded signals, which are crucial in generating synaptic specificity and correct wiring (Vrieseling et al., 2009; Fukuhara et al., 2013). In our *FoxP1* mutant Q tracing, we found that premotor distribution at the level of ipsi-contra ratio is not markedly altered, making it possible that the ectopic motor neurons in the mutant receive a retrograde signal from the muscle, leading to the expression of some surface receptors or secreted molecules that these motor neurons would also express in the wild-type. In this case, the motor neurons have an overall transcriptional profile of an ectopic motor column, but maintain the expression of some genes normally only found in “limb controlling motor neurons”, induced by peripheral signals. In fact, previous work has shown that

retrograde signals from muscles to motor neurons can have profound influences on the transcriptional program of motor neurons. It has been demonstrated that the glial cell line-derived neurotrophic factor (GDNF) produced by specific muscles, controls the expression of *Pea3* in the motor neurons innervating them (Haase et al., 2002). Subsequently, it has then been revealed that *Pea3* in turn regulates dendritic structure of certain motor neurons, influencing the type of monosynaptic input they receive (Vrieseling et al., 2006). Taken together, these studies demonstrate that muscle-derived signals can indirectly influence the input the respective motor neuron receives in the spinal cord. In this perspective, our maintained ipsi-contra ratio and overall pattern for the Q in the *FoxP1* mutant, may reflect the fact that the ectopic motor neurons still receive signals from their target muscle, which in turn leads to currently unknown proteins to be expressed which allow recruitment of the proper spinal premotor interneurons.

5.5. Premotor circuits in the zebrafish and the mouse

Since we succeeded in applying monosynaptic rabies tracing in the zebrafish, various interesting questions can now be addressed. First of all, one can study the structure of axial premotor networks of the fish and compare it to the corresponding mouse premotor circuit to investigate whether some circuit structures are conserved between the two species and where differences may lie. Furthermore, premotor tracing of pectoral- or pelvic fin muscles will reveal the premotor network of a structure, which is the evolutionary precursor of the vertebrate limb. This makes it very interesting to see whether fin premotor circuits exhibit features, reminiscent of what we found in the mouse – for example ipsi-contra ratio.

Besides these evolutionary questions, rabies tracing may not only be used to label premotor neurons, but for example make them express a calcium indicator in order to reveal neuronal activity of the premotor interneurons. Given the fact that zebrafish larvae at early stages are

transparent, creates the unique opportunity to study the temporal activation pattern of premotor neurons *in-vivo* during a certain motor behavior.

Another approach would be to use rabies virus expressing channel- or halorhodopsin in order to activate or silence specific premotor populations to reveal functional effects. Taken together, the premotor circuit tracing in the zebrafish will give interesting insights on networks controlling axial muscles and muscles of the appendages of an aquatic organism. Such experiments will show whether these networks are similar or not, compared to the corresponding neuronal circuits in a terrestrial vertebrate, like the mouse.

6. References

Agalliu, D., Takada, S., Agalliu, I., McMahon, A.P., and Jessell, T.M. (2009). Motor neurons with axial muscle projections specified by Wnt4/5 signaling. Neuron *61*, 708-720.

Alaynick, W.A., Jessell, T.M., and Pfaff, S.L. (2011). SnapShot: spinal cord development. Cell *146*, 178-178 e171.

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

Ampatzis, K., Song, J., Ausborn, J., and El Manira, A. (2014). Separate microcircuit modules of distinct V2a interneurons and motoneurons control the speed of locomotion. Neuron *83*, 1-10.

Arber, S. (2012). Motor circuits in action: specification, connectivity, and function. Neuron *74*, 975-989.

Azim, E., Jiang, J., Alstermark, B., and Jessell, T.M. (2014). Skilled reaching relies on a V2a propriospinal internal copy circuit. Nature *508*, 357-363.

Baek, M., Enriquez, J., and Mann, R.S. (2013). Dual role for Hox genes and Hox co-factors in conferring leg motoneuron survival and identity in Drosophila. Development *140*, 2027-2038.

Bekoff, A., and Lau, B. (1980). Interlimb coordination in 20-day-old rat fetuses. J Exp Zool *214*, 173-175.

Betley, J.N., Wright, C., Kawaguchi, Y., Erdelyi, F., Szabo, G., Jessell, T.M., and Kaltschmidt, J.A. (2009). Stringent specificity in the construction of a GABAergic pre-synaptic inhibitory circuit. Cell *139*, 161-174.

Bonanomi, D., and Pfaff, S.L. (2010). Motor axon pathfinding. Cold Spring Harb Perspect Biol *2*, a001735.

Brink, E.E., Morrell, J.I., and Pfaff, D.W. (1979). Localization of lumbar epaxial motoneurons in the rat. Brain Res *170*, 23-41.

Brink, E.E., and Pfaff, D.W. (1980). Vertebral muscles of the back and tail of the albino rat (Rattus norvegicus albinus). Brain Behav Evol *17*, 1-47.

Brink, E.E., and Pfaff, D.W. (1981). Supraspinal and segmental input to lumbar epaxial motoneurons in the rat. Brain Res *226*, 43-60.

Buchanan, J.T. (1982). Identification of interneurons with contralateral, caudal axons in the lamprey spinal cord: synaptic interactions and morphology. J Neurophysiol *47*, 961-975.

Buchanan, J.T. (1999). Commissural interneurons in rhythm generation and intersegmental coupling in the lamprey spinal cord. J Neurophysiol *81*, 2037-2045.

Bui, T.V., Akay, T., Loubani, O., Hnasko, T.S., Jessell, T.M., and Brownstone, R.M. (2013). Circuits for grasping: spinal dI3 interneurons mediate cutaneous control of motor behavior. Neuron *78*, 191-204.

Cohen, A.H., and Harris-Warrick, R.M. (1984). Strychnine eliminates alternating motor output during fictive locomotion in the lamprey. Brain Res *293*, 164-167.

Conradi, S., and Skoglund, S. (1969). Observations on the ultrastruture and distribution of neuronal and glial elements on the motoneuron surface in the lumbosacral spinal cord of the cat during postnatal development. Acta physiologica Scandinavica *333*, 5-52.

Cowley, K.C., and Schmidt, B.J. (1995). Effects of inhibitory amino acid antagonists on reciprocal inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. J Neurophysiol *74*, 1109-1117.

Crone, S.A., Quinlan, K.A., Zagoraiou, L., Droho, S., Restrepo, C.E., Lundfald, L., Endo, T., Setlak, J., Jessell, T.M., Kiehn, O., and Sharma, K. (2008). Genetic ablation of V2a ipsilateral interneurons disrupts left-right locomotor coordination in mammalian spinal cord. Neuron *60*, 70-83.

Dasen, J.S., Liu, J.P., and Jessell, T.M. (2003). Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature *425*, 926-933.

Dasen, J.S., De Camilli, A., Wang, B., Tucker, P.W., and Jessell, T.M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell *134*, 304-316.

Dasen, J.S., and Jessell, T.M. (2009). Hox networks and the origins of motor neuron diversity. Curr Top Dev Biol *88*, 169-200.

Dasen, J.S., Tice, B.C., Brenner-Morton, S., and Jessell, T.M. (2005). A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell *123*, 477-491.

De Marco Garcia, N.V., and Jessell, T.M. (2008). Early motor neuron pool identity and muscle nerve trajectory defined by postmitotic restrictions in Nkx6.1 activity. Neuron *57*, 217-231.

Demireva, E.Y., Shapiro, L.S., Jessell, T.M., and Zampieri, N. (2011). Motor neuron position and topographic order imposed by β- and γ-catenin activities. Cell *147*, 641-652.

Di Sanguinetto, S.A., Dasen, J.S., and Arber, S. (2008). Transcriptional mechanisms controlling motor neuron diversity and connectivity. Curr Opin Neurobiol *18*, 36-43.

Eberhart, J., Swartz, M.E., Koblar, S.A., Pasquale, E.B., and Krull, C.E. (2002). EphA4 constitutes a population-specific guidance cue for motor neurons. Dev Biol *247*, 89-101.

Eccles, J.C., Eccles, R.M., and Lundberg, A. (1957). The convergence of monosynaptic excitatory afferents on to many different species of alpha motoneurones. J Physiol *137*, 22-50.

Esposito, M.S., Capelli, P., and Arber, S. (2014). Brainstem nucleus MdV mediates skilled forelimb motor tasks. Nature *508*, 351-356.

Falgairolle, M., and Cazalets, J.-R. (2007). Metachronal coupling between spinal neuronal networks during locomotor activity in newborn rat. J Physiol *580*, 87-102.

Fetcho, J.R. (1992). The spinal motor system in early vertebrates and some of its evolutionary changes. Brain Behav Evol *40*, 82-97.

Fukuhara, K., Imai, F., Ladle, D.R., Katayama, K., Leslie, J.R., Arber, S., Jessell, T.M., and Yoshida, Y. (2013). Specificity of monosynaptic sensory-motor connections imposed by repellent Sema3E-PlexinD1 signaling. Cell Rep *5*, 748-758.

Galea, M.P., Hammar, I., Nilsson, E., and Jankowska, E. (2010). Bilateral postsynaptic actions of pyramidal tract and reticulospinal neurons on feline erector spinae motoneurons. J Neurosci *30*, 858-869.

Gallarda, B.W., Bonanomi, D., Muller, D., Brown, A., Alaynick, W.A., Andrews, S.E., Lemke, G., Pfaff, S.L., and Marquardt, T. (2008). Segregation of axial motor and sensory pathways via heterotypic trans-axonal signaling. Science *320*, 233-236.

Gosgnach, S., Lanuza, G.M., Butt, S.J., Saueressig, H., Zhang, Y., Velasquez, T., Riethmacher, D., Callaway, E.M., Kiehn, O., and Goulding, M. (2006). V1 spinal neurons regulate the speed of vertebrate locomotor outputs. Nature *440*, 215-219.

Geisler, H.C., Westerga, J., and Gramsbergen, A. (1993). Development of posture in the rat. Acta Neurobiol Exp *53*, 517-523.

Geisler, H., and Gramsbergen, A. (1998). Motor development after vestibular deprivation in rats. Neurosci Biobehav Rev *22*, 565-569.

Gharbaran, R., and Aisemberg, G.O. (2013). Identification of leech embryonic neurons that express a Hox gene required for the differentiation of a paired, segment-specific motor neuron. Int J Devl Neuroscience *31*, 105-115.

Goulding, M., and Pfaff, S.L. (2005). Development of circuits that generate simple rhythmic behaviors in vertebrates. Curr Opin Neurobiol *15*, 14-20.

Goulding, M. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. Nat Rev Neurosci *10*, 507-518.

Gramsbergen, A., Ijkema-Paassen, Westerga, J., and Geisler, H.C. (1996). Dendrite bundles in motoneuronal pools of trunk and extremity muscles in the rat. Exp Neurol *137*, 34-42.

Gramsbergen, A., Geisler, H.C., Taekema, H., and Eykern, L.A. (1999). The activation of back muscles during locomotion in the developing rat. Dev Brain Res *112*, 217-228.

Greene, E.C. (1935). Anatomy of the rat (New York, Hafner Press).

Grillner, S. (2003). The motor infrastructure: from ion channels to neuronal networks. Nat Rev Neurosci *4*, 573-586.

Grillner, S., and Jessell, T.M. (2009). Measured motion: searching for simplicity in spinal locomotor networks. Curr Opin Neurobiol *19*, 572-586.

Gross, M.K., Dottori, M., and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron *34*, 535-549.

Gutman, C.R., Ajmera, M.K., and Hollyday, M. (1993). Organization of motor pools supplying axial muscles in the chicken. Brain Res *609*, 129-136.

Haase, G., Dessaud, E., Garcès, A., de Bovis, B., Birling, M.-C., Filippi, P., Schmalbruch, H., Arber, S., and deLapeyrière, O. (2002). GDNF acts through Pea3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron *35*, 893-905.

Hellstrom, J., Oliveira, A.L., Meister, B., and Cullheim, S. (2003). Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. J Comp Neurol *460*, 476-486.

Hippenmeyer, S., Vrieseling, E., Sigrist, M., Portmann, T., Laengle, C., Ladle, D.R., and Arber, S. (2005). A developmental switch in the response of DRG neurons to ETS transcription factor signaling. PLoS Biol *3*, e159.

Huber, A.B., Kania, A., Tran, T.S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T.M., Ginty, D.D., and Kolodkin, A.L. (2005). Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. Neuron *48*, 949-964.

Hultborn, H., Jankowska, E., and Lindstrom, S. (1971). Relative contribution from different nerves to recurrent depression of Ia IPSPs in motoneurones. J Physiol *215*, 637-664.

Jankowska, E. (2008). Spinal interneuronal networks in the cat: elementary components. Brain Res Rev *57*, 46-55.

Jankowska, E., Bannatyne, B.A., Stecina, K., Hammar, I., Cabaj, A., and Maxwell, D.J. (2009). Commissural interneurons with input from group I and II muscle afferents in feline lumbar segments: neurotransmitters, projections and target cells. J Physiol *587*, 401-418.

Jankowska, E., and Odutola, A. (1980). Crosses and uncrossed synaptic actions on motoneurones of back muscles in the cat. Brain Res *194*, 65-78.

Jessell, T.M. (2000). Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. Nat Rev Genet *1*, 20-29.

Jessell, T.M., Sürmeli, G., and Kelly, J.S. (2011). Motor neurons and the sense of place. Neuron *72*, 419-424.

Jung, H., Lacombe, J., Mazzoni, E.O., Liem Jr., K.F., Grinstein, J., Mahony, S., Mukhopadhyay, D., Gifford, D.K., Young, R.A., Anderson, K.V., Wichterle, H., and Dasen, J.S. (2010). Global control of motor neuron topography mediated by the repressive actions of a single Hox gene. Neuron *67*, 781-796.

Kania, A., Johnson, R.L., and Jessell, T.M. (2000). Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. Cell *102*, 161-173.

Kania, A., and Jessell, T.M. (2003). Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. Neuron *38*, 581-596.

Kao, T.-J., and Kania, A. (2011). Ephrin-mediated cis-attenuation of Eph receptor signaling is essential for spinal motor axon guidance. Neuron *71*, 76-91.

Kasumacic, N., Glover, J.C., and Perreault, M.-C. (2010). Segmental patterns of vestibular-mediated synaptic inputs to axial and limb motoneurons in the neonatal mouse assessed by optical recording. J Physiol *588*, 4905-4925.

Kiehn, O. (2006). Locomotor circuits in the mammalian spinal cord. Annu Rev Neurosci *29*, 279-306.

Kiehn, O. (2011). Development and functional organization of spinal locomotor circuits. Curr Opin Neurobiol *21*, 100-109.

Kullander, K., Butt, S.J., Lebret, J.M., Lundfald, L., Restrepo, C.E., Rydstrom, A., Klein, R., and Kiehn, O. (2003). Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. Science *299*, 1889-1892.

Ladle, D.R., Pecho-Vrieseling, E., and Arber, S. (2007). Assembly of motor circuits in the spinal cord: Driven to function by genetic and experience-dependent mechanisms. Neuron *56*, 270-283.

Lanuza, G.M., Gosgnach, S., Pierani, A., Jessell, T.M., and Goulding, M. (2004). Genetic identification of spinal interneurons that coordinate left-right locomotor activity necessary for walking movements. Neuron *42*, 375-386.

Ljunggren, E.E., Haupt, S., Ausborn, J., Ampatzis, K., and El Manira, A. (2014). Optogenetic activation of excitatory premotor interneurons is sufficient to generate coordinated locomotor activity in larval zebrafish. J Neurosci *34*, 134-139.

McCrea, D.A., and Rybak, I.A. (2008). Organization of mammalian locomotor rhythm and pattern generation. Brain Res Rev *57*, 134-146.

McHanwell, S., and Biscoe, T.J. (1981). The localization of motoneurons supplying the hindlimb muscles of the mouse. Philosophical transactions of the Royal Society of London *293*, 477-508.

Miles, G.B., Hartley, R., Todd, A.J., and Brownstone, R.M. (2007). Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. Proc Natl Acad Sci U S A *104*, 2448-2453.

Misra, M., Sours, E., and Lance-Jones, C. (2012). Hox transcription factors influence motoneuron identity through the integrated actions of both homeodomain and non-homeodomain regions. Dev Dyn *241*, 718-731.

Muller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron *34*, 551-562.

Murakami, Y., and Tanaka, M. (2011). Evolution of motor innervation to vertebrate fins and limbs. Dev Biol *355*, 164-172.

Musienko, P.E., Deliagina, T.G., Gerasimenko, Y.P., Orlovsky, G.N., and Zelenin, P.V. (2014). Limb and trunk mechanisms for balance control during locomotion in quadrupeds. J Neurosci *34*, 5704-5716.

Parker, H.J., Bronner, M.E., and Krumlauf, R. (2014). A Hox regulatory network of hindbrain segmentation is conserved to the base of vertebrates. Nature 514, 490-493.

Pecho-Vrieseling, E., Sigrist, M., Yoshida, Y., Jessell, T.M., and Arber, S. (2009). Specificity of sensory-motor connections encoded by Sema3e-Plxnd1 recognition. Nature *459*, 842-846.

Peterson, B.W., Pitts, N.G., and Fukushima, K. (1979). Reticulospinal connections with limb and axial motoneurons. Exp Brain Res *36*, 1-20.

Pfaff, S.L., Mendelsohn, M., Stewart, C.L., Edlund, T., and Jessell, T.M. (1996). Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell *84*, 309-320.

Philippidou, P., and Dasen, J.S. (2013). Hox genes: choreographers in neural development, architects of circuit organization. Neuron *80*, 12-34.

Pivetta, C., Esposito, M.S., Sigrist, M., and Arber, S. (2014). Motor-circuit communication matrix from spinal cord to brainstem neurons revealed by developmental origin. Cell *156*, 537-548.

Puskar, Z., and Antal, M. (1997). Localization of last-order premotor interneurons in the lumbar spinal cord of rats. J Comp Neurol *389*, 377-389.

Renshaw, B. (1941). Influence of discharge of motoneurons upon excitation of neighboring motoneurons. J Neurophysiol *4*, 167-183.

Romanes, G.J. (1951). The motor cell columns of the lumbo-sacral spinal cord of the cat. J Comp Neurol *94*, 313-363.

Sapir, T., Geiman, E.J., Wang, Z., Velasquez, T., Mitsui, S., Yoshihara, Y., Frank, E., Alvarez, F.J., and Goulding, M. (2004). Pax6 and engrailed 1 regulate two distinct aspects of renshaw cell development. J Neurosci *24*, 1255-1264.

Scheibel, M.E., and Scheibel, A.B. (1970). Organization of spinal motoneuron dendrites in bundles. Exp Neurol *28*, 106-112.

Scheibel, M.E., and Scheibel, A.B. (1973). Dendrite bundles in the ventral commissure of cat spinal cord. Exp Neurol *39*, 482-488.

Schoenen, J. (1982). Dendritic organization of the human spinal cord: The motoneurons. J Comp Neurol *211*, 226-247.

Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., and Pfaff, S.L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell *95*, 817-828.

Sharma, K., Leonard, A.E., Lettieri, K., and Pfaff, S.L. (2000). Genetic and epigenetic mechanisms contribute to motor neuron pathfinding. Nature *406*, 515-519.

Shirasaki, R., Lewcock, J.W., Lettieri, K., and Pfaff, S.L. (2006). FGF as a target-derived chemoattractant for developing motor axons genetically programmed by the LIM code. Neuron *50*, 841-853.

Sieber, M.A., Storm, R., Martinez-de-la-Torre, M., Muller, T., Wende, H., Reuter, K., Vasyutina, E., and Birchmeier, C. (2007). Lbx1 acts as a selector gene in the fate determination of somatosensory and viscerosensory relay neurons in the hindbrain. J Neurosci *27*, 4902-4909.

Smith, C.L., and Hollyday, M. (1983). The development and postnatal organization of motor nuclei in the rat thoracic spinal cord. J Comp Neurol *220*, 16-28.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol *1*, 4.

Stepien, A.E., and Arber, S. (2008). Probing the locomotor conundrum: Descending the `V`interneuron ladder. Neuron *60*, 1-4.

Stepien, A.E., Tripodi, M., and Arber, S. (2010). Monosynaptic rabies virus reveals premotor network organization and synaptic specificity of cholinergic partition cells. Neuron *68*, 456-472.

Surmeli, G., Akay, T., Ippolito, G.C., Tucker, P.W., and Jessell, T.M. (2011). Patterns of spinal sensory-motor connectivity prescribed by a dorsoventral positional template. Cell *147*, 653-665.

Szokol, K., Glover, J.C., and Perreault, M.-C. (2008). Differential origin of reticulospinal drive to motoneurons innervating trunk and hindlimb muscles in the mouse revealed by optical recording. J Physiol *586*, 5259-5276.

Talpalar, A.E., Bouvier, J., Borgius, L., Fortin, G., Pierani, A., and Kiehn, O. (2013). Dual-mode operation of neuronal networks involved in left-right alternation. Nature *500*, 85-88.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123-1133.

Tresch, M.C., Saltiel, P., d`Avella, A., and Bizzi, E. (2002). Coordination and localization in spinal motor systems. Brain Res Rev *40*, 66-79.

Tripodi, M., Stepien, A.E., and Arber, S. (2011). Motor antagonism exposed by spatial segregation and timing of neurogenesis. Nature *479*, 61-66.

Vanderhorst, V.G., and Holstege, G. (1997). Organization of lumbosacral motoneuronal cell groups innervating hindlimb, pelvic floor, and axial muscles in the cat. J Comp Neurol *382*, 46-76.

Van der Want, J.J.L., Gramsbergen, A., Ijkema-Paassen, J., de Weerd, H., and Liem, R.S.B. (1998). Dendro-dendritic connections between motoneurons in the rat spinal cord: an electron microscopic investigation. Brain Res *779*, 342-345.

Vong, L., Ye, C., Yang, Z., Choi, B., Chua, S., Jr., and Lowell, B.B. (2011). Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. Neuron *71*, 142-154.

Vrieseling, E., and Arber, S. (2006). Target-induced transcriptional control of dendritic patterning and connectivity in motor neurons by the ETS gene Pea3. Cell *127*, 1439-1452.

Westerga, J., and Gramsbergen, A. (1993). Development of locomotion in the rat: the significance of early movements. Early Hum Dev *34*, 89-100.

Wojcik, S.M., Katsurabayashi, S., Guillemin, I., Friauf, E., Rosenmund, C., Brose, N., and Rhee, J.S. (2006). A shared vesicular carrier allows synaptic corelease of GABA and glycine. Neuron *50*, 575-587.

Wu, Y., Wang, G., Scott, S.A., and Capecci, M.R. (2008). Hoxc10 and Hoxd10 regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons. Development *135*, 171-182.

Yakovenko, S., Mushahwar, V., VanderHorst, V., Holstege, G., and Prochazka, A. (2002). Spatiotemporal activation of lumbosacral motoneurons in the locomotor step cycle. J Neurophysiol *87*, 1542-1553.

Zagoraiou, L., Akay, T., Martin, J.F., Brownstone, R.M., Jessell, T.M., and Miles, G.B. (2009). A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. Neuron *64*, 645-662.

Zhang, J., Lanuza, G.M., Britz, O., Wang, Z., Siembab, V.C., Zhang, Y., Velasquez, T., Alvarez, F.J., Frank, E., and Goulding, M. (2014). V1 and v2b interneurons secure the alternating flexor-extensor motor activity mice require for limbed locomotion. Neuron *82*, 138-150.

Zhang, Y., Narayan, S., Geiman, E., Lanuza, G.M., Velasquez, T., Shanks, B., Akay, T., Dyck, J., Pearson, K., Gosgnach, S., Fan, C.-M., and Goulding, M. (2008). V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. Neuron *60*, 84-96.