Organization and connectivity of spinal ascending pathways reporting to brainstem

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Chapter 1

Summary

Movement is the final output of neuronal activity in the spinal cord. In all vertebrates, motor neurons are grouped into motor neuron pools, the functional units innervating individual muscles. Spinal premotor interneurons are the last stage of integration of a variety of inputs from different brain regions and sensory afferents before directing them to motor neurons. For the generation of movement, precise activation of distinct motor neuron pools at the right moment in time is crucial and this precision is likley due to the cohorts of spinal premotor interneurons, connected with specificity to distinct motor neuron pools that regulate motor neuronal activity. However, for accurate generation of movement, motor pathways need to constantly compare action planning to action execution. To achieve this task, motor pathways establish efference copy collaterals at many level of the motor command stream. Spinal ascending signaling systems can be regarded as part of this reporting system and will represent the main focus of the here presented thesis.

I will present the result of my studies on the organization of spinal ascending pathways signaling information to the lateral reticular nucleus (LRN) in the brainstem using a newly-developed mouse genetic tool intersectionally with viruses. I will focus on : (1) the organization of forelimb premotors signaling information to the LRN, (2) their developmental origin and (3) the organization of spinal premotor and non-premotor input to the LRN. In the following chapters, I will also present preliminary

behavioral data on the role of the LRN in reaching behavior before and after the learning of the motor task, as well as on the inhibitory inputs to spinocerebellar neurons in the lumbar spinal cord and differences among them.

The last part of my thesis concerns my contribution to the project of Cyrill Goetz. The aim is to elucidate the structural organization and composition of premotor neurons controlling muscles with distinct biomechanical functions, axial- versus limb muscles. I will show the results obtained in the premotor distribution of axial muscle innervating motor neurons and my contribution in corroborating these findings by means of an anterograde viral approach.

Chapter 2

Introduction

The interaction with the external word relies on the ability to move and this distinguishes the animal from the plant kingdom. Some movements are induced by external stimuli whereas others are the result of planning and may also dependent on learning to perform a certain task. One aspect that is crucial for any movement is the precise and coordinated activation of group of muscles executing a movement. Among different forms of movements, basic locomotion and reaching/grasping are of fundamental importance when an animal is looking for food, escaping dangerous situations or in order to find a partner. Animals develop different motor strategies according to the environment they inhabit. Locomotion is a movement paradigm performed also by the more simple organisms without limbs whereas reaching/ grasping requires a more fine control and can be observed in evolutionarily more advanced organisms. Independent of whether it is locomotion or the more complex reaching behavior concerned, all animals need a nervous system that can orchestrate the temporally appropriate contraction of muscles to generate the desired movement output.

Aquatic animals and terrestrial ones adopt different strategy to move but more importantly, the support of the body weight evolutionary have been joined through the

development of limbs by the terrestrial ones. Limbs support the body weight and allow the animals to experience the word outside but at the same time, limbs can be use to catch a pray or reach for food. In both cases, reciprocal and coordinated movements of the appendages (flexion and extension of a joint) are required. Similarly, in all vertebrate the muscles are innervated by motor neurons whose cell bodies reside in the spinal cord. Specific genetic programs and signaling molecules allow motor neurons to innervate appropriate muscle groups. Muscles are innervated as well by sensory neurons with cell bodies residing in the dorsal root ganglia. Activation of motor neurons leads to muscle contraction. Input converging on motor neurons originates from different sources: sensory input from receptors outside the spinal cord, from interneurons in the spinal cord or from supraspinal centers as brainstem, midbrain or cortex. It is thought that every animal species developed or reinforced specific neuronal circuits according to the way they interact with the external word.

Nevertheless, no movement could be accomplished or learned correctly if the nervous system wasn't able to detect and adjust on line its own commands. To be able to accomplish this, the nervous system generates copies of its motor output pathways (efference copy) at many levels in the motor command stream, in order to integrate it with the sensory information generated upon movement.

The main topic of my thesis is to elucidate the nature and organization of efference copy signaling systems. In fact, although much has been done through electrophysiological recording in cat or monkey, a precise description of how this information is organized at the circuit level is still missing. We have taken advantage

of the progress in mouse genetic and viral tracing in conjunction with the fine genetic characterization of interneuronal population in the spinal cord, to start to investigate spinal ascending pathways relaying efference copy signal to supraspinal centers. In the following chapters, I will present the genetic dissection of ascending spinal pathways relaying efference copy information to the cerebellum.

2.1 Efference copy pathways in the motor system

The interaction of an animal with the external word relys on the ability of self generated movement and sensory perception. The muscular system acts according to instructions supraspinal centers convey by descending pathways to the spinal cord, where motor neurons ultimately elicit muscles contraction. Muscles contraction can generate sensory feedback derived from its own movement in the form of "reafference". However the sensory channel delivering such information can convey as well information about environmental changes in the form of "exafference"(Holst and Mittelstaedt 1950). In other words, sensory channels would convey both exafference and reafference equally and downstream processing would proceed identically for both. Such an organization would generate a sensory and interpretative problem due to the inability to distinguish the two sensory inputs (self-generated vs external world-induced). The animal's nervous system developed a uniform strategy to solve this ambiguity (Crapse and Sommer 2008; Poulet and Hedwig 2007; Sommer and Wurtz 2008; Miall and Wolpert 1996). Animals keep track of their own movement commands and inform the sensory channels about movement that is imminent to occur. von Holtz and Mittelstaedt referred to that strategy as "efference copy signal". To identify neurons conveying such information, one must demonstrate

that they signal movement-related activity and establish collaterals upstream, away from motor neurons, instead of downstream towards motor neurons. In other words, their activity must transmit information about movement without causing movement (Sommer and Wurtz 2002). The motor command stream destined for an effector (muscles), generates a coincident copy delivered to the sensory stream (Figure 1).

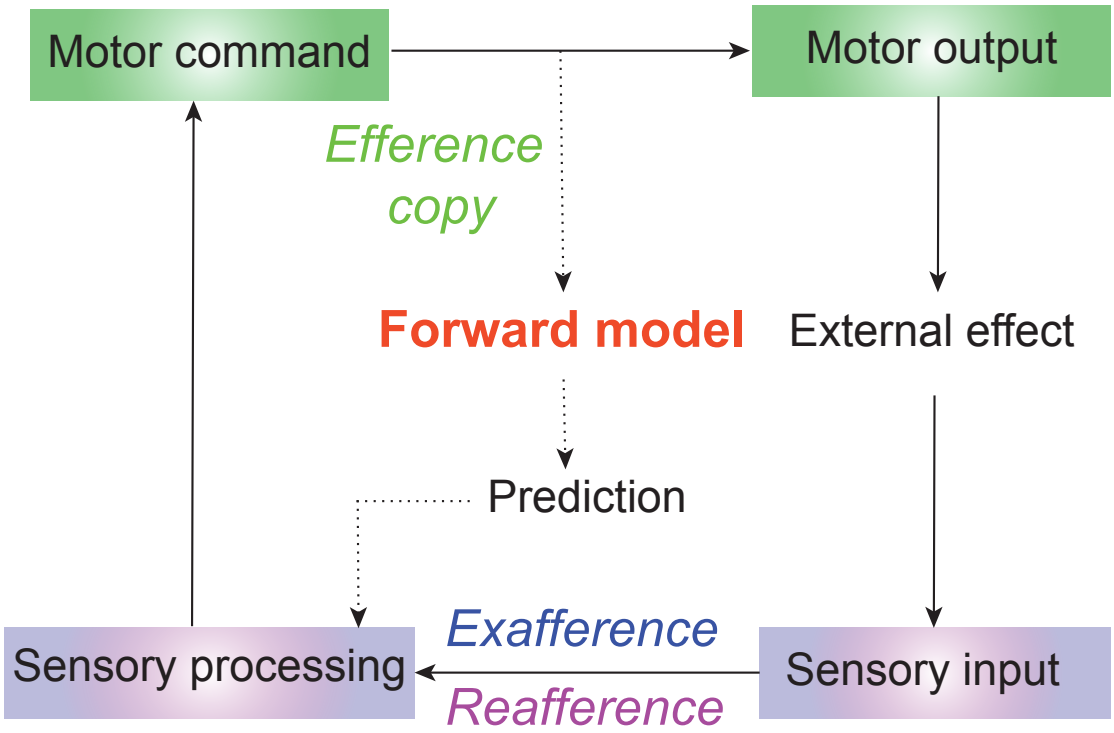


Figure 1. Forward model in the sensory motor system. Motor command translated into motor output generates some effects on the external word and this causes sensory input. Forward models take the copy of the motor command (efference copy) and predicts the expected sensory input. The actual sensory input is an heterogeneous information (exafference and reafference). The confusion among the two types of sensory information is then solve comparing the actual sensory input with the predicted one generated by the forward model.

The loop described above is generally referred to as "forward model" in the literature(Webb 2004; Wolpert, Ghahramani, and Jordan 1995). Besides the ability to distinguish sensory inputs, the forward model helps to adjust and modify the

descending motor commands for accurate motor performance. A well characterized circuit that incorporates an efference copy to generate a correct motor behavior is the songbirds basal ganglia circuit of vocal learning (Fee 2012). Young songbirds acquire their song by vocal imitation using a reinforcement learning mechanism (Fee and Goldberg 2011). An essential brain area underlying vocal learning in the songbird is AreaX, a basal ganglia circuit that has high homology with the mammal one. AreaX receives glutamatergic input from two distinct area: the lateral nucleus of the anterior nidopallidum-LMAN and the HVC, a cortical region controling the temporal structure of the song. The LMAN is a cortical area whose function is the generation of vocal babbling and exploratory variability in learning birds. LMAN neurons project to a nucleus of the archipallium-RA, homologous of the primary motor cortex in mammals and they produce a collateral that terminates in AreaX. During singing, LMAN neurons generate highly variable activity patterns that drive variability in the vocal motor pathway. Therefore in this view, the LMAN input to AreaX can be seen as an efference copy of the ongoing motor signal that drives vocal exploration. Lesions of the basal ganglia circuitry in this system have little effect on the generation of the vocal exploration during learning (Goldberg and Fee 2011) whereas cooling down leading to inactivation of the LMAN results in slowing the timescales of vocal babbling (Aronov et al. 2011). These experiments suggest that LMAN is the variability generator that drives vocal exploration during learning. An additional input to the AreaX comes from the VTA, a dopaminergic input that might carry real-time information about song performance (Fee and Goldberg 2011). Therefore, with the above mentioned song evaluation signal and the efference copy from the LMAN leading to vocal variability, AreaX would be in a position to determine which

variations lead to a better song outcome (Fee 2012). Despite the major role played in the generation of correct motor responses, little is known about their structure, composition, synaptic organization and genetic identity.

2.2 Ascending spinal pathways

Ascending spinal pathways are the main route through which supraspinal centers are constantly informed about internal conditions, position and movement of the body and input from the external word. Generally speaking, propriospinal neurons project to spinal segments caudally or rostrally in relation to their cell body position and/or to supraspinal centers. From the spinal cord, propriospinal axons can reach different brain areas, conveying information about the activity of peripheral receptors (e.g. Ia propioceptor, cutaneous, mechanoreceptive) or of spinal circuits (mainly spinal CPGs). The cerebellum receives information about peripheral events but also central processes through numerous precerebellar systems, predominantly in two different flavors as mossy fibers (MFs), treated in more detailed in following paragraphs, and climbing fibers (CFs). A major difference between the two types of inputs is the direct or indirect regulation of Purkinje cells (PCs). Mossy fibers have an indirect influence on PCs through granule cells-parallel fibers synapses, whereas climbing fibers have a potent direct effect on PCs. Bursts of action potential known as complex spikes in PCs can be triggered by a single inferior olivary neuron (Eccles, Llinás, and Sasaki 1966). Granule cells receive up to 4 mossy fibers inputs, potentially of different origin (Huang et al. 2013). Each of them can therefore exhibit only a small excitatory effect on Purkinje cells compared to climbing fibers. Purkinje cells are GABAergic, and are the sole output of the cerebellar cortex reaching deep cerebellar and the vestibular nuclei. The cerebellar output can influence in turn

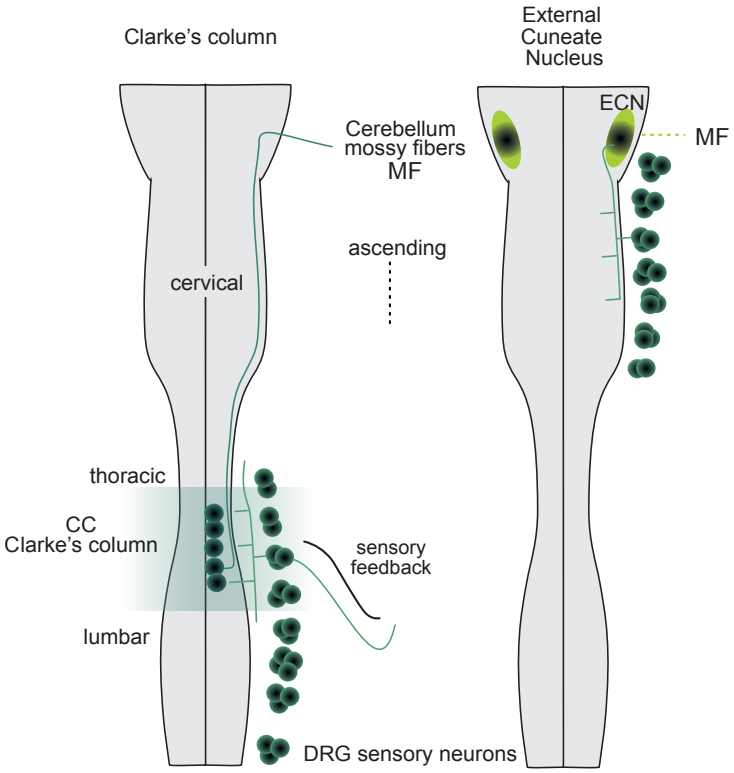
descending pathways as for example the red nucleus or the reticular formation. Climbing fibers arise only from neurons of the inferior olivary nucleus and their terminal field topography perfectly overlaps with PCs zones (Sillitoe 2012). The Inferior olive is organized into subnuclei: the dorsal and medial accessory olive and the principal one plus other several smaller ones. Spinal projections to the olive arise mainly from the thoracic and lumbar spinal cord and they target the caudal part of both accessory olives (Armstrong 1974). The inferior olive have been implicated in learning in respect to the adaptation of the vestibulo-ocular reflex (VOR) (Ito 1982; Ito 2013; De Zeeuw et al. 1998) and in timing of motor behavior (Chen et al. 1995; Welsh et al. 1995). In 1995, Chen suggested that motor dis-coordination observed in PKCγ mutant mice might be due to the persistent multiple innervation of PCs by CFs. In 1995, Welsh suggested that the olivocerebellar control of movement derives from populations of olivary neurons operating as a distributed system whose collective activity is rhythmic and temporally related to specific parameters of movement activity in absence of sensory input. Despite the anatomical description of the input (Brown, Chan-Palay, and Palay 1977) the IO receives from the spinal cord, is currently unknown its contribution to the suggested roles of the IO. Only in the vestibulo-ocular reflex, the visual signal derived from the accessory optic system (Simpson 1984; Ito 2013) and vestibular sensory input from the nucleus prepositus hypoglossi (De Zeeuw, Wentzel, and Mugnaini 1993) to the IO have been investigated. Other areas, receiving spinal input, encompass the caudal ventrolateral medulla (CVLM), the lateral parabrachial area (LPb), the periacqueductal grey matter (PAG) and extensively the thalamus (Gauriau and Bernard 2002; Gauriau and Bernard 2003; Todd 2010). These pathways convey pain information and might play a role in

higher cognitive function such as affective-motivational aspects of pain through the somatosensory and insular cortex. An other proposed brain area receiving ascending information about ongoing spinal activity is the red nucleus in the hindbrain (Vinay et al. 1993; Vinay and Padel 1990).

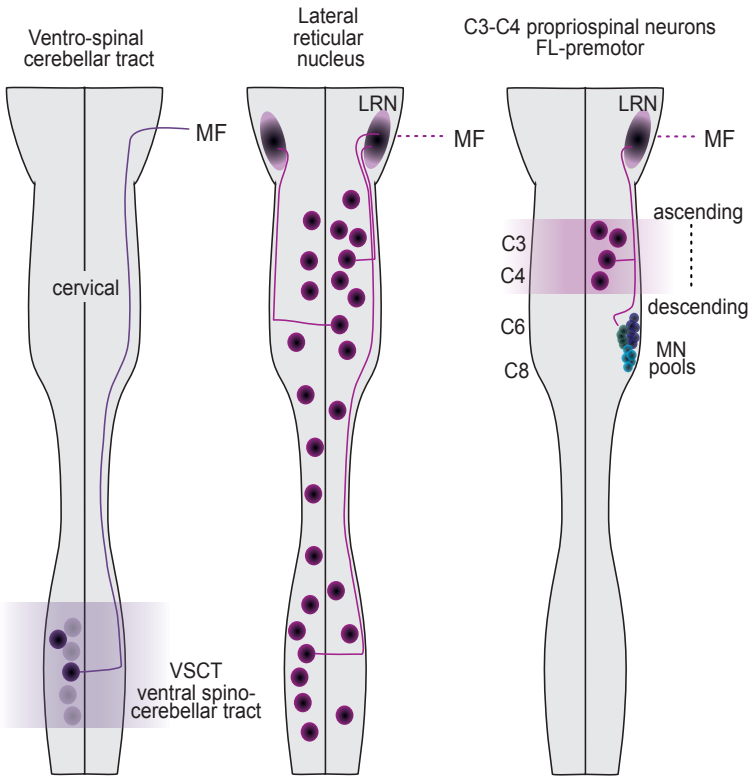
In the following paragraphs, the attention will be moved on spinal ascending information delivering mossy fibers input to the cerebellum. Mossy fiber input can roughly be divided into a system for processing forelimb (FL)-related information residing mostly in the brainstem and another one for handling hindlimb (HL)-related information located at lower thoracic and rostral lumbar spinal levels ((Oscarsson 1965; Orlovsky et al. 1999)). Each of these two systems is subdivided into further modules that can be considered to process functionally equivalent information for the FL and HL dedicated systems respectively. In particular, one module transmits efference copy signals of ongoing spinal activity (FL: lateral reticular nucleus, LRN; HL: ventral spino-cerebellar neurons, VSCN), and another module is primarily concerned with sensory feedback information reporting exafference and reafference infomation (FL: external cuneate nucleus, ECN; HL: Clarke's column, CC) (Figure 2).

Figure 2. Spinal ascending pathways. Sensory information (exafference and reafference) is conveyed through two parallel system. Sensory lumbar information is delivered through Clarke's column cells to the cerebellum whereas the ECN in the brainstem delivers sensory brachial information. These two pathways are mirrored to the system delivering efference copy information the cerebellum, from lumbar spinal cord VSCT and from brachial the LRN.

Exafference and reafference



Efference copy

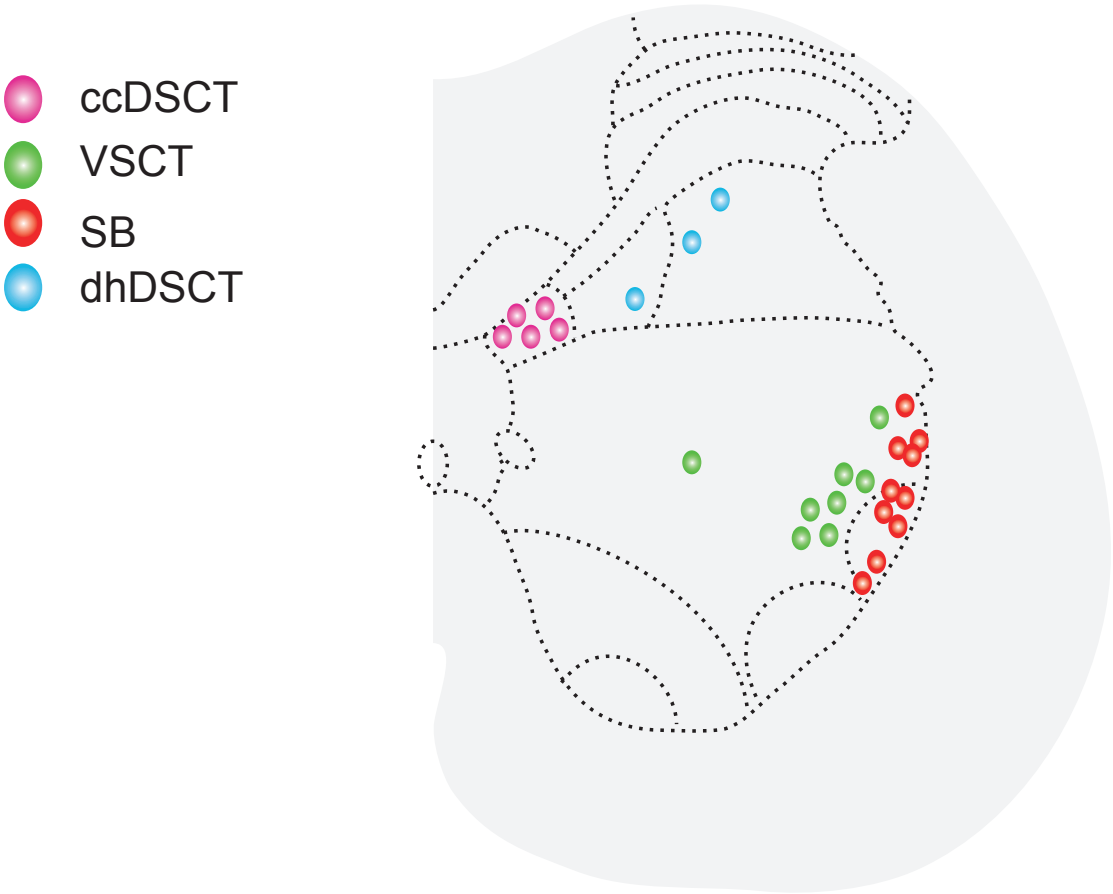


2.2.1 Hindlimb related ascending pathways

2.2.1.1 Dorsal-spinocerebellar tract (DSCT)

Dorsal spinocerebellar tract (DSCT) neurons can be subdivided in two main groups: Clarke's column (ccDSCT) and dorsal horn dorsal spino-cerellar neurons (dhDSCT). Their axons ascend almost exclusively on the ipsilateral side (uncrossed) reaching the cerebellum through the inferior cerebellar peduncle and terminating on the ipsilateral side in the anterior lobule I-IV and in the posterior pyramis (central lobule 8) and paramedian lobule. They were primarily found to carry information about peripheral events, subpopulations of these neurons monitoring input from different types of receptors including muscle spindles, Golgi tendon organs, joint and cutaneous receptors (Oscarsson 1965; Edgley and Jankowska 1988). Information carried by the whole population of these neurons has been found to reflect whole limb kinematics (BOSCO and POPPELE 2001). ccDSCT have their own origin in Clarke's column spinocerebellar neurons that span from mid-thoracic to the beginning of lumbar spinal cord (Hongo et al. 1987; Walmsley 1991) and receive their main excitatory input from Ia afferents and inhibitory input from group Ib and II afferents. ccDSCT have been found to receive direct excitatory input from cortico-spinal tract neurons (Hantman and Jessell 2010) but only indirect excitatory input from reticulospinal neurons (Hammar et al. 2011). The dhDSCT neurons located in the dorsal horn receive excitatory and inhibitory input from group II and cutaneous afferents but not group I afferents (Edgley and Jankowska 1988)(Figure 3).

Figure 3. Hindlimb related ascending pathways. Representation of laminar cell body location for ccDSCT, dhDSCT, SB and VSCT neurons in the lumbar spinal cord. The lumbar level is only indicative.



2.2.1.2 Ventro-spinocerebellar tract (VSCT)

The ventro-spinocerebellar tract (VSCT) neurons can be divided into two main groups: VSCT neurons and spinal border neurons (SB). Together, these neurons are rather scattered and extend more caudally than the Clarke's column. The VSCT axons cross to the contralateral side at a level close to the cell bodies and they reach the cerebellum through the superior cerebellar peduncle terminating mainly in the anterior lobe contralaterally. VSCT neurons locate in the medial part of lamina VII and receive excitatory peripheral input mainly from group Ib afferents. VSCT neurons are excited both directly and indirectly by reticulospinal, vestibulospinal,

corticospinal and rubrospinal neurons (Hammar et al. 2011; Jankowska, Nilsson, and Hammar 2011; Baldissera and Roberts 1976). SB neurons, located at the border between the white and the gray matter in the ventral horn, can receive excitatory peripheral input mainly from Ia afferents or be devoid of such input and inhibitory input from Ia, Ib, II and high-threshold muscle, skin and joint afferents. SB neurons with input from Ia afferents have been recently suggested to forward information about the probability of activation of motor neurons by descending commands (Jankowska, Nilsson, and Hammar 2011).

Recently, the input to DSCT and VSCT neurons has been investigated by immunohistochemistry (Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012a; Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012b). The authors used a similar approach in both studies comparing cat versus rat spinocerebellar neurons backlabelled from the cerebellum. They evaluate synaptic contacts upon immunostaining of vesicular glutamate transporter 1 (VGLUT1) to label primary afferent terminals and corticospinal tract, VGLUT2 to mark axon terminals of spinal interneurons and most descending tract neurons with the exception of the corticospinal tract (Liguz-Lecznar and Skangiel-Kramska 2007), VGAT and GAD65 and 67 (Kaufman, Houser, and Tobin 1991; Todd et al. 1995). The quantitative analysis of the excitatory and inhibitory input on the different subpopulation revealed striking differences. Regarding VGLUT1-immunoreactive terminals on VSCT and SB neurons, these are significantly less abundant than on ccDCST and dhDSCT neurons and among them, the majority was found in ccDSCT neurons, known targets of the corticospinal tract and Ia afferents. The inhibitory axonal (VGAT positive) contacts onto SB and VSCT neurons were described to be

significantly more on these populations compared to the DSCT neurons, providing a morphological substrate for the differences in inhibition of these neurons found in electrophysiological studies. In fact, electrophysiological studies revealed particularly strong inhibitory inputs to SB and VSCT neurons. Inhibitory input to these neurons is evoked to great extent by collateral actions of premotor interneurons mediating disynaptic inhibition from group Ia, Ib and II afferents (Jankowska, Krutki, and Hammar 2010), Renshaw cells, high threshold muscle, cutaneous and joint afferents (Baldissera and Roberts 1976). Although informative, clear evidence of the nature and specificity of the input these different spinocerebellar neurons receive is still missing, as well as clear evidence of the behavioral relevance of the feedback information they convey to the cerebellum by these neurons are currently only speculative.

2.2.2 Forelimb related ascending pathways

2.2.2.1 Cuneocerebellar tract (CCT)

The cuneocerebellar tract (CCT) is considered to be the functional equivalent of the DSCT. CCT cell bodies are located in the Dorsal column nuclei (DCN) in the brainstem and are a relay station in the dorsal column medial leminiscal somatosensory pathways and one of major sources of cerebellar somatosensory mossy fibers (Cerminara, Makarabhirom, and Rawson 2003)besides the spinal cord (CC and VSCT). The DCN is generally subdivided into four parts, the Cuneate nucleus (CuN), the Gracile nucleus (GN) and the External Cuneate nucleus (ECuN) and the nucleus Z (Quy et al. 2011). Only the ECuN, is projecting to the cerebellum. The information these nuclei convey, is forelimb cutaneous (CuN), hindlimb cutaneous (GN), forelimb proprioception (ECuN) and hindlimb proprioception (Nucleus Z) (LANDGREN and

Silfvenius 1971) although minor in respect to CC. The caudal part of the CuN and the GN in the cervical spinal cord do not contain cerebellum-projecting neurons whereas the ECuN is entirely composed of cerebellum-projecting neurons (Quy et al. 2011). The DCN can receive ascending axons of primary somatosensory neurons or secondary neurons of the spinal cord delivering sensory information. Mechanoreceptors mainly innervate the GN and a dorso-medial domain of the CuN, while the ECuN receives direct input from proprioceptive sensory neurons with their body in dorsal root ganglia from upper to T6 (Niu et al. 2013) and it projects to the cerebellum through the inferior cerebellar peduncle. The tract terminates almost exclusively in the ipsilateral part of the cerebellar cortex (Oscarsson 1965). Ascending proprioceptive axons from below T6 travel for a few segments before terminating at thoracic levels whereas all the mechanoreceptors project to the medulla regardless of their soma position (Niu et al. 2013).

2.2.2.2 The Lateral reticular nucleus (LRN): inputs and output

The lateral reticular nucleus (LRN) is a precerebellar nucleus of the reticular formation residing in the caudal medulla and receiving most of its input from the spinal cord. Its main output are the cerebellar cortex and deep cerebellar nuclei and the non cerebelellar structure cochlear nucleus (Zhan and Ryugo 2007) . The first description of the spinal input to the LRN dates back to Cajal who in 1909 described fibers terminating or giving off collaterals to the LRN. Although most of the following studies employed staining of fibers degenerated upon lesion of spinal tracts in the cat as entry point of their characterization, it have been clear that the LRN was one of the major terminations of ascending spinal input for many years. In cat and rabbit, Brodal (BRODAL 1943) first subdivided the nucleus according to cell size,

density of cells and synaptic terminals into a magnocellular (mLRN-dorsolateral large division), a parvicellular (pLRN- ventrolateral small division) and a subtrigeminal part (stLRN- rostrally from the main portion of the nucleus). In 1949 Brodal (BRODAL 1949) notably noticed that the terminal area of the spinal afferents does not comprise the entire nucleus but it is limited to its caudal and ventro-lateral parts (magnicellular and parvicellular), thus sparing most of the nucleus and suggesting that the intact rest of the nucleus most likely receives at least a majority of its fibers from higher levels of the brain. He then tried to correlate the termination pattern of the spinal afferents with the termination in the cerebellum. The differential distribution of spinal afferents in the nucleus was further analyzed through both electrophysiology and staining techniques in the cat. In particular, it was shown that the magnocellular part of nucleus receives the bulk of the spinal afferents from the cervical and high thoracic spinal segments and these terminations are mainly ipsilateral whereas the lumbar projections where restricted to the small neurons of the parvicellular part. It was suggested that the LRN, as well as other brainstem nuclei are not purely relay nuclei for specific pathways but that they may represent higher order of convergence from different sources (Künzle 1973). Whereas it was pretty much clear from these old studies in cat and more recently in rat (Shokunbi, Hrycyshyn, and Flumerfelt 1985; Garifoli et al. 2006) that spinal input was topographically organized, only attempts were done in correlating this topography with the projections pattern to the cerebellum (Clendenin, Ekerot, Oscarsson, and Rosén 1974a; Payne 1987; Wu, Sugihara, and Shinoda 1999). The cell body location of propriospinal neurons projecting to the LRN was assessed with injection of retrograde tracer in rat and cat (Shokunbi, Hrycyshyn, and Flumerfelt 1985; Corvaja et al. 1977; Menétrey, Roudier,

and Besson 1983; Koekkoek and Ruigrok 1995). These studies revealed similar patterns of distribution in the two species. In the cervical spinal cord, propriospinal neurons with axons extending to the LRN are mainly ipsilateral located (broadly from lamina III to VII) with the contribution of a contralateral population in lamina VIII, whereas in the lumbar spinal cord, the main component resides contralaterally in lamina VIII and VII, with only a minor contribution from the ipsilateral lamina IV and V. Among these propriospinal neurons projecting to the LRN, a particular population mainly investigated in cat has received much of attention: the C3-C4 PNs will be subject of detailed analysis in the following paragraph (for an extensive review- (Alstermark et al. 2011)). The nature of the spinal input was first assessed thought recordings of the LRN activity during fictitious and actual scratching in cat (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a). The firing pattern of the LRN neurons was similar with (actual scratching) and without (fictitious scratching) sensory input. Therefore the activity of LRN neurons was attributed to central mechanisms rather than to the rhythmical sensory input. Importantly, the spinal cord is not the only input the LRN receives and delivers to the cerebellum. Supraspinal inputs have been investigated through retrograde and anterograde tracer dye in cat, rat and monkey (Corvaja et al. 1977; Walberg 1958; Marini and Wiesendanger 1987; R. Wiesendanger and Wiesendanger 1987; Rajakumar, Hrycyshyn, and Flumerfelt 1992). In these studies, the three main areas found with projections to the LRN are the contralateral red nucleus, the contralateral fastigial nucleus, motor cortex and partially somatosensory cortex although not as clearly as the red nucleus.

Despite the significant amount of studies on input and output of the LRN only one tentatively addressed its function on posture and reflex movement (Santarcangelo,

Pompeiano, and Stampacchia 1981). Cats injected with kainic acid showed postural asymmetry with ipsilateral hypertonia and contralateral hypotonia of the limb extensor muscles, transient depression of proprioceptive placing reaction and a persistent deficit of the tactile placing reflex. The behavioral relevance of the spinal input was addressed only for the C3-C4 PNs population in cat or monkey more recently (Kinoshita et al. 2012), through lesion studies (Alstermark, Lundberg, et al. 1981).

2.2.2.2 Spino-reticulocerebellar tract (SRCP)

The spino-reticulocerebellar tract (SRCT) (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a), having its last order neurons in the LRN in the caudal medulla, has been physiologically divided into 3 components: the bilateral ventral reflex tract (bVFRT), the ispilateral forelimb tract (iFT) and the C3-C4 PNs propriospinal system. The SRCT reaches the cerebellum through the inferior and superior cerebellar peduncle (Oscarsson 1965). The bVFRT is one of the major components of the SRCT to the LRN (Grant, Oscarsson, and Rosén 1966). bVFRT neurons have wide, often bilateral, peripheral receptive fields and are monosynaptically activated by the vestibulospinal tract (Clendenin, Ekerot, Oscarsson, and Rosén 1974b). It has been suggested that the bVFRT carries information about activity in spinal motor centers influenced by segmental afferents and descending motor paths (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a). Besides that, the bVFRT has been shown through intracellular recordings from the LRN in cat to consist of approximately equally large groups of excitatory and inhibitory neurons making both monosynaptic connections to LRN neurons and having similar segmental distributions, peripheral receptive fields and termination area in the LRN (Ekerot 1990a). It was suggested that bVFRT

neurons are mainly active during the extensor phase, in contrast to the ventral spinocerebellar tract (VSCT) neurons, which are active during the flexor phase, suggesting that the two systems signal activity about complementary populations of spinal interneurons controlling rhythmic activity like scratching (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a; Arshavsky, Gelfand, Orlovsky, and Pavlova 1978b). However, this view was challenged by (Ezure and Tanaka 1997) who showed that during fictive locomotion, LRN neurons fired both during flexion and extension phase of the scratch. Both studies agree however on the fact that the rhythmic activity of LRN neurons is of central but not of peripheral origin. In fact, during actual and fictitious scratching (in absence of sensory input), the rhythmical burst firing of LRN neurons is similar.

(Clendenin, Ekerot, and Oscarsson 1974) demonstrated the existence of a parallel tract to the main bVFRT ascending to the LRN and denoted as ipsilateral forelimb tract (i-FT). The i-FT is polysynaptically activated by cutaneous afferents and high threshold muscle afferents (group II and III) and can be subdivided in an excitatory and inhibitory component similarly organized (Ekerot 1990b). i-FT neurons are located in the forelimb innervating segments, their axons ascend ipsilaterally in the ventral part of the lateral funiculus, and they terminate in the dorsal part of the magnicellular LRN, segregating from the bVFRT. It is not known to what extent the iFT neurons are activated by descending pathways, but it has been suggested that some may receive input from the cortico- and rubrospinal tracts (Ekerot 1990c).

2.3 C3-C4 PNs system

Early studies in monkey (LAWRENCE and KUYPERS 1968; Lawrence and Kuypers 1968), demonstrate how skilled finger movement was dependent on the cortico-motor neuronal (CM) pathway. Much of the attention in the motor control of forelimb movement (reaching and grasping) was directed to the motor cortex with studies in cat and monkey. Initially, (Illert, Lundberg, and Tanaka 1977) investigated the cortical input to motor neurons (MNs) in cat. The stimulation of the pyramidal tract elicited disynaptic EPSPs in forelimb motor neurons. This response remained after transection of the corticospinal tract at C5 but abolished after a C2 segment transection. The same result was obtained for input from the rubrospinal tract. It was concluded that disynaptic cortico-motor neuronal and rubro-motor neuronal excitation must have been relayed by a population of propriospinal neurons originating in the C3-C4 segment of the spinal cord. Subsequent studies (Illert et al. 1978) further characterized this population through direct recordings from the cells body in C3-C4 segments and defined the inputs delivered to MNs as monosynaptic from not only the motor cortex but also from the rubrospinal and tectospinal tract and oligosynaptic from cutaneous and muscular afferents of the ipsilateral forelimb. Shortly after (Illert and Lundberg 1978) demonstrated that stimulation in the LRN antidromically evoked monosynaptic responses in forelimb MNs and postulated that they activated neurons with their cell body in C3-C4 segments projecting to lower cervical segments. Since then, the “C3-C4 PNs system” built of bifurcating propriospinal neurons (one ascending branch to the LRN and one descending to forelimb MNs), started to be investigated in cat by Alstermark and more recently, in monkey by Isa (Isa 2006; Alstermark et al. 1999) through electrophysiological recordings and lesions of spinal tracts. In particular, this

system received great attention for its supposed role in target reaching movements. First evidence for such a function came from lesion studies (Alstermark, Lundberg, et al. 1981). The authors aimed to investigate the relative contribution of the C3-C4 PNs and the neuronal network within the forelimb segments for precise forelimb movement. The lesions done were the following: 1. Cortico- and rubro-spinal tract in C5 (input to forelimb neurons); 2. Cortico- and rubro-spinal tract in C2 (input to C3-C4 PNs); 3. ventral lesion in C5 of the descending C3-C4 PNs axons to MNs; 4. ventral lesion in C2 to interrupt the bulbospinal fibers and ascending C3-C4 PNs collaterals. The combination of these lesion experiments and others led the investigators to postulate that the "C3-C4 PN" system transmits supraspinal input to forelimb MNs for target-reaching movements but not grasping, which in turn would be dependent on the direct activation of neuronal networks within the segments by cortico- and rubrospinal tracts. Further characterization (Alstermark, Lindström, et al. 1981; Alstermark, Lundberg, and Sasaki 1984) subdivided the population in an excitatory and inhibitory component. Recently a study in monkey (Kinoshita et al. 2012) demonstrated with a new viral approach the involvement of the PN pathway in the control of hand dexterity.

Elecrophysiological studies, dye tracing and tract lesions in rats and cats did not allow experimenters to address the question of the genetic identity of propriospinal neurons. Recently, the advances in mouse genetics and spinal cord neuronal development allow us to investigate the genetic identity of spinal ascending populations. In the following paragraph, I will briefly summarize the current knowledge and progressed done on spinal neuronal subpopulations.

2.4 Spinal neuronal subpopulations

The building blocks of the spinal motor system are local interneurons whose activity is orchestrated in order to control spinal motor neurons. Neuronal networks capable of generating organized patterns of motor activity independent of sensory inputs are generally referred to as central pattern generators (CPGs) and these have first been described in the locust (WILSON and WYMAN 1965). Descending input from cortex, basal ganglia and brainstem engage and shape selective CPG networks during different motor sequences with further modulation of their activity by sensory, vestibular and other descending pathways. However, the CPG are thought to be the key elements to drive the rhythmic activity of motor neurons.

During development, local progenitors give rise to postmitotic neurons during temporally restricted periods. Along the dorso-ventral axis of the neuronal tube, the influence of signaling cues, the dorsal bone morphogenetic proteins (BMPs) and the ventral Sonic-hedgehog (Shh), lead to spatial subdivision of the progenitor domain territory(Jessell 2000). Many of these spinal progenitors can be identified by expression of a unique combinations of transcription factors at early embryonic time points and can be further divided into 11 major subpopulations (Jessell 2000; Grillner and Jessell 2009; Kiehn 2011; Arber 2012). Postmitotic neurons can therefore be subdivided into six dorsal (dI1 to dI6) and 5 ventral (V0 to V3 and MNs) derived main populations. The dorsal populations are generally considered responsible for the transmission of sensory input to motor neurons and brain. Although not all dorsally derived populations play a role in sensory transmission and some, such as the dI6, migrate to finally reside in a ventral position (Alaynick, Jessell, and Pfaff 2011). For example, the dI4 population, defined by the expression of Ptf1a, gives rise to

GABAergic interneurons that mediate presynaptic inhibition of proprioceptive sensory input (Betley et al. 2009; Glasgow et al. 2005), but more generally, make up all inhibitory neurons of the dorsal spinal cord. Recently, dI3 interneurons, defined by the expression of the transcription factor Isl1, have been shown to form excitatory glutamatergic synapses with motor neurons and in turn, receive low-threshold cutaneous afferent input. The elimination of their glutamatergic transmission results in grasping defects in mice (Bui et al. 2013). Although dorsally derived, the dI6 interneurons migrate ventro- medially to laminae VII/VIII of the postnatal spinal cord (Gross, Dottori, and Goulding 2002) and are likely to be part with the ventral subpopulation to the CPG network (Lanuza et al. 2004; Müller et al. 2002). They express, together with dI1 and dI5, Lbx1 at early embryonic time points and are commissural inhibitory interneurons. The lack of a unique molecular marker for these neurons did not allow the evaluation of their hypothetical contribution in the locomotor CPG. An attempt to demonstrate the involvement of the dI6 in the spinal locomotor CPG came from a recent study of (Dyck, Lanuza, and Gosgnach 2012). In this study, the authors tried to isolate the dI6 population through a genetic strategy. Evaluating the membrane properties of the putatively dI6 interneurons, they arrived to the conclusion that the dI6 population is indeed heterogeneous. In part, it is involved in the rhythm generation (spinal CPGs) and in part it regulates motor neuron firing by input from the CPGs.

More attention has been given to interneurons with ventral origin, putative core CPG interneurons. Although till now, no single genetically defined interneuronal population has been shown to be necessary and sufficient for the generation of the rhythmic activity in the locomotor CPG. V0 interneurons, derived from Dbx1

expressing progenitors, are subdivided in an excitatory and inhibitory as well as cholinergic component and are as dI6 commissural inhibitory interneurons located in lamina VIII (Goulding 2009). V0 neurons express the transcription factor Dbx1 necessary for the development of commissural properties. They are necessary for proper coupling of the left and right hindlimb CPG during walking, as isolate spinal cords from *Dbx1-/-* mice exhibited intermittent periods of synchronous hopping-like activity. Normal period of alternation are still occurring, making them not the only source of left and right alternation (Lanuza et al. 2004). The excitatory V3 population is part, with the dI6 and V0, of the commissural populations. It has been proposed that they ensure a normal walking gait by controlling two important aspects of the locomotor rhythm. When fictive walking is induced in isolate spinal cord preparations, these neurons are required for coherent rhythmic bursting of flexor and extensor related motor neurons. Second, they additionally function to balance the locomotor output between both halves of the spinal cord, therefore ensuring a symmetrical pattern of locomotor activity during walking (Zhang et al. 2008). Recently, it was shown that the V3 population in thoracic and upper lumbar segments consists of a dorsal, medial and ventral population recruited in different locomotor activities and characterized by different morphologies and electrophysiological properties (Borowska et al. 2013). Among ipsilaterally projecting interneurons, the V1 population, defined by the expression of Engrailed1 transcription factor, seems to be the most heterogeneous presently described (Sapir et al. 2004; Alvarez et al. 2005). This class comprises two known types of local circuit inhibitory neurons (Renshaw cells-RCs and Ia inhibitory interneurons-IaINs) and one or more undefined inhibitory interneurons. RCs mediate recurrent inhibition of homonymous and synergistic motor

neurons and receive excitatory input from intraspinal motor axons collaterals (RENSHAW 1946). IaINs are characterized by inputs from sensory Ia muscle afferents and provide reciprocal inhibition to antagonistic motor pools (Eccles, FATT, and LANDGREN 1956). V1 neurons are required to regulate locomotor speed (Gosgnach et al. 2006). The ispilaterally projecting V2 population expresses the transcription factor Lhx3, is subdivided into an excitatory V2a Chx10 positive (Crone et al. 2008), an inhibitory V2b GATA3 positive and V2c Sox1 positive component (Panayi et al. 2010). The ablation of the V2a interneurons leads to the disruption of the left and right alternation because of the absence of excitatory drive to the commissural population (Crone et al. 2008). Recently, the homeodomain protein Shox2 have been shown to mark a discrete subset of ventrally positioned glutamatergic neurons with ipsilateral projections and targets. The majority of this population coexpress Chox10 (Dougherty et al. 2013). Therefore the V2 population appears to be more diverse comprising a population of V2d (Shox2+/Chox10+) additional to the V2a (Chx10+) previously reported (Crone et al. 2008).

In summary, much has been described on spinal neuronal subpopulations in term of developmental origin. More importantly, neurons originated from different progenitor domains have been described to have a particular connectivity profile, neurotransmitter phenotype and function in the spinal cord circuits. However, most of the functional work on spinal subpopulations have focused on their contribution to locomotor CPGs and local spinal network processing, but have never addressed their putative influence on supraspinal center. As each of them plays a different function, the hypothetical information delivered to supraspinal center is different. Therefore it is important to assess which of them report information to supraspinal centers: Are the

ascending pathway, originating at forelimb level, composed mainly by one of these spinal subpopulations? Are they spatially segregated from other interneurons? Are they encompassing premotor character? In the present thesis we will take advantage of the knowledge on spinal subpopulations and of recently developed tools to retrogradely labeled premotor interneurons (Stepien, Tripodi, and Arber 2010) to investigate the contribution of each of these populations on the spinal ascending pathways previously described.

Chapter 3

Motor-circuit communication matrix from spinal cord to brainstem neurons revealed by developmental origin

Chiara Pivetta, Maria Soledad Esposito, Markus Sigrist, and Silvia Arber

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3.1 Summary

Accurate motor-task execution relies on continuous comparison of planned and performed actions. Motor-output pathways establish internal circuit collaterals for this purpose. Here we focus on motor collateral organization between spinal cord and upstream neurons in the brainstem. We used a newly developed mouse genetic tool intersectionally with viruses to uncover the connectivity rules of these ascending pathways by capturing the transient expression of neuronal subpopulation determinants. We reveal a widespread and diverse network of spinal dual-axon neurons, with coincident input to forelimb motor neurons and the lateral reticular nucleus (LRN) in the brainstem. Spinal information to the LRN is not segregated by motor pool or neurotransmitter identity. Instead, it is organized according to the developmental domain origin of the progenitor cells. Thus, excerpts of most spinal information destined for action are relayed to supraspinal centers through exquisitely organized ascending connectivity modules, enabling precise communication between command and execution centers of movement.

3.2 Introduction

Movement is the behavioral output of neuronal circuits computing motor commands and performance. The muscular system of the body acts according to instructions supraspinal centers convey by descending pathways to the spinal cord, which in turn delivers these commands to muscles through motor neurons eliciting movement. The central nervous system employs two circuit-level strategies to monitor planned and performed motor actions (Crapse and Sommer 2008; Poulet and Hedwig 2007; Sommer and Wurtz 2008; Miall and Wolpert 1996). First, motor output

pathways establish axon collaterals at many levels, providing internal efference copy signals of planned action to recipient neurons. Second, movement-evoked sensory feedback from the body reaches the central nervous system to report on performed motor actions. Together, these two distinct information streams are used to adjust and modify descending motor commands accordingly. Despite their undisputed role in influencing motor behavior, surprisingly little is known about identity, composition and synaptic organization of core circuit elements encompassing these pathways, undoubtedly fundamental information needed to understand their function.

Efference copy pathways arising from spinal neurons with direct connections to motor neurons represent a suitable entry point to address these challenging questions. Landmark studies by Orlovsky and collaborators in the cat demonstrated that locomotor-related ascending signaling streams from the spinal cord are transmitted to supraspinal centers by two main pathways [for review, see (Arshavsky et al. 1986; Orlovsky et al. 1999)]. Of highest relevance to this study, the lateral reticular nucleus (LRN) in the caudal medulla receives synaptic input strongly correlated with ongoing spinal intrinsic information in a manner independent of peripheral sensory feedback, and its activity is in turn relayed to the cerebellum by mossy fibers (Arshavsky, Gelfand, and Orlovsky 2011; Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a; BRODAL 1949; Orlovsky et al. 1999; Oscarsson 1965). Pharmacological ablation of the LRN in the cat leads to movement deficits related to postural balance and paw placement (Santarcangelo, Pompeiano, and Stampacchia 1981). A spinal subsystem projecting to the LRN comprised of neurons referred to as

C3C4 propriospinal neurons (PNs) is implicated in voluntary forelimb motor control in cat and monkey [for review, see (Alstermark et al. 2011; Alstermark et al. 2007)]. C3C4 PNs are located at cervical levels C3-C4 and have the special feature of bifurcating axonal projections, with one ascending branch to the LRN and a second descending branch establishing direct synaptic connections to cervical motor neurons at C5-C8 spinal levels. Collaterals of C3C4 PNs hence transmit an efference copy signal of premotor information directly to the LRN, leading to coincident regulation of motor neurons and LRN. However, whether this neuronal system is constructed as a single homogeneous reporting channel or monitors motor output pathways more generally through functionally diverse neuronal subpopulations is currently entirely unknown and represents a conceptually important question to address.

Recent work in the mouse has provided a wealth of information about genetic specification of neuronal subpopulations and their function in the spinal cord [reviewed by (Alaynick, Jessell, and Pfaff 2011; Arber 2012; Goulding 2009; Grillner and Jessell 2009; Kiehn 2011)]. These studies demonstrate that spinal neurons can be subdivided into 11 cardinal classes based on their progenitor domain origin (dorsal: dI1 – dI6; ventral: V0 – V3; MN: spinal motor neurons), and genetic mutation or silencing experiments reveal a variety of distinct roles of corresponding neuronal subpopulations [reviewed by ((Arber 2012; Goulding 2009; Grillner and Jessell 2009; Kiehn 2011)]. In addition, transsynaptic virus tools (Wickersham et al. 2007) determined the distribution of spinal interneurons with monosynaptic connections to individual motor neuron pools, revealing broad segmental but highly stereotype patterns (Stepien, Tripodi, and Arber 2010; Tripodi, Stepien, and Arber 2011). The

availability of genetic entry points to virtually any spinal neuron in the mouse and the possibility to visualize neurons with direct motoneuronal connections has opened the door to determine functional diversity and connectivity profiles of ascending spinal populations to the brainstem.

Here we unravel the connectivity profiles of ascending pathways from the spinal cord to supraspinal centers, taking intersectional approaches between mouse genetic and viral tools. We find that bifurcating premotor PNs with collaterals to the LRN surprisingly represent a set of highly diverse neuronal populations with residence throughout the cervical and rostral thoracic spinal cord. Neuronal diversification is uncovered by differential genetic identity based on progenitor domain origin during development, which foreshadows distinct neuronal settling positions in the spinal cord. Moreover, genetically diverse spinal subpopulations establish a highly selective and organized connection map to the LRN. Our findings support a model in which LRN represents a major hub for selective combination of functionally diverse ascending spinal information in order to extract an excerpt of most ongoing motor activity needed for execution of motor tasks.

3.3 Results

3.3.1 FL premotor axons terminate in ipsilateral LRN

To visualize brainstem areas targeted by efference copy information of neurons premotor to limb-innervating motor neurons, we used transsynaptic rabies technology with monosynaptic restriction (Stepien, Tripodi, and Arber 2010; Tripodi, Stepien, and Arber 2011) (Wickersham et al. 2007). These premotor neurons are

defined by their monosynaptic connections to lateral motor column (LMC) motor neurons, the source of motor neurons innervating forelimb (FL; LMC^{FL}; Figure 4A) or hindlimb (HL; LMC^{HL}) muscles. We targeted combined injection of glycoprotein-deficient Rabies-mCherry virus and adeno-associated virus (AAV) expressing glycoprotein broadly into different FL or HL muscles to retrogradely infect and initiate transsynaptic spread from LMC^{FL} or LMC^{HL} motor neurons, respectively (Figure 4A).

In limb muscle injections with monosynaptic rabies viruses, analysis revealed strong and selective targeting of the LRN by FL- but not HL-premotor axons. Notably, the termination site was exclusively in the LRN ipsilaterally to the injected FL muscles (Figure 4A, B). We identified LRN by its location at caudal brainstem levels in a position ventral to the Ambiguus motor nucleus ($ChAT^{ON}$) (Paxinos and Franklin 2012). Moreover, we found it to be surrounded by but entirely devoid of glycinergic neurons (Figure 4B; visualized in *GlyT2*^{eGFP} mice) (Zeilhofer et al. 2005) (Figure 4B).

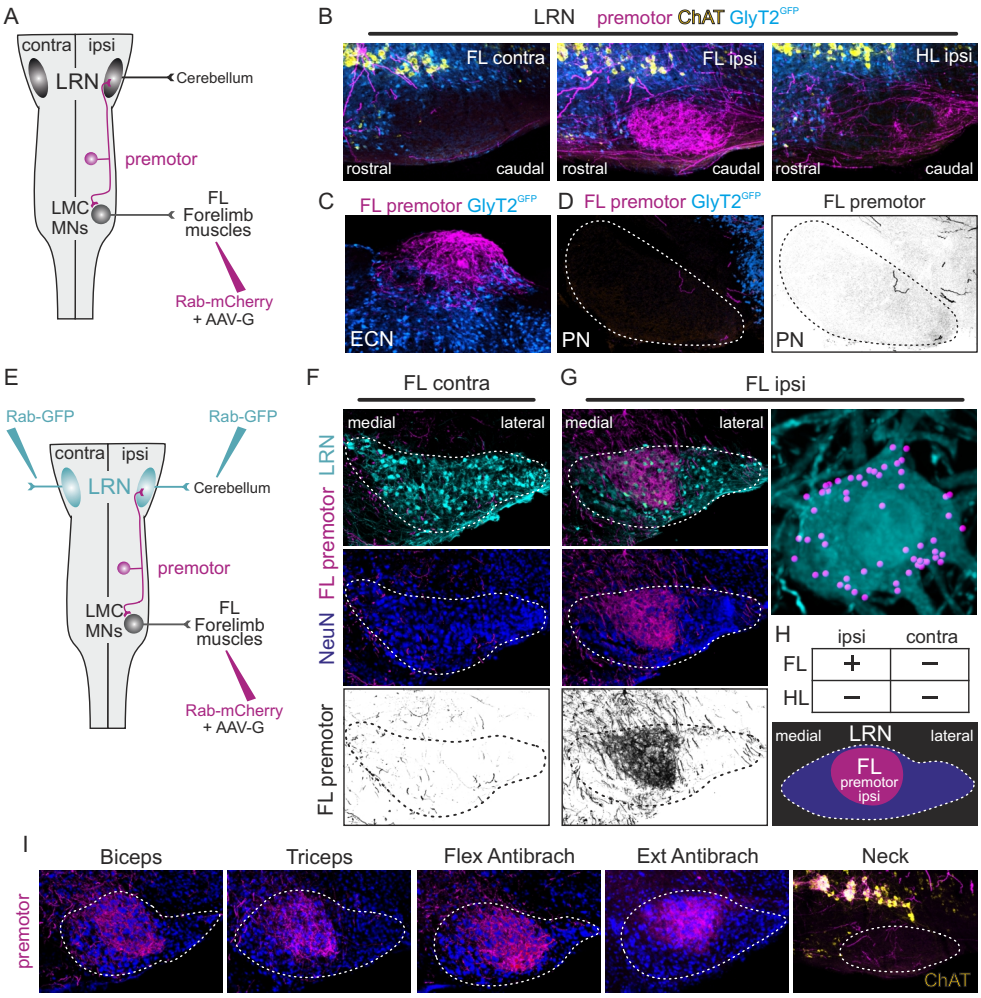

Figure 4. Forelimb premotor axons terminate in ipsilateral LRN

(A) Scheme of experimental setup displaying FL premotor neurons labeled by monosynaptic retrograde spreading from FL LMC motor neurons upon co-injection of FL muscles with Rab-mCherry and AAV-G-protein. Assay is used to determine presence of axonal terminals of premotor neurons in contra- and ipsilateral LRN.(B) Analysis of contra- and ipsilateral LRN on sagittal sections upon injection of monosynaptic rabies virus broadly in FL or HL muscles in *GlyT2*^{GFP} mice. FL premotor terminals (purple) target ipsi- but not contralateral LRN, in an area ventral to ChAT^{on} Ambiguus motor neurons (yellow) and devoid of glycinergic neurons (light blue).(C, D) FL premotor input to ipsilateral external cuneate nucleus (ECN) and pontine nucleus (PN) on sagittal sections upon monosynaptic rabies injections into *GlyT2*^{GFP} mice. Note absence of input to PN.(E) Scheme of experimental setup as in (A), but with additional bilateral retrograde infection of LRN neurons from the cerebellum by Rab-GFP. (F, G) Retrogradely marked LRN neurons (cyan), FL premotor terminals (top, middle: purple, bottom: black) and NeuN (blue) contralateral (F) and ipsilateral (G) to FL muscle injections (coronal sections, midway along rostro-caudal LRN dimension). High resolution panel in (G) to the right depicts apposition of direct premotor input (purple) to LRN neurons (cyan) on the side ipsilateral to FL injection. (H) Summary diagram depicting confined termination of FL premotor neuron input to central core domain of LRN ipsilateral to limb muscle injection (table on top: [+] indicates input; [-] indicates no input by corresponding premotor axons to LRN). (I) FL premotor input to ipsilateral LRN upon monosynaptic rabies injections into Biceps, Triceps, Flexor Antibrachium, Extensor Antibrachium and neck muscles. NeuN (blue; 4 left coronal sections) and Ambiguus motor neurons (ChAT; yellow, right sagittal section) are used as landmarks to delineate LRN.

In addition to the LRN, we found that FL-premotor axons also terminate in most cranial motor nuclei both ipsi- and contralaterally to the injected limb (Figure 5A). FL-premotor axons also projected to the external cuneate nucleus (ECN) located dorsally at caudal brainstem levels, known for its inputs from dorsal root ganglia (DRG) sensory neurons and in turn projecting to the cerebellum through mossy fibers (Campbell, Parker, and Welker 1974; Oscarsson 1965; Rosén 1969) (Figure 4C). In contrast, the more rostrally located pontine precerebellar nucleus also giving rise to cerebellum-projecting mossy fibers was devoid of FL premotor input (Figure 4D).

To determine whether FL premotor axons target the entire LRN, we next combined monosynaptically restricted transsynaptic marking of FL-premotor axons with cerebellar injections to retrogradely label LRN neurons from their target, using two distinct fluorescent colors of Rabies viruses (Figure 4E). We found that FL-premotor axons only target a specific region of the caudal LRN with high axonal density, located in a central but dorsally restricted area (Figure 4F-H). To delineate whether LRN axonal targeting specificity is related to motor neuron pool identity from which the rabies spread is initiated, we injected different FL muscles to label corresponding premotor neurons. We found that premotor axons terminate broadly within the previously mapped FL premotor LRN territory irrespective of muscular identity for four FL muscle groups (Figure 4I). By contrast and as previously described in the cat(Alstermark, Isa, and Tantisira 1991; Alstermark, Pinter, and Sasaki 1985), neck premotor neurons failed to terminate within the LRN (Figure 4I). Analysis of motor pool specific premotor input to ECN revealed more segregated

input specificity (Figure 5B), consistent with previous electrophysiological studies (Campbell, Parker, and Welker 1974; Rosén 1969).

Together, these findings demonstrate that while premotor input to brainstem structures is highly specific, FL premotor neurons associated with individual functionally distinct motor neuron pools do not exhibit profound input specificity within the LRN premotor FL targeted area.

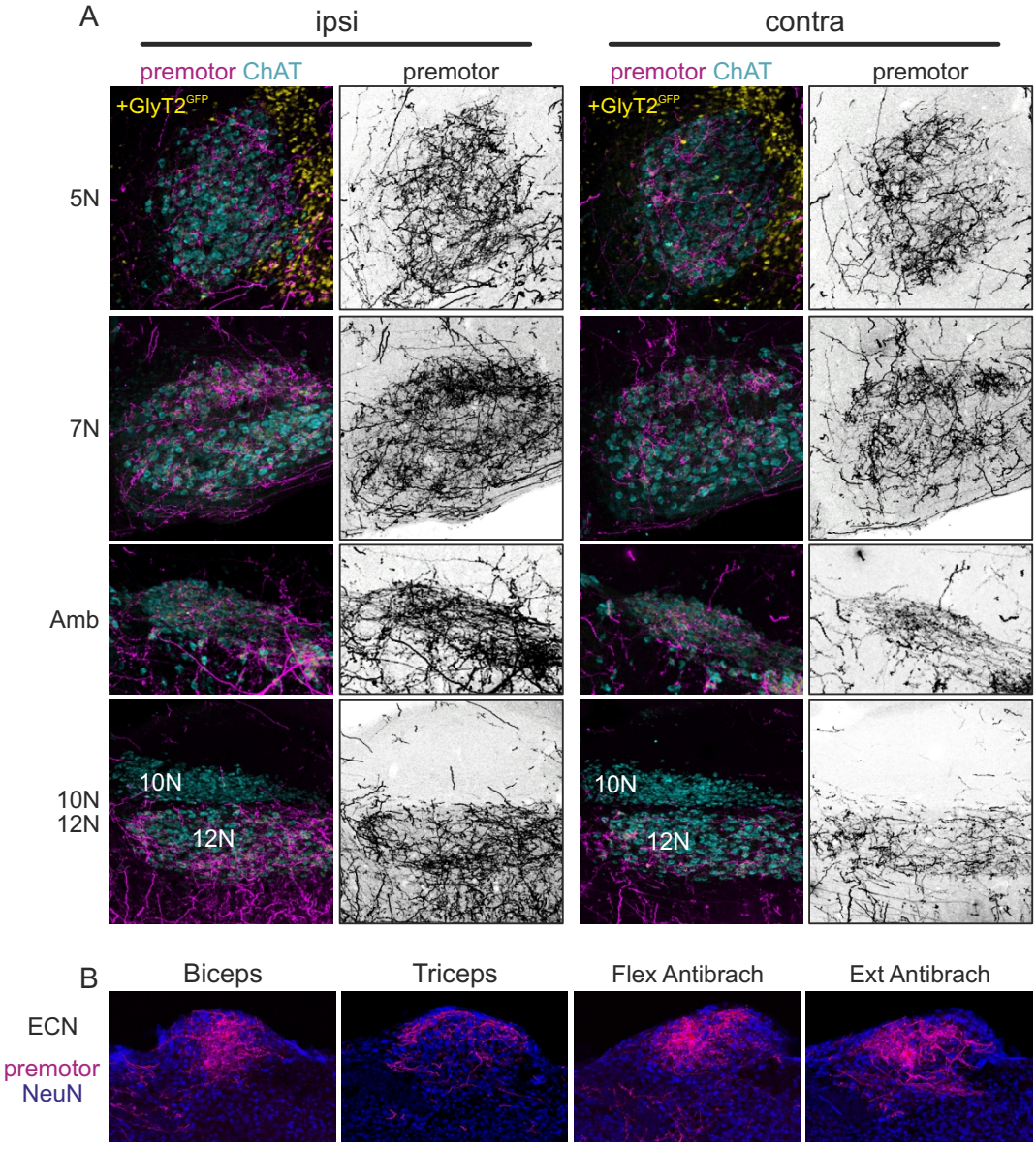

Figure 5. Analysis of FL premotor input to the brainstem

(A) FL premotor axons target several (5N, 7N, Amb, 12N) but not all (10N) cranial motor nuclei both ipsi- and contralaterally to limb injection.(B) FL premotor input to ipsilateral ECN upon monosynaptic rabies injections into Triceps, Biceps, Extensor Antibrachium and Flexor Antibrachium muscles. NeuN staining (blue) is used as landmark to delineate ECN on coronal sections.

3.3.2 Spinal cord provides prominent source of synaptic input to LRN

To delineate possible sites of cellular origin contributing to synaptic input to the LRN, we carried out experiments initiating monosynaptic transsynaptic spread selectively from LRN neurons, using a triple virus injection approach. We first targeted LRN neurons from the cerebellum by Cav-Cre injection, followed by sequential intra-LRN injection with AAV-flex-TVA/G and EnvA-coated Rabies-mCherry (Figure 6A). This approach led to high targeting specificity of LRN neurons at high efficiency (Figure 6B).

We found many neurons marked throughout the spinal cord and at cervical levels, the majority of LRN connecting neurons resided ipsilaterally to injection, whereas contralateral neurons were confined to Rexed's lamina 8 (Figure 6C). Supraspinally, we observed only few additional structures labeled dominantly using this approach. Most notably, the contralateral red nucleus contained many labeled neurons, in agreement with previous studies (HINMAN and CARPENTER 1959; Walberg 1958). We also noted a distinct cluster of neurons in the rostral ventral respiratory group (rVRG) dorsal to the contralateral LRN and in close proximity to $ChAT^{ON}$ Ambiguus motor neurons (Figure 2D) (Ezure and Tanaka 1997). In agreement with these findings, we also found that contralateral phrenic motor neurons

in the spinal cord received highly selective synaptic input by Pre-LRN rabies marked axons (Figure 6D). Motor cortex provided minor input to LRN, with a low density of terminals mainly surrounding the LRN (Figure7), consistent with previous experiments in the rat (Rajakumar, Hrycyshyn, and Flumerfelt 1992). Together, these data demonstrate that LRN neurons receive prominent synaptic input from spinal neurons (Figure 6E), thus directing our further analysis to the spinal cord.

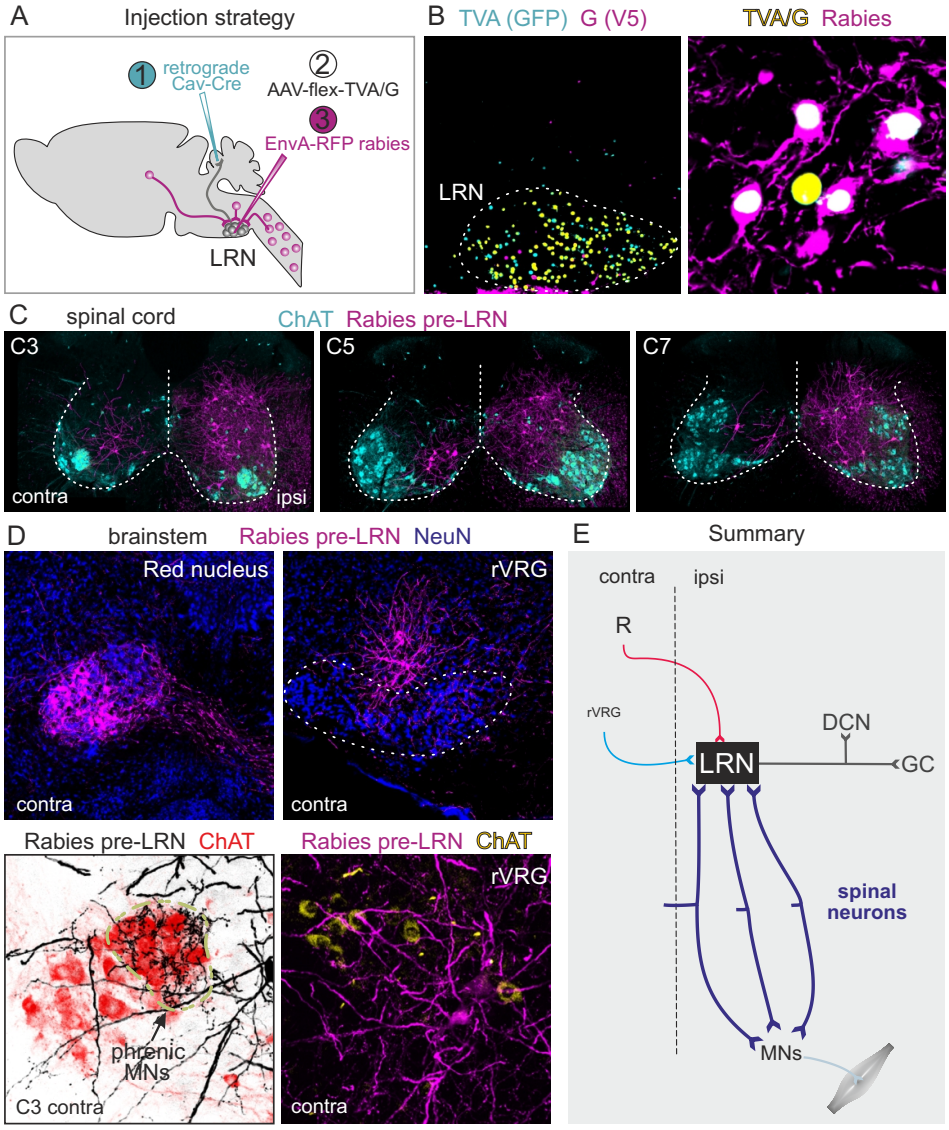

Figure 6. Synaptic input to LRN revealed by transsynaptic rabies

(A) Scheme for sequential injections to initiate monosynaptic transsynaptic rabies spread from LRN neurons (Retrograde Cav-Cre; AAV-flex-TVA/G; EnvA-RFP).(B) Coronal LRN section analysis at the end of an experiment to determine infection (left: low-resolution overview depicting high co-infection

rate of TVA and G viruses; right: triple infected neurons white).(C) Pre-LRN neurons on spinal cord sections (C3, C5 and C7 spinal levels) are located both ipsi- and contralaterally to injection, and less numerous contralateral neurons are confined to Rexed's lamina 8.(D) Supraspinal labeled pre-LRN populations include neurons in the red nucleus (R) and rostral ventral respiratory group (rVRG). Input of pre-LRN axons to phrenic motor neurons (bottom left) in the contralateral spinal cord and intermingling of pre-LRN neurons with ChAT^{ON} Ambiguus motor neurons (bottom right) confirms rVRG identity. (E) Summary of synaptic input to LRN, depicting major input sources from the spinal cord, as well as supraspinal input from R and rVRG.

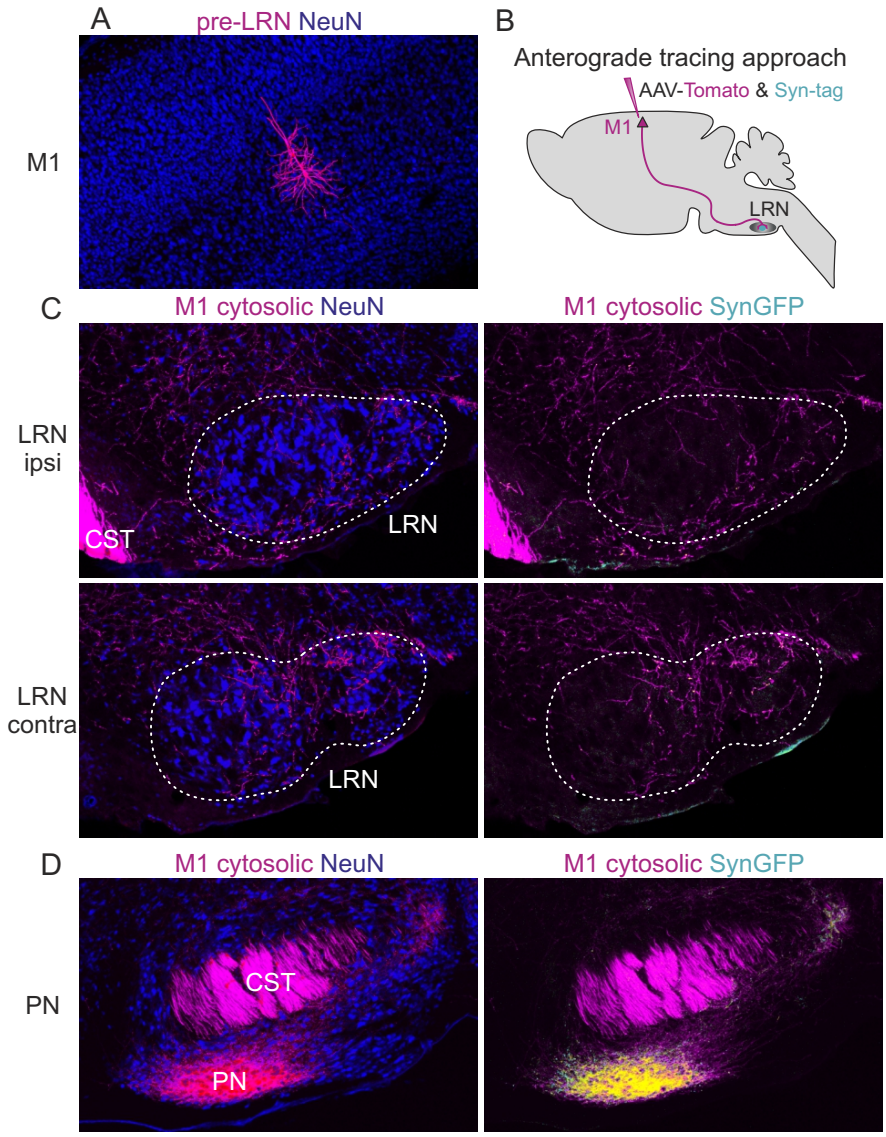

Figure 7. Motor cortex input to LRN

(A) Retrograde transsynaptic labeling of M1 cortex layer 5 pyramidal neurons from LRN (strategy see Figure 2) is detected at very low frequency.(B) Anterograde AAV tracing strategy from M1 cortex to the brainstem to determine axonal (Tomato) and synaptic (SynGFP) distribution elaborated by M1 pyramidal neurons.(C, D) Ipsi- and contralateral LRN receives only minor input from M1 motor cortex with sparse axonal arborization around the LRN. In contrast, the pontine nucleus (PN) receives very strong input. Cortico-spinal tract is indicated (CST).

3.3.3 Broad spinal residence of neurons providing FL premotor input to LRN

To identify the source of premotor axons in the LRN, we combined retrograde axonal infection from the LRN and transsynaptic marking of premotor neurons by FL muscle injections with monosynaptic rabies tools (Figure 8A). While such a strategy is not suitable to quantify the absolute number of dually connecting neurons due to the limited time window during which two different rabies viruses can co-infect the same neuron (Ugolini 2010), it has been used successfully before to visualize the overall distribution of dual-connection neurons (Stepien, Tripodi, and Arber 2010). We found that at cervical levels, the distribution of spinal neurons projecting to the LRN was similar to that of FL premotor neurons (Stepien, Tripodi, and Arber 2010), with a majority of neurons located ipsilaterally and with contralateral neurons mainly confined to Rexed's lamina 8 (Figure 9). The distribution pattern of spinal neurons projecting to the LRN morphed gradually into one with a contralateral dominance at lumbar levels (Figure 9), in agreement with previous studies (Koekkoek and Ruigrok 1995; Shokunbi, Hrycyshyn, and Flumerfelt 1985).

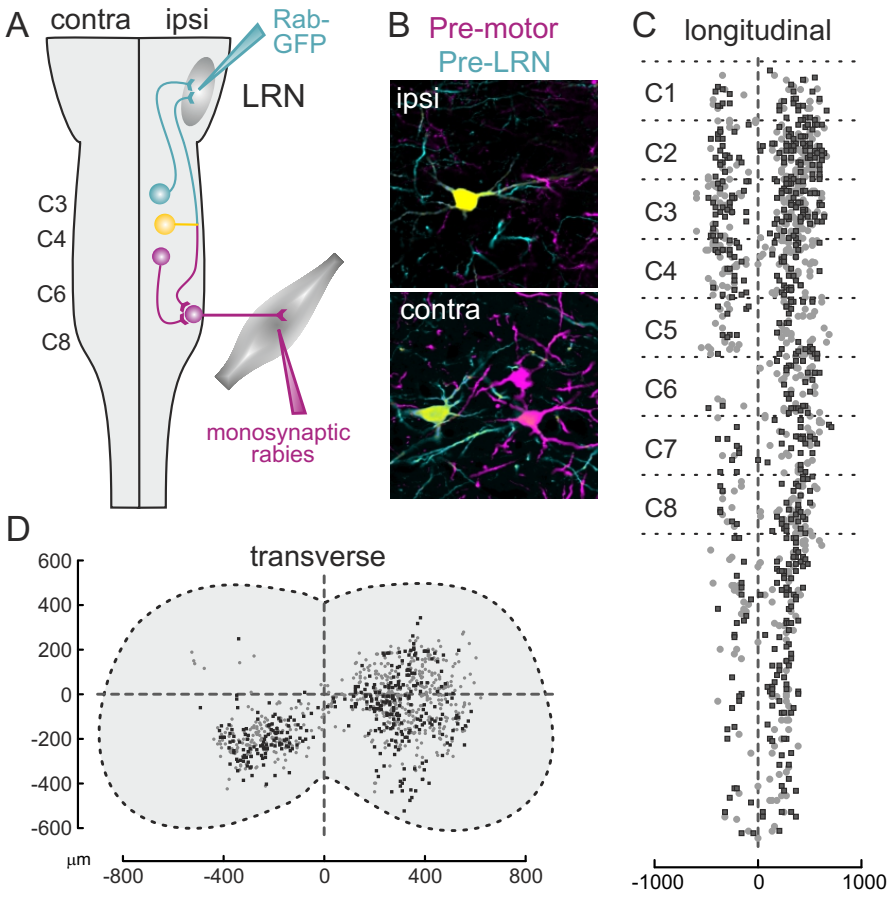

Figure 8. Source of premotor neurons to LRN is distributed across spinal cord

(A) Scheme of experimental setup displaying retrograde infection of FL LMC motor neurons from FL muscles by co-injection of Rab-mCherry (purple) and AAV-G-protein to label FL premotor neurons and injection of Rab-GFP (cyan) into LRN to retrogradely label neurons with projections to LRN. Assay is used to analyze position of neurons with projections to LRN and connections to FL motor neurons in the spinal cord (yellow).(B) Examples of neurons with dual rabies infection ipsilateral (top) and contralateral (bottom) to limb muscle injection.(C) Longitudinal position of neurons with dual projections along the cervical and thoracic spinal cord (two representative reconstructions are shown by black square and grey round symbols). Cervical spinal levels are indicated and scale depicts μm.(D) Transverse position analysis of neurons with dual projections, compiled projection along spinal levels analyzed (see C). Scales depict μm.

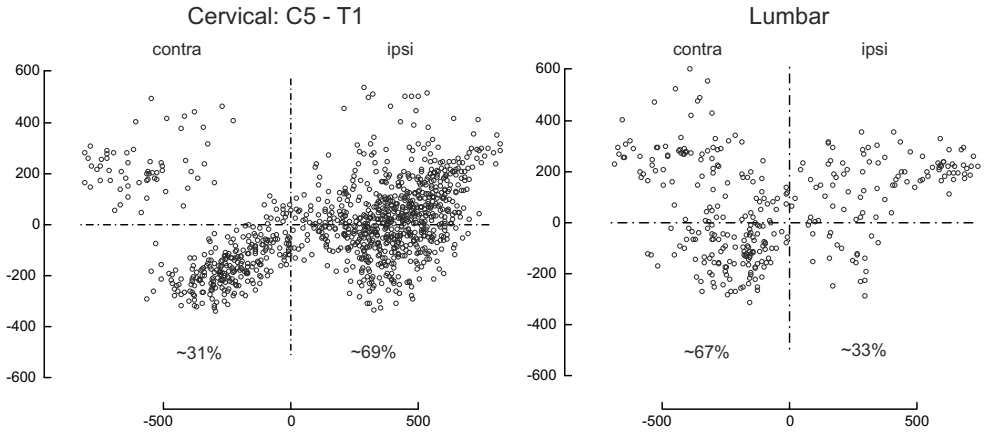

Figure 9. Spatial distribution of LRN-connecting cervical and lumbar spinal populations

Comparative analysis of pre-LRN neuron distributions (left: C5-T1; right: L1-L6) as determined by retrograde labeling from the LRN with targeted rabies injection. Note that ipsi- and contralateral contributions are distinct.

We next mapped the three-dimensional distribution of dually rabies labeled neurons in the spinal cord, projecting to the LRN and connecting to FL motor neurons (Figure 8A). We found that such neurons were distributed broadly over cervical spinal segments, with double rabies labeled neurons not only found ipsilaterally to LRN and FL injection, but also in the contralateral spinal cord in Rexed's lamina 8 (Figure 8B-D). In the longitudinal dimension, we observed a higher density of neurons at rostral spinal levels but a continuing presence of these neurons to anterior thoracic spinal levels (Figure 8C). Thus, dually connected neurons are not only restricted to cervical levels C3/C4. Moreover, in the transverse dimension, the observed pattern was reminiscent of the overall FL premotor distribution profile in the spinal cord (Figure 8D) (Stepien, Tripodi, and Arber 2010), including the specific locations of ipsi- and contralateral populations.

In summary, these findings demonstrate that propriospinal neurons with projections to the LRN and connections to FL motor neurons in the mouse distribute across many spinal segments and locate both ipsi- and contralaterally in an overall pattern resembling the FL premotor distribution. Thus, the LRN appears to sample the activity of a diverse set of premotor interneurons connecting to FL motor neurons as defined by position in the spinal cord. These observations raise the important question of whether and how this diversity of LRN projecting neurons by spinal location is matched at the level of neuronal subpopulations with distinct genetic identity.

3.3.4 Distinct LRN termination zones by spinal location and neurotransmitter fate

To directly address these questions, we next set up a strategy to visualize axon terminals in the LRN derived from spinal neurons of different origin. We made use of unilateral intra-spinal injections of double-inverted-orientation-LoxP-flanked AAVs (AAV-flex) conditionally expressing a Synaptophysin-GFP fusion protein (AAV-flex-SynGFP) upon Cre recombination, confined to either cervical (C4-C7) or lumbar (L1-L4) levels (Figure 10A; Figure 11). To distinguish input to the LRN derived from excitatory (vGlut2^{ON}) and inhibitory (vGAT^{ON}) spinal neurons, we performed unilateral spinal coinjections of AAV-flex-SynGFP and AAV-flex-H2BGFP into either *vGlut2*^{Cre} or *vGAT*^{Cre} knock-in mice (Vong et al. 2011) (Figure 10A; Figure 11). This strategy allows separate mapping of excitatory or inhibitory ascending input from ipsi- or contralateral cervical and lumbar spinal cord to the LRN, independent of premotor character.

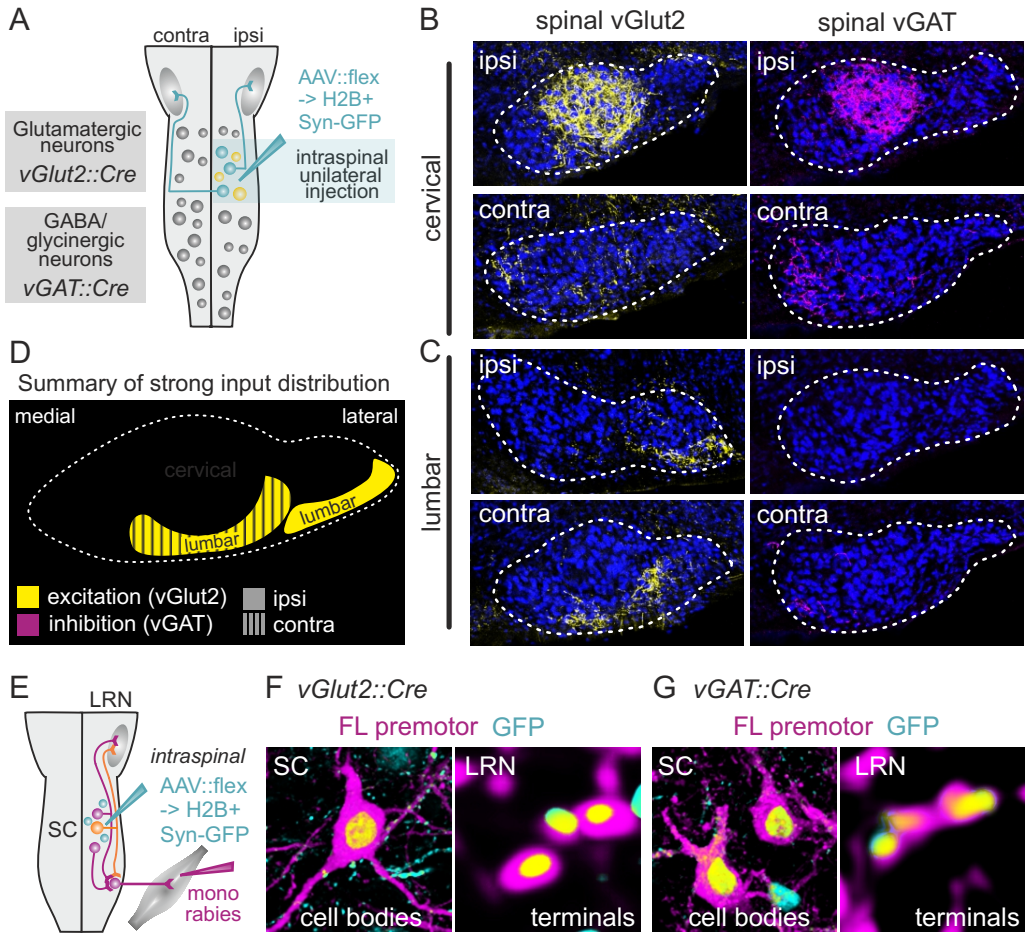

Figure 10. Differential spinal ascending pathways by neurotransmitter and spinal origin

(A) Scheme of experimental setup for unilateral intraspinal injections of flexed AAVs (H2B and SynGFP) into *vGlut2*^{Cre} mice to mark glutamatergic spinal neurons and *vGAT*^{Cre} mice to mark GABA and glycinergic spinal neurons. (B, C) Coronal LRN sections are shown displaying NeuN (blue) and SynGFP (purple for vGAT, yellow for vGlut2) on side ipsilateral (top) or contralateral (bottom) to spinal injections. Dashed lines delineate outer LRN border. Cervical (B) and lumbar (C) injections are shown separately (medial LRN to the left, lateral LRN to the right in all panels). (D) Summary diagram of results displayed in this Figure (only strong anatomical input depicted in model). Note fractionation of LRN into different territories by spinal origin and neurotransmitter identity of neurons.(E) Scheme of experimental setup identical to (A) but with broad unilateral monosynaptic rabies injection into FL-muscles.(F, G) FL premotor neurons (purple) co-infected by flexed AAV (H2B and SynGFP) at the level of the spinal cord (left) and resulting high-resolution terminals in the LRN (right) are shown to confirm premotor status of ascending vGlut2 (F) and vGAT (G) positive neurons.

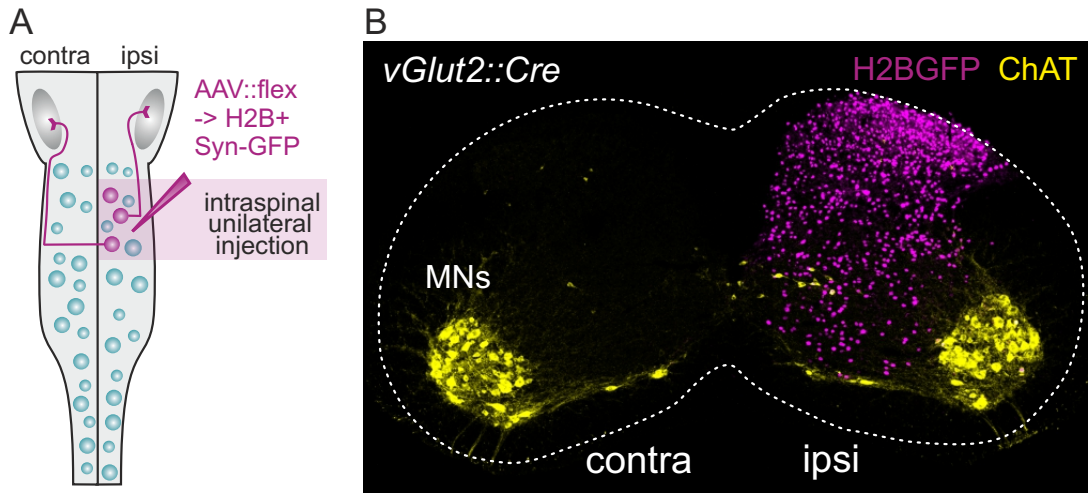

Figure 11. Neurotransmitter tagging by unilateral spinal injection

(A) Scheme depicting injection strategy to unilaterally and spinally segmentally confined label neuronal populations of different neurotransmitter identity. Unilateral injections of AAV-flex-SynGFP and H2B are confined to one side of the spinal cord, thus allowing analysis of axonal terminals targeting ipsi- and contralateral LRN within the same animal.(B) Representative unilateral intraspinal injection in *vGlut2*^{Cre} mouse line (yellow: ChAT^{ON} motor neurons; purple: H2B-GFP-tag).

We found that unilateral cervical injections of AAV-flex-SynGFP into *vGlut2*^{Cre} or *vGAT*^{Cre} mice resulted in pronounced marking of axonal terminals in the entire core LRN ipsilateral to injection (Figure 10B), corresponding to the domain also marked by FL premotor terminals. Within this domain, cervical-derived excitatory and inhibitory synapses were found at approximately equal density (Figure 10B). Combined marking of cervical neurons by intraspinal injection and FL premotor neurons by monosynaptic rabies tools revealed the existence of both vGlut2^{ON} and vGAT^{ON} premotor terminals in the FL dominated core area of the LRN (Figure 10E-G), supporting their coincidence within this domain. Contralateral to spinal injection, only a low density of synaptic terminals compared to ipsilateral density was detected in the LRN of *vGlut2*^{Cre} and *vGAT*^{Cre} mice (Figure 10B). These

sparse terminals were located both in the ipsilateral FL dominated LRN domain but also extended more medially into a domain not targeted by ipsilateral neurons (Figure 10B). In summary, the major targeting domain for cervical spinal neurons within the LRN is an ipsilateral centrally located FL premotor LRN domain, within which no preferential distribution of excitatory and inhibitory terminals can be observed (Figure 10D).

Upon lumbar spinal cord injections, we detected almost exclusively glutamatergic terminals at high densities in the LRN with targeting domains spatially distinct from the FL population (Figure 10C). Both ipsi- and contralateral vGlut2^{ON} terminals were restricted to a highly confined area ventrally to the one targeted by cervical neurons (Figure 10C, D). Contralateral lumbar interneurons targeted the domain just ventral to the ipsilateral cervical territory, and ipsilateral lumbar interneurons targeted the adjacent more lateral domain (Figure 10C, D). The very sparse contralateral vGAT^{ON} terminals were confined to the most medial and ventral corner of the LRN (Figure 10C).

Together, these findings reveal the existence of a spatial map within the LRN. Input from cervical and lumbar segments of ipsi- and contralateral sides is confined to distinct territories within the LRN (Figure 10D). The LRN area targeted by ipsilateral FL spinal input represented the most dominant input to the LRN from the spinal cord. It exhibits shared occupation by excitatory and inhibitory terminals and coincides with the premotor domain. These findings raise the question of whether within this

domain of intermingled terminals, spatial input segregation might occur according to the identity of functionally distinct spinal subpopulations.

3.3.5Distinct LRN termination zones by progenitor domain identity

Our analysis of bifurcating spinal neurons with projections to the LRN uncovered a much more widespread population of neurons than previously anticipated. To determine whether these fractionate into functionally distinct spinal populations as defined by developmental origin from different progenitor domains, we concentrated specifically on the cervical spinal cord. This focus would allow us to address diversity of origin and to establish a possible correlation between genetic identity and axonal targeting within an LRN subdomain. Most genes expressed specifically in identified spinal progenitor domains or in early postmitotic neuronal subpopulations are downregulated rapidly at embryonic stages (Figure 12A) (Alaynick, Jessell, and Pfaff 2011), preventing the direct use of Cre mouse lines at postnatal stages to mark neurons derived from these progenitor domains by intraspinal viral injections. To overcome this limitation, we implemented a novel intersectional mouse transgenic system combined with intraspinal viral injections for our experiments (Figure 12B-D).

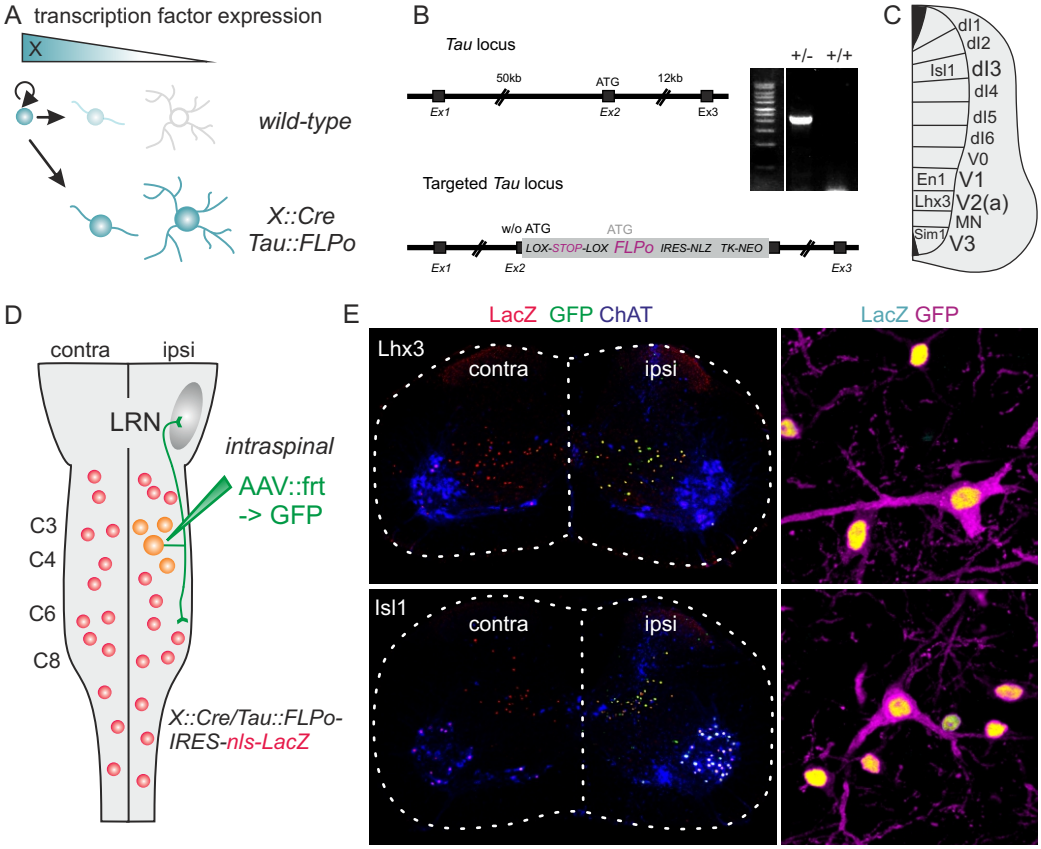

Figure 12. Intersectional genetic tool for targeting developmentally marked subpopulations

(A) Scheme depicts frequently observed downregulation of transcription factor (X) expression by genetically defined progenitors or early postmitotic neurons. Conditional activation through Cre-mediated intersectional breeding between *X::Cre* and *Tau:: FLPo-IRES-nls-LacZ* mice allows permanent marking of corresponding neurons by NLS-LacZ and FLP recombinase expression. (B) Generation of knock-in mouse for conditional expression of FLP recombinase from the pan-neuronal *Tau* locus, by integration into Exon 2 of the locus. PCR to confirm positive recombination event is displayed next to DNA ladder on gel. (C) Scheme displaying subdivision of embryonic spinal cord in 11 distinct progenitor domains and emergent neuronal subpopulations. Transcription factor code is displayed for populations studied here (dI3: Isl1; V1: En1; V2(a): Lhx3; V3: Sim1).(D) Scheme of experimental setup for unilateral intraspinal injections of FRT-flanked AAVs into *X::Cre / Tau:: FLPo-IRES-nls-LacZ* mice to mark synapses of neurons derived from defined spinal progenitor domain. (E) Representative pictures of unilateral spinal injections of FRT-flanked AAVs into $Lhx3^{Cre}$ and $Isl1^{Cre}$ mice crossed with *Tau:: FLPo-IRES-nls-LacZ* mice (high-resolution example neurons are depicted to the right).

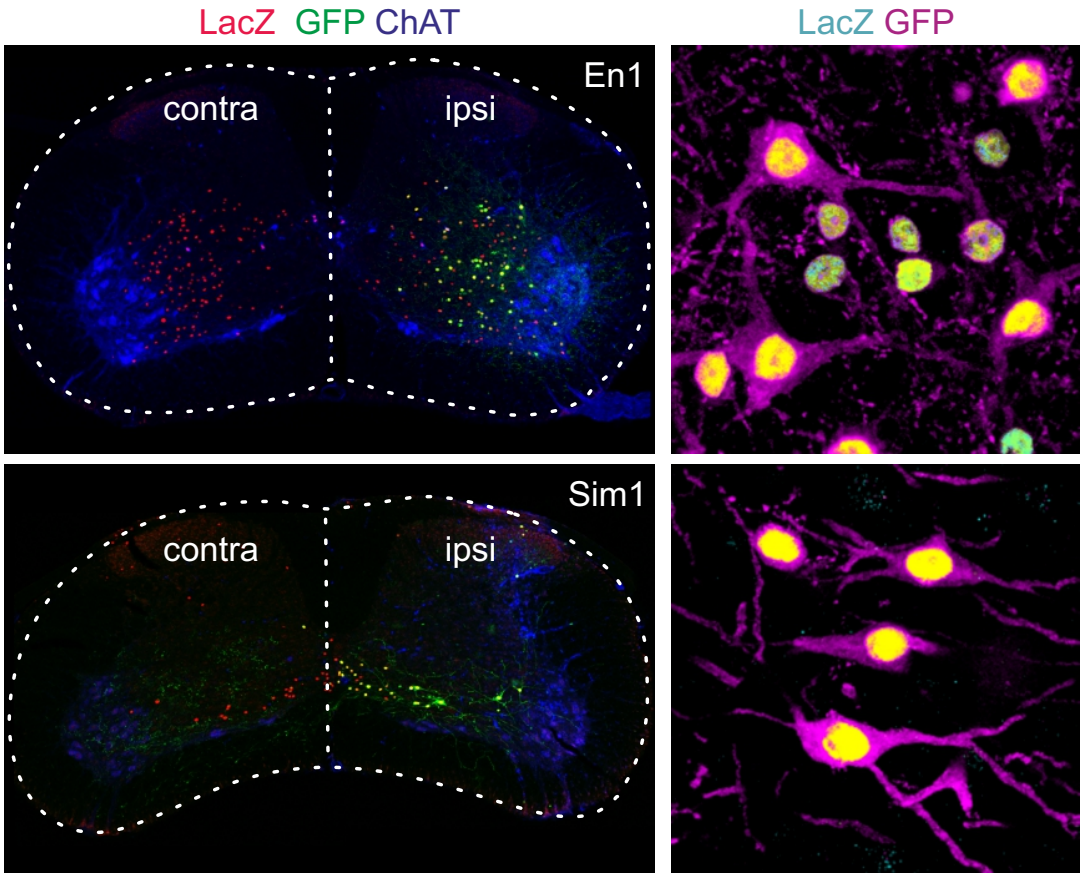

Figure 13. Genetic tool for postnatal manipulation of spinal subpopulations

Representative pictures of unilateral spinal injections of FRT-flanked AAVs into *En1*^{Cre} and *Sim1*^{Cre} mice crossed with *Tau:: FLPo-IRES-nls-LacZ* mice (high-resolution example neurons are depicted to the right).

Our approach involved the generation of a new transgenic mouse line to conditionally express Flp recombinase from the pan-neuronal *Tau* locus (*Tau*$^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ or *Tau*$^{lsl\text{-}FLP}$ mice; Figure 5B), a locus previously used successfully to express transgenes in specific neuronal subpopulations (Hippenmeyer et al. 2005; Stepien, Tripodi, and Arber 2010; Tripodi, Stepien, and Arber 2011). We found that intersectional breeding between Cre lines with transient embryonic expression and *Tau*$^{lsl\text{-}FLP}$ mice led to permanent nls-LacZ and Flp recombinase expression in derivative neurons (Figure 12E, Figure 13). Intraspinal injection of double-inverted-

orientation-FRT-flanked AAVs (AAV-frted) to conditionally express GFP in these mice at postnatal stages can thus be used to visualize mature neuronal subpopulations (Figure 12D, E, Figure 13) and their axon terminals, despite only transient expression of Cre recombinase during development at embryonic stages (Figure 12A, C).

Using this approach (Figure 14A), we first selected two Cre lines marking the neuronal descendants of single progenitor domains giving rise to excitatory vGlut2^{ON} spinal subpopulations. These were the ventrally derived V2 (*Lhx3*^{Cre}; mostly V2a – see Experimental Procedures) and the dorsally derived dI3 (*Isl1*^{Cre}) population (Figure 6B). Unilateral intraspinal injection of AAV-frted-GFP into *Lhx3*^{Cre}*/Tau*$^{lsl\text{-}FLP}$ or *Isl1*^{Cre}*/Tau*$^{lsl\text{-}FLP}$ mice marked selectively respective neuronal subpopulations at the level of the spinal cord (Figure 12E). Analysis of SynGFP fluorescence in the LRN in these mice demonstrated that both V2 and dI3 subpopulations establish ipsilateral termination zones (Figure 14C), but whereas Lhx3-SynGFP synapses are found ventro-medially, Isl1-SynGFP terminals preferentially target a lateral domain (Figure 14C).

We next conducted a similar analysis with a mouse line expressing Cre recombinase in a single progenitor domain giving rise to inhibitory spinal neurons, the ventrally derived V1 population (*En1*^{Cre}) (Figure 14B; Figure 13). We found that En1-SynGFP tagged neurons establish dense and widespread axonal terminals within the FL-territory of the LRN ipsilateral to spinal injection, overlapping with and unlike the

confined termination zones observed for V2 and dI3 neurons in the LRN (Figure 14C).

To assess whether spinal neurons projecting to contralateral LRN territory can be distinguished by their progenitor domain origin as well, we chose the V3 population known to establish excitatory commissural projections at the level of the spinal cord (Zhang et al. 2008) (Figure 14B). Intraspinal injection of AAV-frted-GFP into *Sim1*^{Cre}/*Tau*$^{lsl\text{-}FLP}$ mice revealed that these neurons establish a selective termination zone in the contralateral LRN located in the ventral FL-targeted LRN territory (Figure 14C), providing further evidence for specific targeting of functionally distinct spinal populations to LRN subdomains.

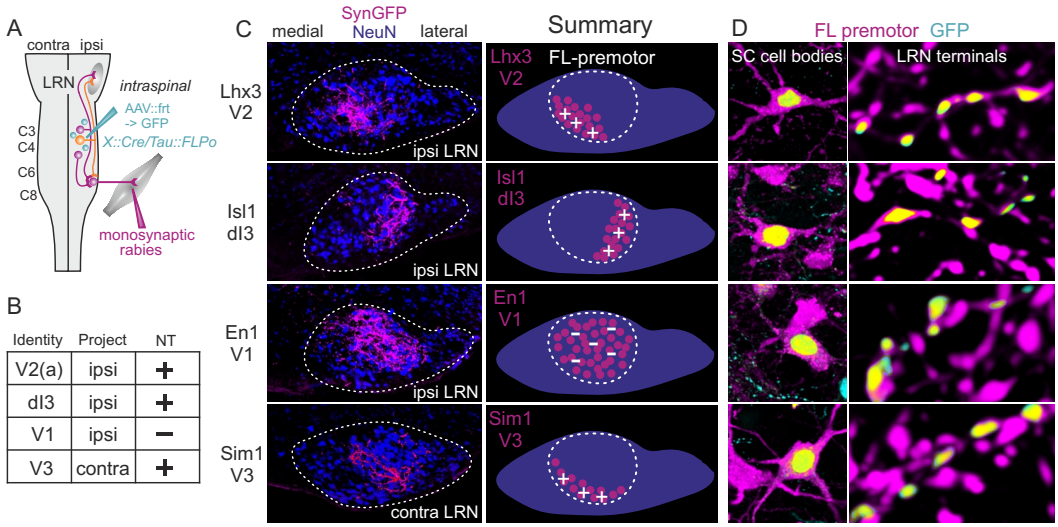

Figure 14. Genetically identified spinal populations exhibit distinct LRN termination zones

(A) Experimental setup with unilateral intraspinal injection of FRT-flanked AAVs (C), and with unilateral monosynaptic rabies injection into FL-muscles (D).(B) Summary diagram of neuronal identity, projection target and neurotransmitter (NT) identity (+: vGlut2^{ON}; -:

vGAT^{ON}) of studied spinal subpopulations.(C) Visualization of axonal terminals (SynGFP) derived from cervical spinal neuron subpopulations marked by progenitor domain origin (Lhx3, Isl1, En1 and Sim1) on coronal caudal LRN sections. Left panels depict LRN ipsilateral to spinal injection for Lhx3, Isl1 and En1 and contralateral LRN for Sim1 experiments. Right panels depict summary diagram of synaptic input observed to different domains of the FL-premotor LRN territory by different spinal populations.(D) Dual labeling experiments with monosynaptic rabies viruses to determine premotor status of labeled spinal neurons (left; H2B-GFP marked) and high-resolution synaptic terminals in LRN (right; SynGFP marked). For Sim1 experiments, monosynaptic rabies viruses were injected contralaterally to spinal injections.

To determine whether ascending terminals of different progenitor domain origin encompass FL premotor populations, we combined intraspinal AAV injections with monosynaptic rabies injections into FL muscles (Figure 14A, D). We subsequently assessed whether we could detect synaptic terminals in the LRN, marked both by the spinally expressed SynGFP tag and by FL premotor rabies virus fluorescence. In experiments carried out in *Lhx3*^{Cre}, *Isl1*^{Cre}, *En1*^{Cre} and *Sim1*^{Cre} genetic backgrounds, we found double labeled terminals for all four conditions (Figure 14D). In summary, these findings establish that spinal neurons of different single progenitor domain origin establish distinct axonal terminations in the LRN, including FL premotor populations. However, excitatory spinal subpopulations marked by these criteria target more confined LRN subdomains than inhibitory counterparts.

3.3.6LRN projecting genetic subpopulations are spatially confined in the spinal cord

The striking spatial organization of synaptic terminals in the LRN derived from distinct spinal subpopulations raised the complementary question of whether also at the level of the spinal cord, LRN projecting neurons are found in spatially restricted domains, a finding that could have important functional consequences.

We first assayed the spatial distribution of neurons derived from different progenitor domains in the mature cervical spinal cord, using intersectional breeding between $Lhx3^{Cre}$, $Isl1^{Cre}$, $En1^{Cre}$ and $Sim1^{Cre}$ with $Tau^{lsl\text{-}FLP}$ mouse lines. This strategy permanently marks corresponding neurons by LacZ expression ($Lhx3^{LacZ}$, $Isl1^{LacZ}$, $En1^{LacZ}$, $Sim1^{LacZ}$) allowing reconstruction of cell body position of these populations in the spinal cord. We found that both at C4/5 and C7/8 levels, $Lhx3^{LacZ}$ and $Isl1^{LacZ}$ neurons distributed to largely non-overlapping spatial territories, with V2-derived neurons found in a more ventral position than dI3 neurons (Figure 15A, B). $En1^{LacZ}$ neurons distributed more broadly along the dorso-ventral axis, but overall were located in closer proximity to LMC motor neurons than $Lhx3^{LacZ}$ or $Isl1^{LacZ}$ neurons, whereas $Sim1^{LacZ}$ neurons were located in an extreme medial and ventral position (Figure 15A, B). Quantitative analysis further demonstrated that V1 neurons were much more abundant than V2, dI3 or V3 (Figure 15C), suggesting that perhaps V1 neurons fail to demonstrate LRN targeting specificity due to further division into distinct subpopulations beyond single progenitor domain origin.

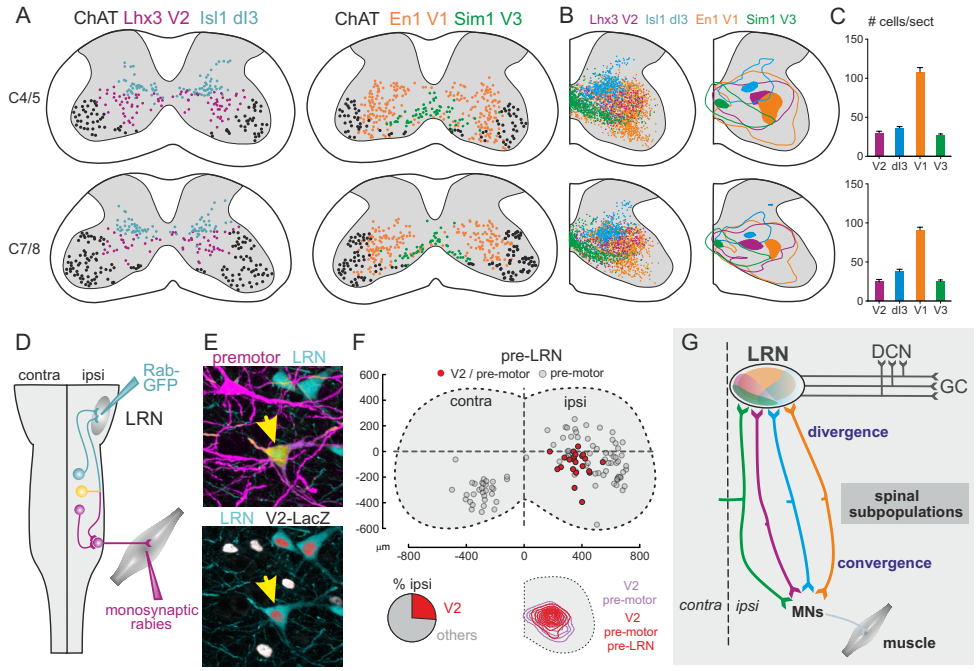

Figure 15. Spinal location of LRN projecting subpopulation reveals spatial segregation

(A) Spatial distribution of LacZ^{ON} spinal neurons in *Lhx3::Cre* (purple), *Isl1::Cre* (blue), *En1::Cre* (orange) and *Sim1::Cre* (green) mice crossed with *Tau:: FLPo-IRES-nls-LacZ* mice. Representative sections at C4/5 and C7/8 spinal levels are shown, and LMC motor neurons are shown in black. (B, C) Overlay of scatter plots of neuronal position (left), isolines of neuronal distributions (middle; filled territory: 20%; outer line: 90% of neurons around highest density), number of neurons per 40µm section (average over 12 sections each shown; n=3 mice) for data shown in (A). (D) Scheme of experimental setup in analogy to Figure 3A. Assay is used to determine position of neurons with projections to LRN and connections to FL motor neurons in the spinal cord (yellow). (E) Representative pictures of experiments carried out in *Lhx3::Cre* mice crossed with *Tau:: FLPo-IRES-nls-LacZ* mice to depict LacZ (white), LRN projection and FL premotor status. Yellow arrow depicts exemplary triple positive neuron. (F) Positional analysis of pre-LRN/premotor (grey) and V2/pre-LRN/ premotor (red) neurons shown on transverse projection. Bottom shows that V2 neurons make up approximately 30% of ipsilateral pre-LRN/premotor population (left) and distribution analysis depicts that the two populations are not distinctly localized (right). (G) Summary diagram depicting main findings. Spinal neuron subpopulations diversify by developmental genetic identity and spatial distribution in the spinal cord. Both ipsi- and contralaterally located subpopulations establish dual projections to cervical motor neurons and LRN in the brainstem. Subpopulation input convergences on spinal motor neurons and diverges to different territories in the LRN. LRN neurons in turn project to deep cerebellar nuclei (DCN) and granule cells (GC) in the cerebellum.

The observed spatial segregation of neurons derived from individual progenitor domains at the level of the spinal cord raises the question of whether LRN

projecting premotor neurons of a given progenitor domain identity follow the same organizational principle. To address this issue we chose V2 neurons as an exemplary population. We crossed *Lhx3*^{Cre} and *Tau*$^{lsl\text{-}FLP}$ mice to visualize corresponding spinal neurons and carried out the dual rabies labeling approach marking spinal neurons with connections to FL motor neurons and projections to LRN (Figure 15D). We found that LacZ^{ON} neurons in this experiment made up approximately 30% of the dual rabies-labeled ipsilateral population and that these neurons were confined to a medial domain in the intermediate spinal cord (Figure 15E, F). The distribution of these neurons was similar to the distribution of the overall Lhx3^{ON} premotor population (Figure 15F), suggesting that dual connecting premotor-LRN neurons make up a subset of the general population, transmitting an excerpt of ongoing activity of premotor neurons to the LRN.

In summary, these findings provide evidence that developmental progenitor domain origin in the spinal cord prefigures stereotype and spatially confined spinal settling positions of derivative neuronal subpopulations, as well as the establishment of specific ascending axonal targeting domains to the LRN in the brainstem.

3.4Discussion

Motor circuit collaterals are the internal neuronal substrate for corrective signaling during the execution of motor tasks. Here we unravel the synaptic organization and origin of such collaterals carrying motor-related information from the spinal cord to supraspinal centers. We demonstrate the existence of a precisely organized connectivity matrix between diverse and genetically distinct spinal populations and stereotype LRN subdomains. We discuss our findings in the context of organizational and functional properties of ascending spinal signaling systems and their role in the control of motor behavior.

3.4.1Ascending signals to the LRN established by distinct spinal subpopulations

Our work demonstrates that FL-premotor LRN projecting PNs fractionate into many diverse spinal populations based on genetic criteria. In the past, “C3C4 PNs” were thought of as a singular neuronal population located at segmental levels C3/C4 in the ipsilateral spinal cord, only divided into an excitatory and inhibitory population (Alstermark et al. 2011; Alstermark et al. 2007; Alstermark, Lundberg, and Sasaki 1984). In fact, evidence for the existence of an inhibitory subpopulation has been sparse due to the inherent difficulty to separate excitatory from inhibitory axons by antidromic stimulation experiments from the LRN (Alstermark, Lundberg, and Sasaki 1984).

We show that the overall distribution of these dual-connection neurons in the spinal cord is much broader than just C3C4 spinal levels, with cell bodies positioned throughout cervical and extending into the thoracic spinal cord. This discovery was made possible by the combination of novel mouse genetic tools and connectivity-based circuit mapping approaches of high sensitivity, as compared to electrophysiological recordings in the *in vivo* spinal cord in the past. These novel methods also enabled us to uncover that premotor neurons in the spinal cord giving rise to LRN-projecting axons encompass both ipsi- and contralateral populations. We note that the overall density of FL premotor LRN projection neurons is higher at rostral than caudal spinal levels, as well as on the ipsi- than contralateral spinal side, providing a possible explanation for why it was too challenging to detect the overall distribution of these neurons using electrophysiological techniques.

3.4.2Functional implications of genetically diverse spinal channels to the LRN

Our results demonstrate that subpopulations of cervical spinal neurons with distinct genetic identities by developmental ontogeny establish axons terminating in stereotype territories within the LRN FL domain. These neuronal populations also exhibit distinct spatial distributions in the spinal cord. Together, our findings raise the question of possible implications of such highly organized efference copy signaling arrangements for the function of these circuits in controlling motor behavior.

It is well established that neurons derived from different progenitor domains exhibit distinct intraspinal functions [reviewed by (Alaynick, Jessell, and Pfaff 2011; Arber 2012; Goulding 2009; Grillner and Jessell 2009; Kiehn 2011)]. For example, a recent study demonstrated the importance of dI3 interneurons in the execution of grasping behavior in mice (Bui et al. 2013). Thus, genetic programs initiated at early developmental stages in specific spinal subpopulations prefigure their functional properties in the adult spinal cord, including the control of neuronal settling position as well as synaptic input and output patterns. Our study demonstrates that subpopulations with distinct intraspinal functions transmit these differences to the brainstem in highly organized ascending information channels to different neurons in the LRN. This signaling setup has the strong advantage of coincident and faithful transfer of premotor information to the LRN without synaptic intermediary. Most importantly, while an individual motor neuron pool unlikely discriminates the identity of a premotor population providing synaptic input, our findings demonstrate that much in contrast, the ascending axonal branches of these same premotor neurons segregate and combine according to their spinal subpopulation identity. As an important functional consequence, synaptic information convergent at the motor pool level diverges in the LRN (Figure 15G).

In addition to the functional diversity of neuronal subpopulations revealed through genetic entry points, we found that the spatial distribution of dual connection neurons is highly reminiscent of the overall FL premotor interneuron distribution (Stepien, Tripodi, and Arber 2010). This observation lends further supports to a model

in which ascending LRN signaling pathways are composed of many functionally distinct premotor spinal populations, transmitting an excerpt of perhaps even almost all ongoing motor output related spinal activity to the LRN. It also provides an explanation for why synaptic terminals of premotor neurons connected to one motor pool as a whole do not exhibit a discriminatory LRN axonal targeting pattern. Instead, each FL premotor population linked to an individual motor neuron pool fractionates into many genetically and functionally distinct subpopulations following separate rules for LRN axon targeting, resulting in an overall targeting map based on spinal subpopulation identity.

3.4.3LRN information in the cerebellar loop and influence on descending pathways

Our findings on the exquisite synaptic organization of spinal input to the LRN raise the question of the further transmission of this information through the cerebellar loop. The LRN is composed exclusively of projection neurons to the cerebellum and thought to be a pure integration nucleus with no local computation. LRN mossy fibers project to cerebellar granule cells but at the same time give off a collateral to deep cerebellar nuclei (Wu, Sugihara, and Shinoda 1999; Shinoda et al. 2000). Granule cells transmit information in highly divergent connectivity patterns to Purkinje cells, which in turn provide the output of the cerebellum to deep cerebellar nuclei (Arshavsky, Gelfand, and Orlovsky 2011; Ito 2006).

Previous work trying to disentangle the projection specificity of LRN neurons to cerebellar target lobules provides evidence that a dorsally located “region A”

within the LRN may exhibit preferential ipsilateral cerebellar projections, whereas a ventral “region B” projects bilaterally (Clendenin, Ekerot, Oscarsson, and Rosén 1974a). While crude due to technical limitations, in light of the differential spatial targeting of the LRN by distinct spinal populations shown here, these findings might imply that transmission of the separate spinal channels may be carried on by the LRN mossy fiber system to deep cerebellar nuclei and the cerebellar cortex. In this context, it will be interesting to assess how deep cerebellar nuclei combine excitatory collateral LRN mossy fiber and inhibitory Purkinje cell information to determine how the spatial map observed in the LRN is transformed at this level. Deep cerebellar nuclei exert profound synaptic influence on several descending brainstem nuclei (reticulo-spinal; vestibular nucleus, red nucleus) (Arshavsky, Gelfand, and Orlovsky 2011; Ito 2006; Orlovsky et al. 1999), which deliver this information updated through the cerebellar loop to the spinal cord. From this point of view, it can be expected that the spinal cord-LRN-cerebellum-deep cerebellar nuclei loop is closed and provides means to update descending pathways in a timely fashion with ongoing activity from the spinal cord.

3.4.4General principles of ascending signaling system organization

LRN projecting neurons in the spinal cord also exhibit striking organization beyond the cervical populations. Cervical and lumbar spinal neurons establish separate terminations within the LRN, a finding in line with previous electrophysiological recordings and anatomical experiments (BRODAL 1949; Clendenin, Ekerot, Oscarsson, and Rosén 1974b; Ekerot 1990a; Künzle 1973).

Interestingly, we find that ipsilateral cervical terminals reside immediately dorsal to contralateral lumbar terminals within the LRN. This arrangement is intriguing considering the normal rodent two-beat gait exhibited during locomotion, with coincident activity of diagonal limbs and parallel action patterns of ipsilateral FL and contralateral HL. Nevertheless however, the HL dominated ventral territory in the LRN does not receive input from lumbar premotor neurons, raising the question of whether alternative channels for transmission of HL premotor information exist.

Electrophysiological studies in the cat lumbar spinal cord demonstrate that inhibitory premotor interneurons connect to neurons projecting through the ventral spino-cerebellar tract (VSCT) (Jankowska and Hammar 2013; Jankowska, Krutki, and Hammar 2010; Lundberg 1971). VSCT neurons establish direct mossy fiber projections from the lumbar spinal cord to the cerebellum and are proposed to be functional equivalents of LRN neurons monitoring spinal cord intrinsic activity in the lumbar spinal cord (Orlovsky et al. 1999; Oscarsson 1965). Recent anatomical work retrogradely labeling lumbar neurons with projections to the cerebellum demonstrates that VSCT neurons are targeted predominantly by inhibitory neurons (Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012b; Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012a). Whether and how synaptic targeting specificity also exists for input to VSCT neurons in a manner similar to LRN will be an important question to address in the future. The overall picture that emerges is one of a binary transmission system for spinal information related to FL or HL motor output to the cerebellum. The most universal one with a dedicated FL premotor

domain is the LRN, and a second one is embedded within the lumbar spinal cord as VSCT neurons transmitting more local lumbar events to the cerebellum.

In summary, our findings provide evidence for precise organization of ascending spinal information to the brainstem, encompassing many functionally distinct spinal subpopulations, which can be divided by site of residence in the spinal cord, developmental origin and neurotransmitter fate. We provide first insight into the genetic complexity of the spinal efference copy signaling system, lending support to the notion that listening attentively to ongoing activity of the spinal cord at supraspinal levels provides an important prerequisite for accuracy in motor control.

3.5Experimental Procedures

3.5.1 Generation of mice and mouse genetics

ES cell recombinants for the generation of *Tau*$^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ mice (129/ Ola) were screened by Southern blot and PCR analysis as previously described (Hippenmeyer et al. 2005). *GlyT2*^{GFP} (Zeilhofer et al. 2005), *vGlut2*^{Cre} (Vong et al. 2011), *vGAT*^{Cre} (Vong et al. 2011), *Lhx3*^{Cre} (Sharma et al. 1998), *Isl1*^{Cre} (Srinivas et al. 2001), *En1*^{Cre} (Sapir et al. 2004), and *Sim1*^{Cre} (Zhang et al. 2008) mouse strains have been described and were maintained on a mixed genetic background (129/C57Bl6). During development, Lhx3 expression is rapidly extinguished in V2b neurons but maintained in V2a (Peng et al. 2007), leading to preferential recombination in V2a neurons using *Lhx3*^{Cre} transgenic mice intercrossed with reporter strains. In addition, V2b neurons in the spinal cord establish descending axons (M. Goulding, personal communication), thus allowing the use of *Lhx3*^{Cre} mice to study ascending projections of specifically V2a neurons to the LRN. Intraspinal injections in *Sim1*^{Cre} (Zhang et al.

2008) mice crossed with *Tau*$^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ mice also labeled a minor population locally projecting neurons in the dorsal horn not further analyzed or included in the reconstructions in this study.

3.5.2 Virus production and injections

Rabies virus experiments with monosynaptic restriction to label premotor neurons were carried out as described previously for both production and injection (Stepien, Tripodi, and Arber 2010; Tripodi, Stepien, and Arber 2011). Rabies viruses used in this study (Rab-mCherry and Rab-GFP, as well as EnvA coated versions) were amplified and purified from local viral stocks following established protocols (Wickersham, Sullivan, and Seung 2010). Cav-Cre amplification and purification were carried out following established protocols (Kremer et al. 2000). Limb muscle injections targeted many muscles in single experiments by multiple focal injections and for FL injections consistently included Triceps and Biceps as representative extensor and flexor muscle groups respectively (n≥6 for FL and HL muscle injections; n≥3 for individual muscle injections). Experiments for retrograde marking of spinal neurons projecting to the LRN were carried out at p10 (injection coordinates: A/P 2.4mm from Lambda, 0.9mm L, 4.75mm V), 4 days subsequent to muscle injections, and terminated by perfusion fixation (4% PFA) at p14 (n=3 reconstructions). Confined unilateral intraspinal injections used vertebrae position as landmarks (cervical injection: C4-C7; lumbar injections: L1-L4; volume injected: 100nl; coinjection of AAV-flex or FRT-SynGFP and -H2BGFP), were initiated at p12 and terminated at p22 unless otherwise stated (n≥3 independent unilateral injections for each condition analyzed). Experiments in which unilateral intraspinal injections were combined with monosynaptic rabies virus tracing were performed at p3, followed by

muscle injections targeting Triceps and Biceps at p5 and terminated 8 days thereafter (n=2 mice each). For overall mapping of synaptic input to LRN, we used a sequential triple injection approach. The first injection targeted LRN neurons retrogradely from the cerebellum (Cav-Cre) at p3, followed by a coinjection of AAV-flexGly/V5 and flexTVA/H2BGFP in the LRN (A/P 2.4mm from Lambda, L1.15mm, V4.8mm) at p18 and a third injection at p35-50 with Rabies-EnvA coated in the LRN (A/P 3mm, L 1.2mm, V 4.6mm). Experiments were terminated 7 days subsequent to the final injection (n=3). Coordinates for M1 injections were -0.1mm antero-posterior from Bregma, 1.7mm lateral and 0.8mm ventral (analysis 14 days after injection; n=2).

All CNS injection experiments were executed with a Kopf Stereotaxic apparatus (Model 962 Dual Ultra Precise Small Animal Stereotaxic) and included fluorescently marked beads (Fluoro-Max Blue Fluorescent Polymer Microsphere; Thermo Scientific) to confirm precision of injection site in addition to expression driven by AAVs or rabies viruses upon section analysis of corresponding tissue samples. Design of new AAV vectors used an AAV backbone vector provided by Allen Brain Project containing CAG promoter elements and WPRE sequences (AAV.CAG.FLEX.tdTomato.WPRE.bGH) as backbone vector to integrate additional cDNA sequences (details available upon request). Double inverted LoxP or FRT flanked constructs for Synaptophysin-GFP (Pecho-Vrieseling et al. 2009), H2B-GFP as nuclear marker, TVA and G-protein were ordered as synthetic DNAs (Blue Heron) and corresponding AAVs (serotype 2.9) produced following standard procedures (genomic titers ≥2x10^13/ml).

3.5.3 Immunohistochemistry, imaging and statistical analysis

Antibodies used in this study were: chicken anti-GFP (Invitrogen), goat anti-ChAT (Chemicon), goat anti-LacZ (Biogenesis), guinea-pig anti-vGlut1 (Chemicon), mouse anti-NeuN (Chemicon), rabbit anti-GFP (Invitrogen), rabbit anti-LacZ (Invitrogen), and rabbit anti-RFP (Chemicon). Images were acquired with an Olympus confocal microscope (FV1000) or a custom-made dual spinning-disk microscope (Life Imaging Services GmbH, Basel, Switzerland) (Tripodi, Stepien, and Arber 2011). For identification of cell body position of dual-labeled spinal neurons, sections were acquired using the 10x objective (12 focal planes/40μm section). Files were decomposed to individual channels and planes, aligned in Amira and subsequently opened in Imaris XT for spot detection (Bitplane). Matlab codes implemented in Imaris translated the coordinates for the spot detection. Rostro-caudal alignment of spinal cords from different mice was achieved by assigning cutaneous maximus (C7/8) and phrenic (C3-5) motor neuron pool position in each 3D reconstruction. Isoline plots and projections were obtained using Matlab. Cell position analysis for $LacZ^{ON}$ neurons from progenitor domain-Cre line crosses was done in Image J and analyzed as previously described (Tripodi, Stepien, and Arber 2011). Coordinates were plotted using R (R Foundation for Statistical Computing, Vienna, Austria, 2005, http://www.r-project.org) and for plots shown, error bars represent SE.

3.6 Acknowledgements

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Chapter 4

4. Role of the lateral reticular nucleus (LRN) in target reaching movement

In previous investigations described in chapter 3, we demonstrated that the input to the LRN is heterogeneous in terms of genetically different spinal subpopulations and in terms of regional spinal input distribution to the nucleus. We found that each spinal subpopulation analyzed delivers a faithful copy of the activity to the cerebellum through the LRN, besides playing a role in local spinal local circuits function. Moreover, we have shown that the LRN receives input from the rubrospinal tract descending to the spinal cord and previously identified as important in performance of fine motor tasks (Jarratt and Hyland 1999) and locomotion (Muir and Whishaw 2000). The LRN does not seem to be a pure relay station but a site of additional integration of information it receives in a combinatorial manner. Previously, the input from the C3-C4 population has been implicated in target reaching movements in cat (Alstermark et al. 2011) as well as in grasping in monkey (Kinoshita et al. 2012). These studies did not take into account the diversity and specificity of input to the LRN. We can therefore formulate the following questions: (1) which is the contribution of an efference copy delivered to the LRN from the spinal cord? (2) which is the contribution of an individual spinal channel as defined by subpopulation identity?, (3) which is the contribution of non-premotor ascending information?, (4) which is the contribution of the supraspinal information to the

overall information the LRN transmits to the cerebellum? (5) Finally, how is mossy fiber input integrated with other input to influence descending motor pathways after cerebellar computation? Although interesting, it is technically challenging to answer these questions and development of new genetic and viral tools might be necessary to disclose the behavioral weight of each circuit component.

In my thesis, I tried to address the basic question of the behavioral relevance of the LRN mossy fiber input to the cerebellum. Up to now, only one study assessed the role of the LRN, by direct local injection of kainic acid in cat and thereby killing these neurons (Santarcangelo, Pompeiano, and Stampacchia 1981). We used a viral approach with two sequential injections to target the LRN from the cerebellum similar to the one described in chapter 3. Although the effect in cat of the LRN killing was quite dramatic, the sole observation of mice in which the LRN neurons were killed, did not result in any clear impairment. Therefore we decided to move to a more refined analysis. We took advantage of previous studies on C3C4 PNs population and on the red nucleus. Both of them were described to be involved in forelimb fine movement and therefore we tested the animals in a single pellet reaching task as described in previous experiments (Xu et al. 2009). In the following paragraphs, I will present preliminary behavioral results on the role of the LRN.

4.1 Experimental procedure (single pellet reaching task)

We used an approach similar to the one described in chapter 3 to target specifically the LRN from the cerebellum. Shortly, wild type mice were injected a P3 bilaterally in the cerebellum with a CAV2-cre virus (Kremer et al. 2000) which can be

taken up by axonal terminals and travels retrogradely to the neuronal cell body, in this case labeling precerebellar nuclei. A following bilateral injection was performed at P18-P20 targeting the LRN with an AAV-flex-DTR (diphtheria toxin receptor) in the experimental animals and an AAV-flex-mTomato in control animals (Figure 16). Control and experimental animals were injected with DTA (diphtheria toxin A) after 2 weeks in the first behavioral paradigm (before learning) and after training (after learning) in the second behavioral paradigm. The single pellet reaching task was carried out following previously established procedures with slight modifications (Xu et al. 2009). Briefly, handling of mice was performed only by one experimenter throughout the procedure. Mice were kept in food restriction 4 to 5 days before and during the training time. Body weight was recorded every day and kept between 85% to 90% of the starting weight. The training chamber (modified from(Xu et al. 2009)) was built from clear Plexiglas (1 mm thickness; dimensions 20x8.5x15 cm). One vertical slit (0.5 cm wide; 13 cm high) was located on the front wall of the box. Single reachable chocolate pellets (dustless precision pellets, 20 mg, Bioserv) were located outside the slit, on a platform of 1.5 cm height. After one day of habituation to the box without presentation of pellets outside the slit, the assay consisted of 2 phases: shaping and training. During the shaping phase (day 1), mice are allowed to reach for multiple pellets presented to them outside the box to determine the preferred limb. During the training phase (day 2-8), individual pellets are placed in front of the slit and mice are video-recorded at 100 Hz while reaching for a maximum of 50 pellets within 20 minutes. We classified the reaching accuracy with slow motion video surveillance according to 4 categories: Miss (no contact with the pellet during reach), Touch (the pellet is touched but the position of the limb/paw does not allow the

animal to grasp the pellet) both of these categories are regarded as problem in the correct localization of the pellet, Drop (the pellet is retrieved but falls before taking it into the mouth), Success (the mouse retrieves the pellet directly to its mouth). Success rate was calculated as the percentage of successful reaches over total reaching attempts. Animals consistently using the tongue instead of the FL to retrieve the food pellet or animals that did not show a preferential limb were excluded from further analysis. The training was performed for 8 consecutive days every day. When the LRN was killed after mice learned the task (according to the rate of success), the training was performed twice a week for 2 weeks after the injection of DTA (diphtheria toxin). Postmortem analysis was performed to assess the efficiency of neuronal loss.

4.2 Result: ablation of the LRN before learning

Three groups of mice were pooled in the following analysis. DTA injection was performed 2 weeks after the last surgery. The start of the training was after 2 weeks in order for the toxin to exert its full effect in neuronal ablation. Mice were trained for 6 to 8 days and analysis of the movies was carried out as described in the experimental procedure paragraph for the last day of training. In Figure 17A and B, the percentage of success was calculated on the total amount of attempts. No difference could be noticed among the control and the experimental group of animals. Once the pellet was correctly localized, they were able to successfully retrieve it. The 2 groups were statistically different (unpaired T-test) concerning the errors in the correct localization of the pellet (misses and touches were considered together).

Post mortem analysis of the experimental animals, revealed great variability in the efficiency of neuronal killing although specific to the LRN. The LRN of each side was not equally affected and this made the correlation with the preferred limb difficult to address. All trained mice, with the exclusion of the ones using consistently the tongue, were considered in the analysis although the efficiency could be low.

A different experimental paradigm was used in order to clarify the result above described. This new scheme described in detailed in the following paragraph will allow us to: 1. Eliminate from the analysis animals that do not learn the task, isolating the animals with a real deficit, 2. Evaluate the eventual change of limb usage according to the neuronal loss of the LRN, considering the projections from the spinal cord to be mainly ipsilateral, 3. Assess eventually a learning versus non learning role of LRN in performing the task.

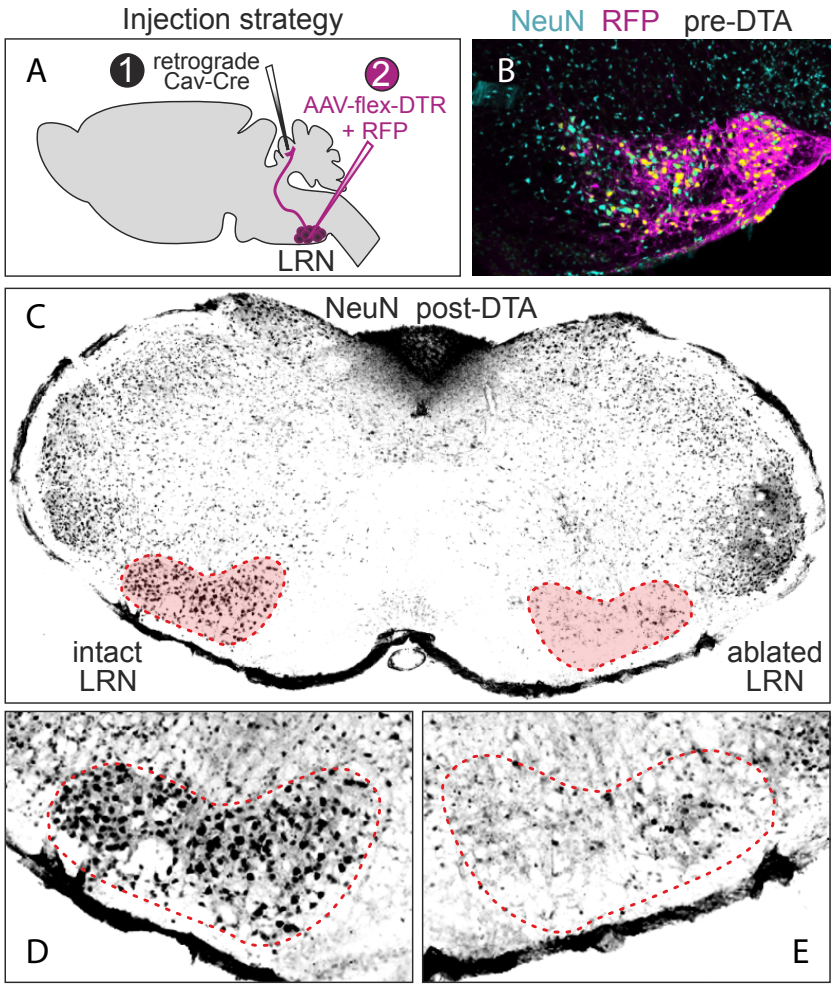


Figure 16. Experimental procedure scheme.

A)The injection strategy: a first injection in the cerebellum with Cav-cre retrogradely infect LRN neurons, a second unilateral injection with AAV-flex-DTR and RFP in the LRN at adult stages allow specific target of LRN neurons with DTR as in (B). C)After injection of DTA neuronal loss in LRN is visible (LRN ablated side and intact side enlarged in pannels D and E)

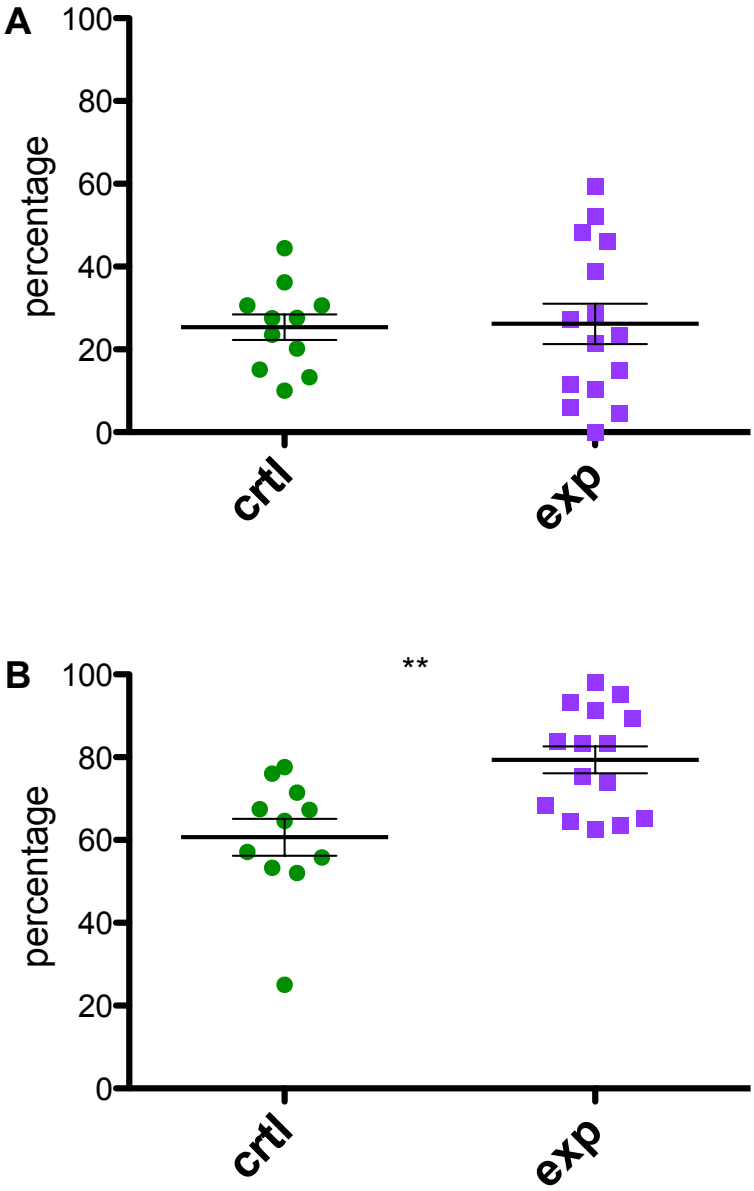


Figure 17. LRN ablation before learning.

Injection of DTA before the training starts. (A) Percentage of success over total attempts in experimental group (exp) and control group (crtl). (B) Percentage of misses plus touches over total mistakes.

4.3 Result: ablation of the LRN after learning

Two sets of animals were trained together. The training was started 2 weeks after the last injection. Mice were trained for 8 consecutive days. Training was stopped for two days and then performed twice a week keeping the animals constantly under food restriction as described in the experimental procedure paragraph. Analysis of the movies was carried out as described in the experimental procedure paragraph for the last day of training before DTA injection (day11) and after the injection of the toxin (day14-corresponding to 2 weeks after day11). Before DTA injection, figure 17A, the control and the experimental group of animals have successfully learned the task according to previous criteria (Xu et al. 2009). Both groups presented the same amount of errors in the correct localization of the pellet (misses plus touches) as shown in figure 17B. After injection of the toxin DTA, no difference in success rate or in the rate of errors was revealed in the experimental group, as in Figure 17C and D. Post mortem analysis reveal great viability across animals in term of efficiency of cell ablation. The viability was also observed in the left and right LRN. Again correlation with limb usage and ablation efficiency was difficult to address. The absence of behavioral phenotype could not be ascribed to actual neuronal loss in the LRN.

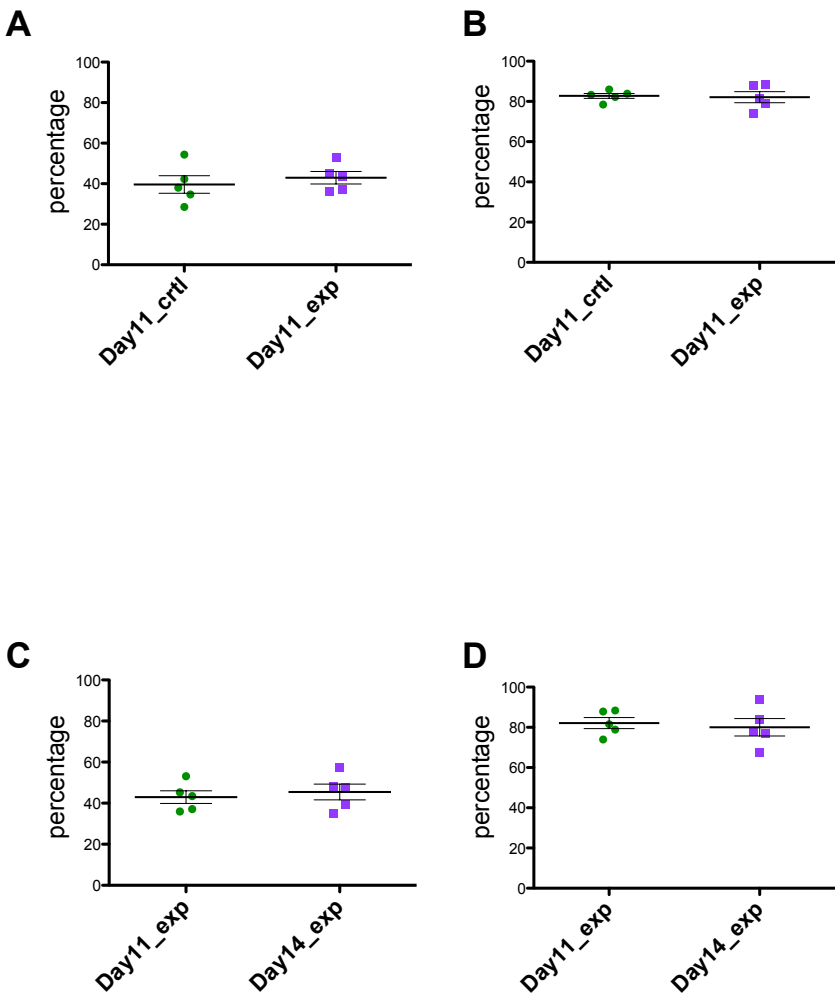


Figure 18. LRN killing after learning.

(A) Percentage of success over attempts and in (B) Percentage of misses plus touches over total mistakes before the injection of the toxin in the control (crtl) and experimental (exp) group. (C) Percentage of success over total attempts and of misses plus touches over attempts (D) before (day11) and after the injection of the toxin (day14) in the experimental group.

4.3 Discussion

Which is the contribution the information, transmitted by the LRN mossy fibers, has on the cerebellum? To address this question, we set up a strategy to specifically target the LRN through a double injection approach. This approach

allowed us to achieve high level specificity in targeting the LRN sparing nearby structure like the inferior olive. We decided to perform a reaching behavioral task since the input to the LRN comes from pathways putatively involved in the control of forelimb movement (Jarratt and Hyland 1999; Alstermark et al. 2011). In light of the still ongoing debate on the role of the cerebellar computation in motor learning and motor consolidation (Galliano et al. 2013; Gao, van Beugen, and De Zeeuw 2012; Raymond, Lisberger, and Mauk 1996), we first evaluated the behavioral output before training. In the experimental group, the animals showed more errors in correctly placing the limb with respect to the pellet position and only after several attempts the reach was successful. Similar deficits during target reaching phase were observed when the supposed input to the LRN from the C3-C4 PNs was blocked through lesion of the ascending tracts (Alstermark, Lundberg, et al. 1981). These effects were transient and long term recovery was observed . If we exclude the ascending input delivered by the LRN to cerebellum, the only spared ascending input from forelimb indirectly routed to the cerebellum is sensory information carried by the External cuneate nucleus which carries mixed information (exafference and reafference). The assumption is that correct movement can only be generated if sensory information is matched with the copy of the descending motor command. Forelimb premotor interneurons receive input from different motor descending pathways and deliver this command to motor neurons and at the same time indirectly to the cerebellum. Absence of this source of information might explain the difficulties in correctly localizing the limb in space as observed in the experimental animals analyzed in this study. Although fascinating this explanation was not conclusively demonstrated in my thesis due to the post mortem analysis of neuronal ablation efficiency. Experimental

animals varied in terms of left-right neuronal losses in the LRN and more experiments would be needed to consolidate the preliminary findings. In particular, it is difficult to assess whether an animal attempts compensatory behavioral strategies with the current experimental design where animals learn to perform the task only after LRN neuron ablation. Different scenarios are possible: 1. An animal with one of the LRNs severely ablated cannot use correctly the corresponding limb, 2 An animals with one of the LRNs severely ablated cannot use the preferred limb and adapts to use the non-preferred limb. This would lead to the possible incorrect use of the non-preferred limb, 3 An animal with the LRN ablation on the side of the non-preferred limb will use correctly the preferred limb. The uni-laterality of the task combined with the differential left and right ablation efficiency resulted in difficulties to narrow down the real contribution of the LRN in the phenotype observed. To sort out the before mentioned dilemmas, we decided to ablate LRN neurons after the task was learned. Assuming the LRN is important in motor execution, the animals with impaired LRN on the side of the preferred limb would have to show defects or might adapt to use the non-preferred limb. However, we observed no change in limb preference after DTA administration and no difference between control and experimental animals in our experiments. Finally, an important consideration is also that the LRN is organized in partially overlapping domains receiving different input matched by progenitor domain origin. Therefore ablation of a particular domain might have a different impact on the information it delivers to the cerebellum, leading to potential differences in the behavioral outcome. In conclusion, ablation experiments of the LRN must be achieved through more reliable and consistent approaches in the future to allow to

address the previous mentioned questions on the contribution of the information it delivers to motor execution and/or motor learning.

Chapter 5

5. Inhibitory input to spinocerebellar neurons

Many populations of spinal interneurons may affect VSCT and SB neurons, but the actions of premotor interneurons in reflex pathways to motor neurons have been documented most thoroughly (Jankowska and Hammar 2013; Jankowska, Krutki, and Hammar 2010). Evidence was first provided for the actions of interneurons mediating reciprocal and recurrent inhibition of motor neurons (Ia interneurons and Renshaw cells) on VSCT neurons but not on dorsal spino- cerebellar tract (DSCT) neurons (Hongo et al. 1983). The DSCT neurons have been regarded as a relay of peripheral sensory input to the cerebellum during rhythmic movements such as locomotion and scratching. In contrast, the VSCT was seen as conveying a copy of the output of spinal neuronal circuitry, including those generating rhythmic motor activity (for extensive review see-(Stecina, Fedirchuk, and Hultborn 2013) (Jankowska and Hammar 2013)). During rhythmic motor actions, both DSCT and VSCT neurons were found to be active, however DSCT activity ceased while activity of VSCT cells persisted following partial removal of sensory input by deafferentation (fictive scratching) (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978b). Parallel to the LRN system, the VSCT was already suggested to convey an efference copy of the ongoing spinal activity and reafference information, as consequence of limb movement, delivered by the DSCT in a parallel circuit in respect to the LRN/ECN

circuit(Stecina, Fedirchuk, and Hultborn 2013). Again, most studies were based on electrophysiological recordings in cat and only recently, excitatory and inhibitory input was evaluate through immunohistochemistry in rat as well (Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012b; Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012a). Although informative, the precise nature and the clear anatomical evidence of the spinal origin of such inputs is still missing. Moreover is not clear whether the complexity of the forelimb circuits in terms of specificity and heterogeneity applies also to the hindlimb system. From the previous mentioned studies, it was clear that inhibition represents a major input to the VSCT and SB neurons compared to the input to CC and the DSCT. Besides, the observation that inhibitory lumbar interneurons project only sparsely to the LRN and the brainstem made us wonder whether they contact preferentially spinocerebellar neurons. In the following paragraph, I will show preliminary results on a quantification of inhibitory input to SB, VSCT, CC, dDSCT retrogradelly labeled from the cerebellum on combination with intraspinal injections, allowing to specifically assess spinal input to these populations.

5.1 Experimental procedure

To assess inhibitory synaptic contacts to different spinocerebellar neurons, we made use of *vGatCre*+/- mouse line. We performed spinal cord injections at lumbar levels of an AAV-flex-SynGFP to label synaptic terminals of these neurons and and coinjected an AAV-flex-nlsGFP to check for unilaterality of the injection at P11. At P14, a bilateralcerebellar backlabelling was performed with rabies virus mCherry and

the animal were killed at P22. Thoracic and lumbar spinal cords were cut and the different populations of precerebellar neurons were identify according to position and morphology as shown in previous studies (Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012b). Images were acquired with a spinning disk microscope at 60x (the z-stack 0.2um) and synaptic inputs evaluated on soma and proximal dendrites on neurons with complete cell bodies in the analyzed section, using Imaris software.

5.2 Results

Inhibitory input was quantified on 3 types of precerebellar neurons according to their rostrocaudal position and laterality to spinal cord injection. We found that SB neurons located in thoracic spinal cord ipsilaterlally to the spinal cord injection receive higher synaptic input than SB neurons located contralaterally (Figure 19A). ccDSCT neurons were found predominantly at thoracic level, ipsilaterally located, show less inhibitory input than ipsilaterally located thoracic SB neurons (Figure 19B). SB neurons ipsilaterally located in the lumbar spinal cord in proximity of injection site showed higher synaptic input than SB neurons at thoracic levels (Figure 19C). Finally, input density analysis to VSCT neurons showed a similar level than to SB neurons, of synaptic input at lumbar level than farther away from the site of injection at sacral level (Figure 19D). SB and VSCT neurons therefore receive inhibitory input from interneurons located at lumbar levels at higher levels than to other populations of precerebellar neurons. Moreover, we found that the synaptic input is higher when cell bodies of these neurons is closer to the ones of the inhibitory neurons in the rostrocaudal dimension. These findings resonate with previous findings (Jankowska

and Hammar 2013) suggesting a rather local nature of the lumbar relay to the cerebellum.

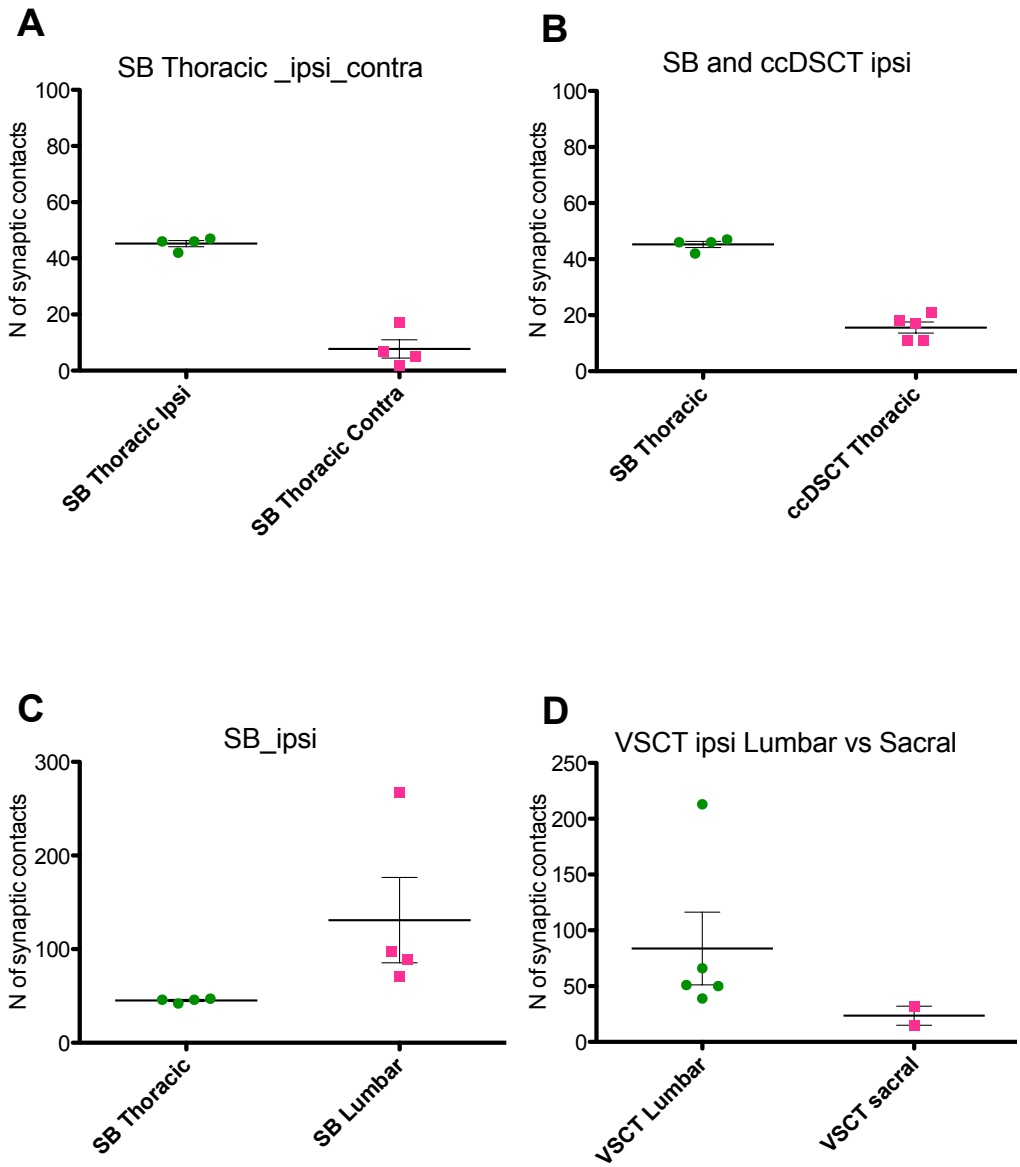


Figure 19.Synaptic input to different spinocerebellar neurons.

(A) Number of synaptic inhibitory inputs to SB located at thoracic level contra and ipsilateral to the spinal cord injection site.(B) Number of synaptic inhibitory inputs to SB and ccDSCT neurons in at thoracic level ipsilateral to the injection site.(C) Number of synaptic inhibitory inputs to SB at thoracic or lumbar level ipsilateral to the spinal cord injection site.(D) Number of synaptic inhibitory inputs to VSCT neurons ipsilateral to the spinal cord injection at lumbar or sacral level.

5.6 Discussion

Inhibitory interneurons located in the lumbar spinal cord have local projections rather than long distance ascending ones. In the brainstem, the contribution of inhibitory input to the LRN derived from lumbar levels is minor compared to the excitatory one. Our results support this view and in particular they suggest that inhibitory interneurons contact precerebellar neurons (SB and VSCT) already shown previously to be target of strong inhibitory inputs (Shrestha, Bannatyne, Jankowska, Hammar, Nilsson, and Maxwell 2012b) and potential target of inhibitory premotor interneurons in the cat (Jankowska, Krutki, and Hammar 2010). Precerebellar neurons receive strong input the closer they are to the cell bodies of the inhibitory neurons. Precerebellar neurons might therefore be selectively recruited by inhibitory lumbar circuits (VSCT/SB versus ccDSCT). These preliminary results raise the following questions: Do spinocerebellar neurons receiving an equal amount of excitatory input from lumbar interneurons? Do they receive premotor information? Which is the role of such information in motor behavior? Does the input have similarly complex organization as we describe here to be the case for the LRN? Why is only premotor inhibitory information forwarded to the VSCT neurons, contrary to the one delivered to the LRN? Our preliminary results represent just a small step in the direction to unravel the complexity of these circuits.

Chapter 6

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

Cyrill Goetz, Chiara Pivetta and Silvia Arber

(Neuron *85*, January 7th, 2015)

6.1 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.

6.2. 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; Vanderhort 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; Vanderhort 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 (Jankoswska, 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; Lanunza 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 (Arber, 2012; Goulding and Pfaff, 2005; Kiehn, 2011; Alaynick et al., 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, 2009; McCrea and Rybak, 2008; Renshaw, 1941) and recent work begins to align functional subtypes to genetic identity (Arber, 2012; Kiehn, 2011; Alaynick et al., 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., 2011; 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.

6.3 Results

6.3.1 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., 2011; Tripodi et al., 2011). Making use of their differential columnar organization and associated peripheral trajectories (Figure 20A, 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 20C). 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 20D, 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 20E, H). Both LMC and MMC cohorts were broadly distributed in the spinal cord ipsilateral to muscle injection (Figure 20E, 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 20E), contralateral MMC-premotor neurons distributed much more broadly (Figure 20H). 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 20F), 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 20I). 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 20J). 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 20K).

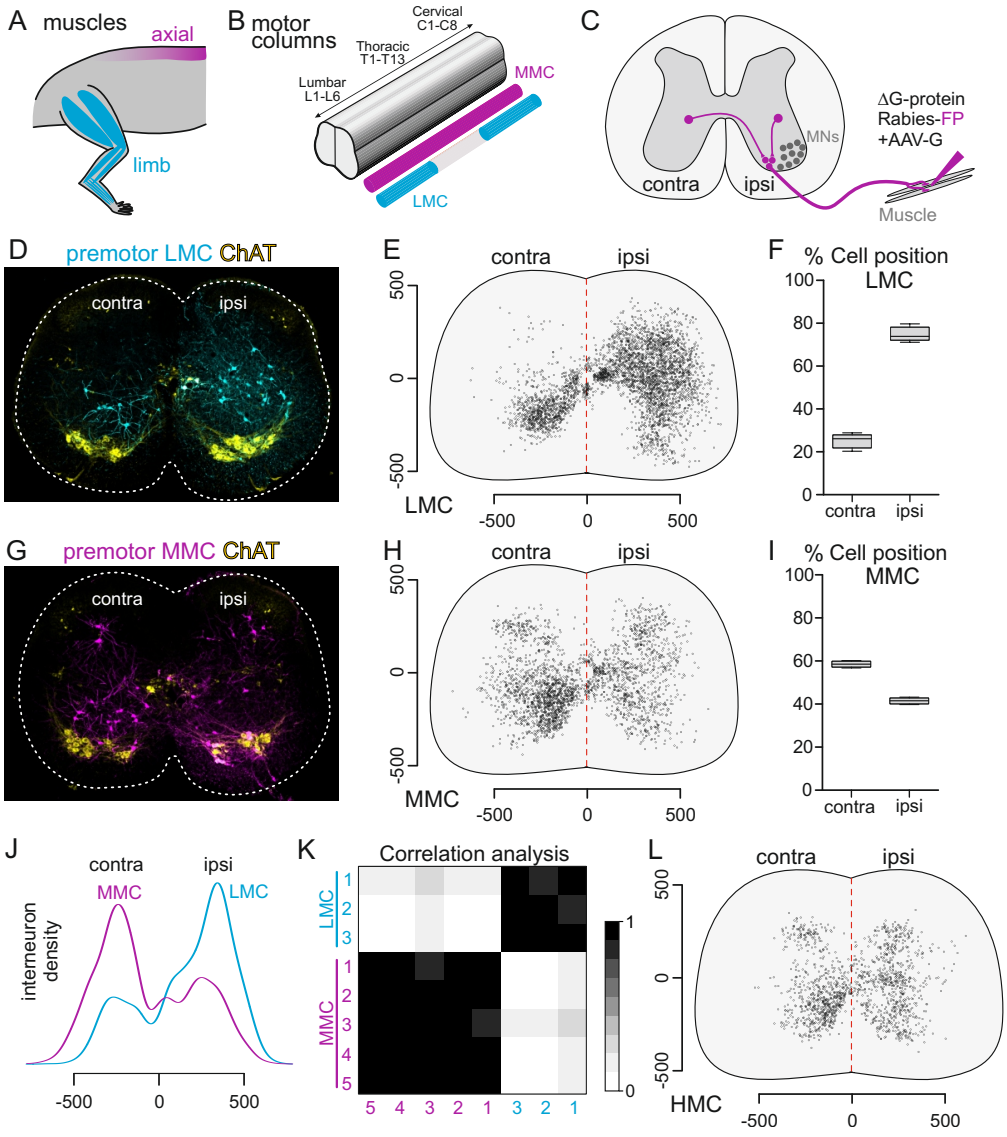


Figure 20. Symmetrical distribution of axial premotor network

(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 20L). 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.

6.3.2. 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 21A). 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 21A).

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 21A-E).

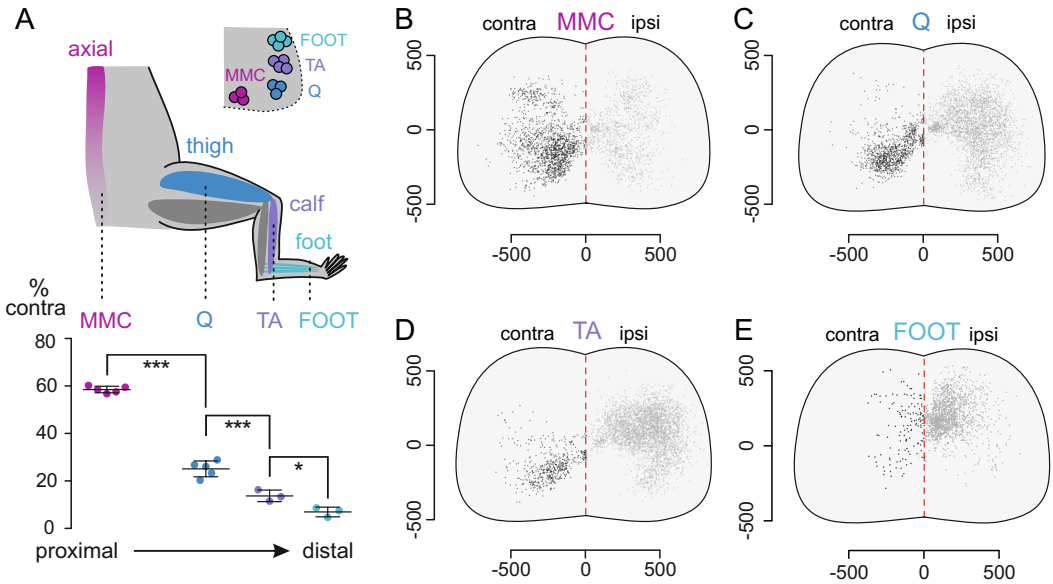


Figure 21. Differential control of LMC motor pools by contralateral premotor network

(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 22).

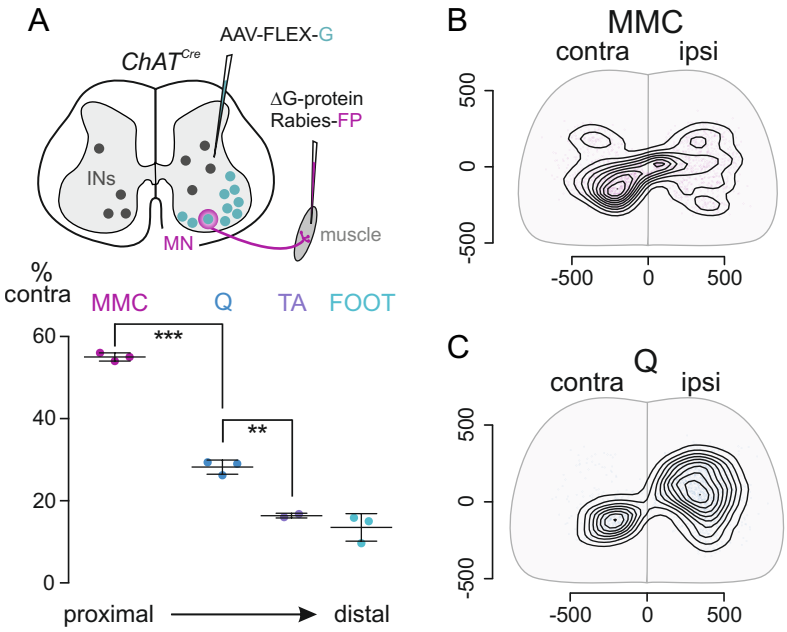


Figure 22. Premotor mapping by use of central motor neuron infection to express G

(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.

6.3.3. 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 (Conrandi 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 23A). 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 23B-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 23E). 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.

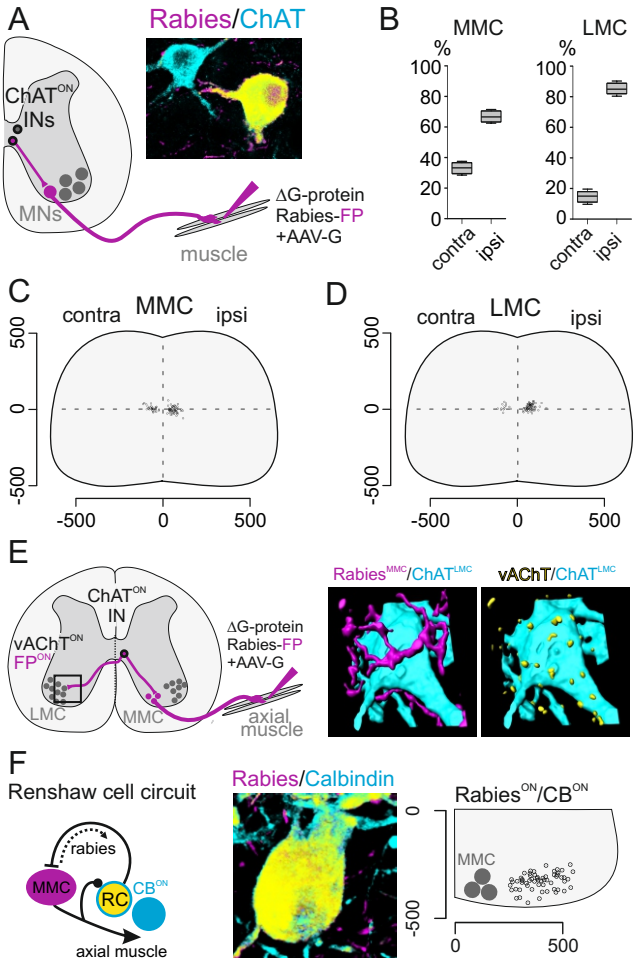
We next assessed the distribution of Calbindin^{ON} Renshaw cells connected to MMC motor neurons (Figure 23F) (Alvarez et al.,2005; Renshaw, 1941). We found that MMC-premotor virus marked Renshaw cells resided in proximity to motor neurons

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.

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.

Figure 23. 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).



6.3.4. 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 24A) (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 24A-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 24D). 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 24B, 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 24C-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 24B-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 24A-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 24D, E). Much in contrast, Lbx1-derived LMC premotor interneurons provide the most pronounced contribution from the ipsilateral intermediate spinal cord to motor neurons (Figure 24D, 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.

6.3.5 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 24F).

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.

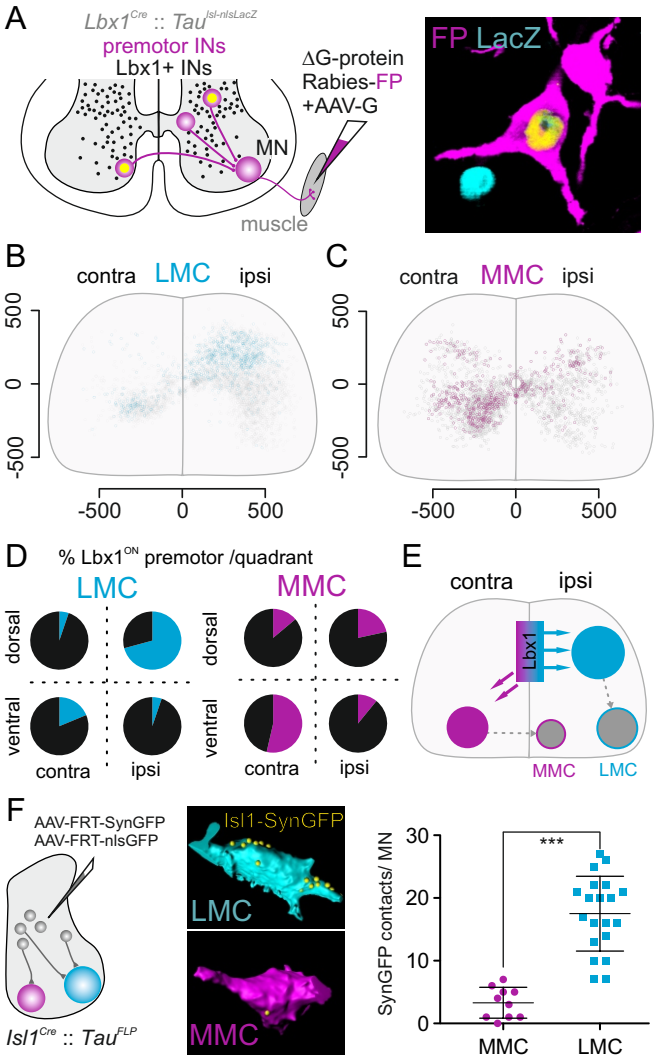


Figure 24. Premotor populations with motor column preferences

(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).

6.3.6. 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 25A, B), a strategy targeting both GABAergic and glycinergic interneuron populations (Wojcik et al., 2006).

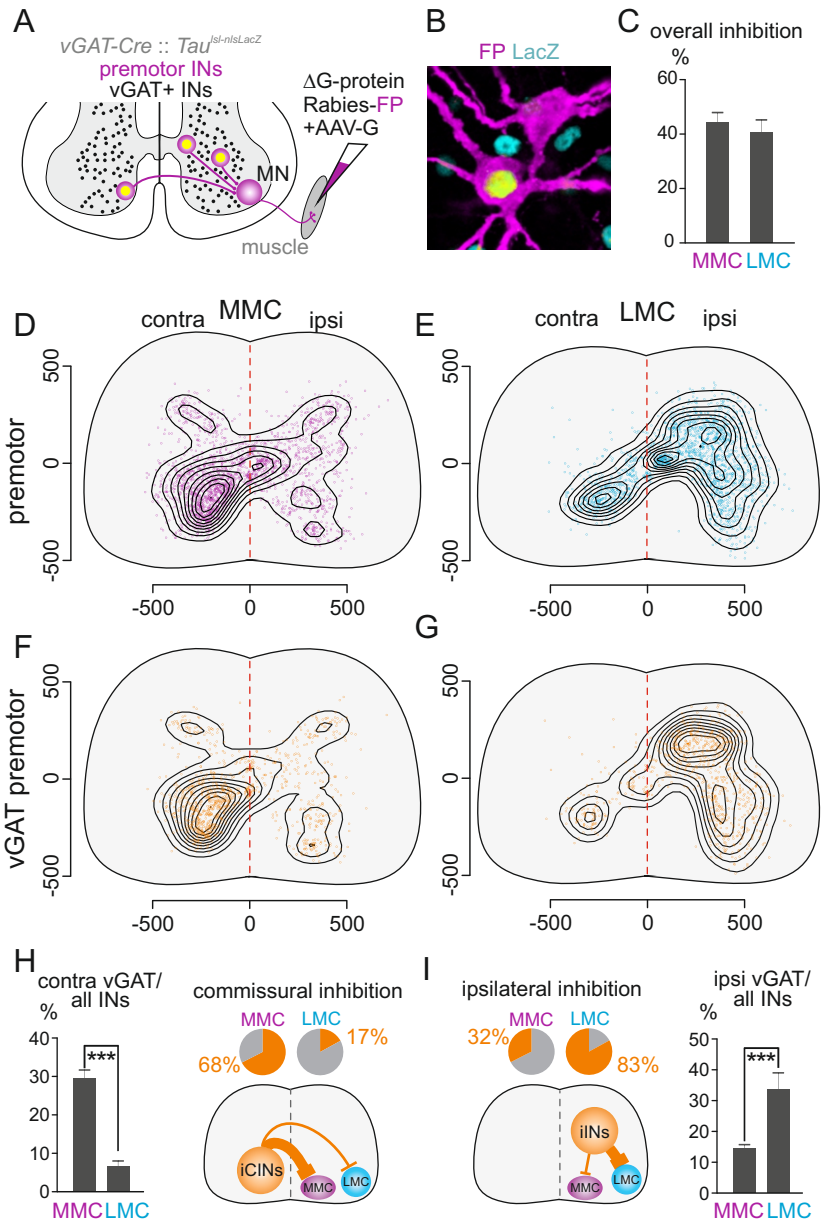
We first assessed the overall inhibitory component within the premotor network, including ipsi- and contralateral populations. We found that ~40% of all marked neurons were vGAT^{ON} for both MMC and LMC premotor populations (Figure 25C), demonstrating that E/I balance at the overall premotor level is 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 25D-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 25H, I). We found that of all inhibitory MMC premotor neurons, ~68% were located in the contralateral spinal cord (Figure 25H, I). In contrast, ~83% of inhibitory LMC premotor interneurons were located ipsilaterally (Figure 25H, I).

Figure 25. 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 26).

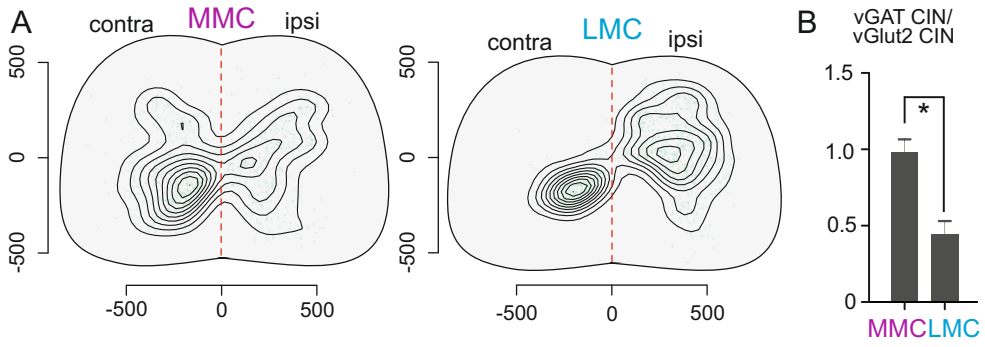


Figure 26. Control of MMC and LMC by putative excitatory interneurons

(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 25H, I).

6.3.7. 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 27A).

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 27A). 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 27B). We found that the highest synaptic input derived from vGAT^{ON} commissural interneurons was targeted towards MMC motor neurons (Figure 27B, 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 27B, 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 27C).

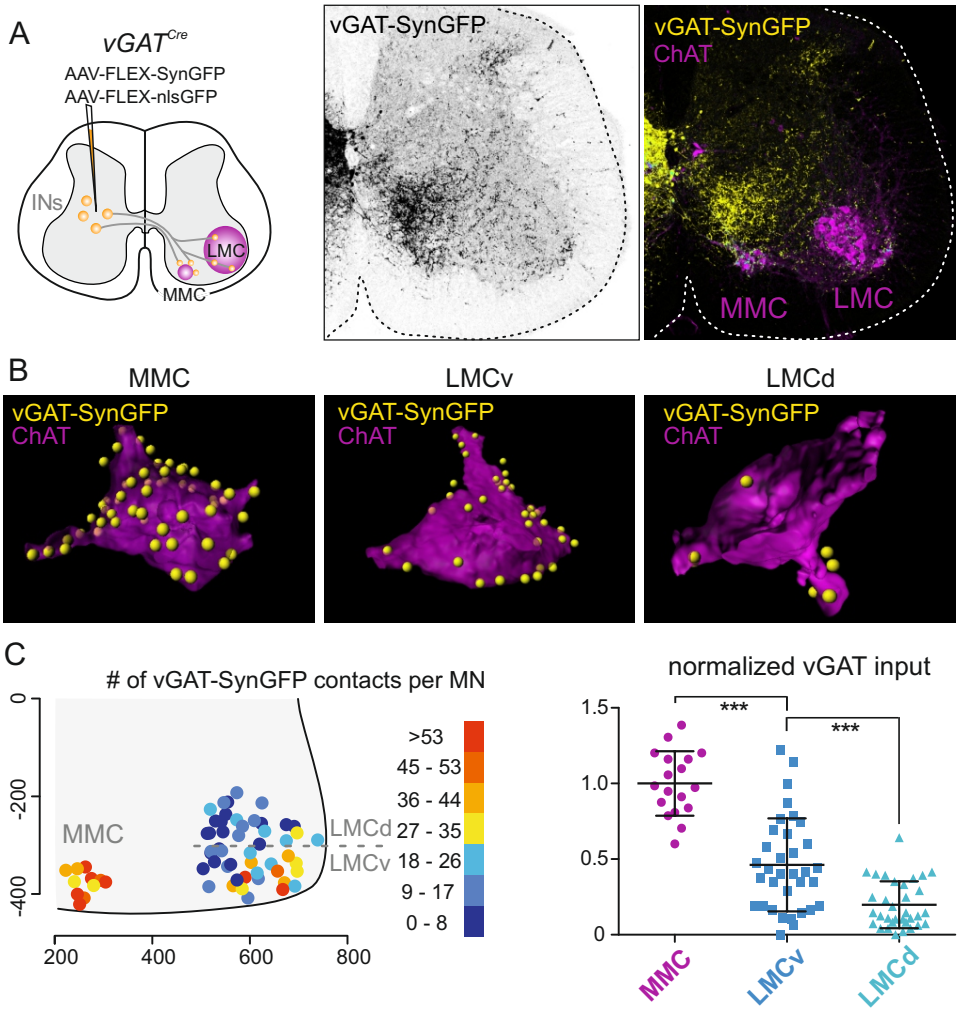


Figure 27. Motor neuron cell body position influences access to contralateral premotor interneurons

(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 28).

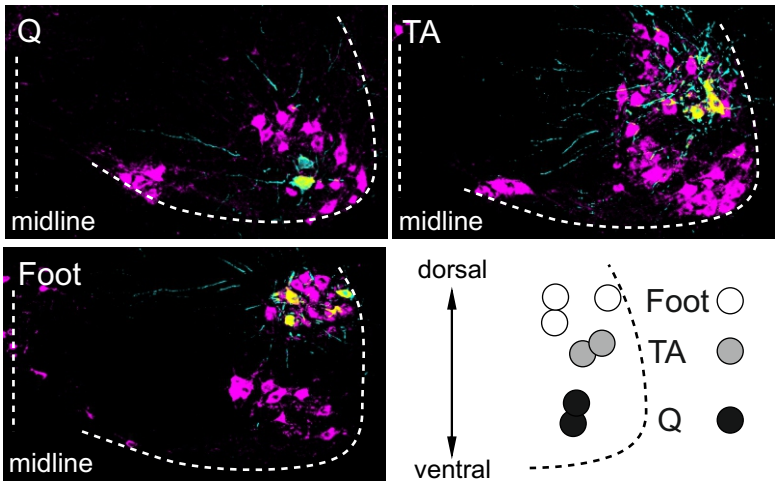


Figure 28. Distinct dorso-ventral positions of LMC motor neuron pools

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.

6.3.8. 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 30A). 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 30A). 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 29A).

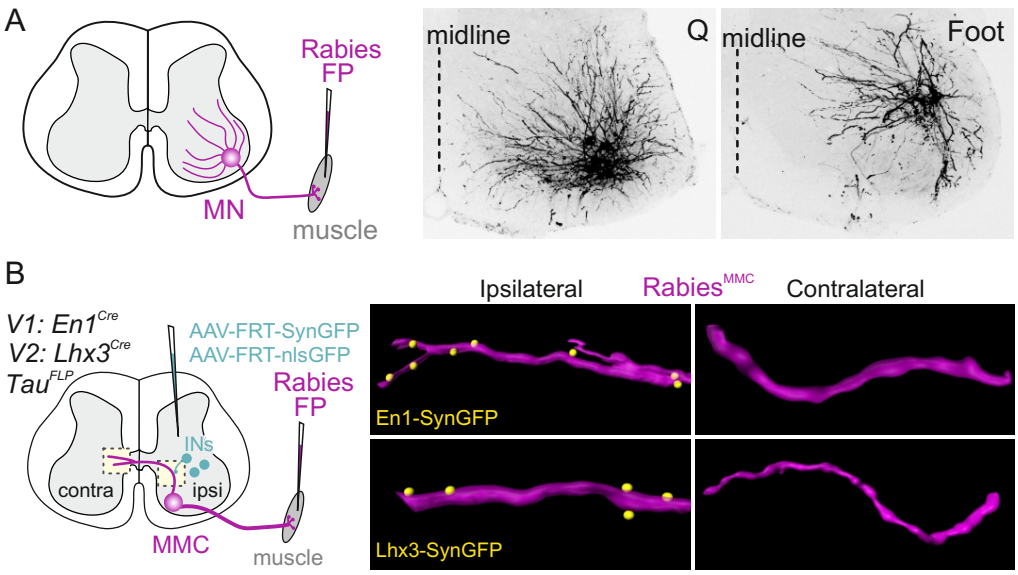


Figure 29. MMC but not LMC motor neurons exhibit midline-crossing dendrites

(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 30B). 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 30C). 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 29B). 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 30C, Figure 29B). 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.

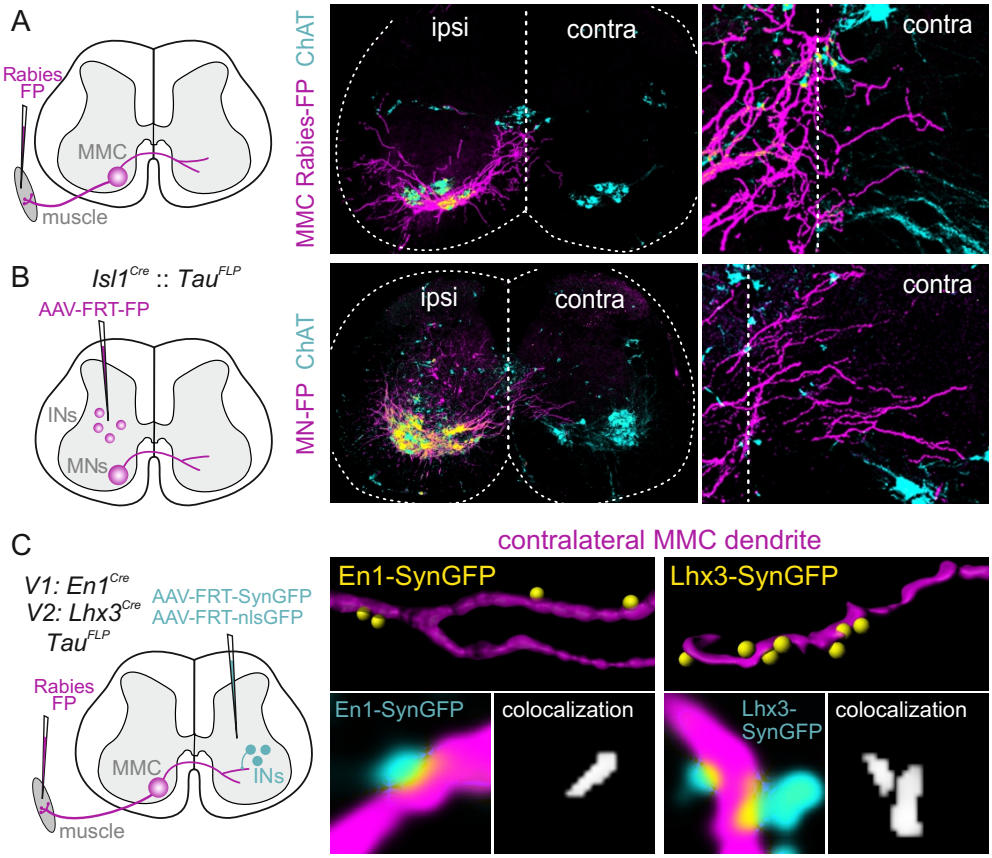


Figure 30. Motor neuron dendrites influence accessibility to contralateral interneurons

(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.

6.4. 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.

6.4.1. 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 31A). 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 31A). 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 31B), 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 31C).

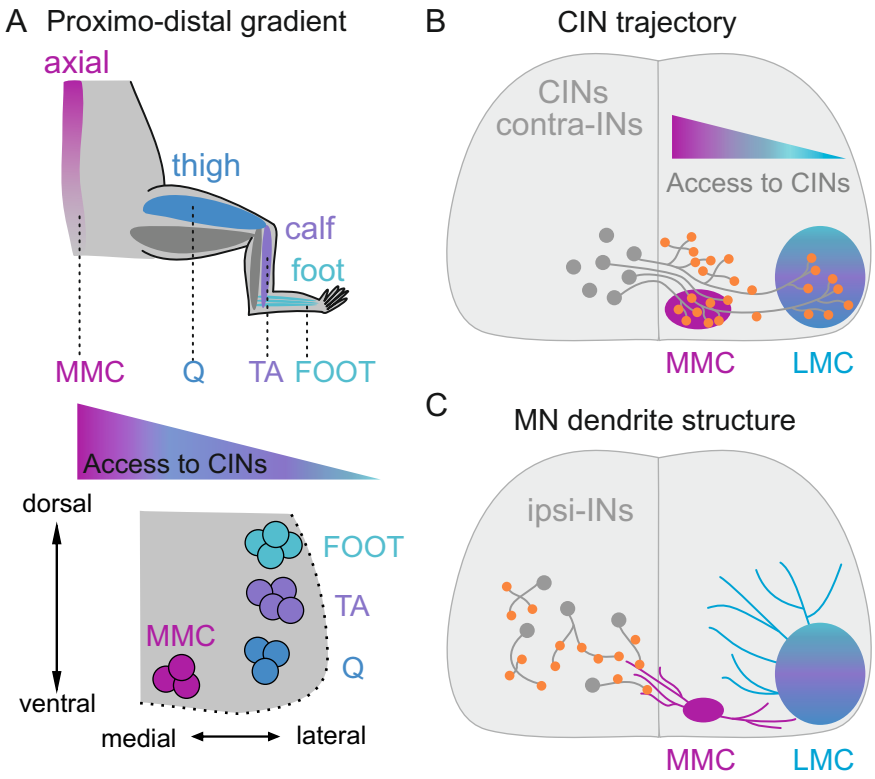


Figure 31. Motor neurons exhibit distinct premotor connectivity profiles

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.

6.4.2. 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.

6.4.3. 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 (Grillner and Jessell, 2009 ; Buchanan, 1982). 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.

6.5. Acknowledgements

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6.6. 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 40um 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.

Chapter 7

7. Final Discussion

Spinal neuronal network regulation is crucial for the generation of accurate motor behavior. During development, intrinsic genetic programs and secreted molecules determine the correct hardwiring of spinal circuits. Previous studies have focused on development of different spinal subpopulations and interneuronal connectivity. Recent advances in motor circuit tracing (Stepien, Tripodi, and Arber 2010) allowed to investigate premotor neuronal networks (Tripodi, Stepien, and Arber 2011). Importantly these studies revealed the specificity of such premotor networks according to the motor neuron pool investigated and start to link circuit organization and development to different executive motor programs. In part of my studies, I contributed to corroborate these findings by means of an anterograde tracing approach through the analysis of premotor network specificity for biomechanically different muscles compared to the previous studied limb muscles. In this work, we revealed a completely new premotor organization following different rules of circuit assembly. However spinal networks do not communicated unidirectionally to motor neurons but indeed also connect to supraspinal centers to update them about spinal neuronal network activity. The main focus of my PhD thesis was to unravel the genetic identity and connectivity profiles of spinal pathways signaling to supraspinal centers opening a new view on spinal subpopulation functionality.

7.1 Input to the Lateral reticular nucleus

Spinal networks receive input from descending pathways and sensory information from the periphery. This information converges on motor neurons that mediate muscle contraction. Different spinal subpopulations have been shown to participate in neuronal networks controlling motor neurons. These diverse populations are not only responsible for unidirectional information transfer but a subset of them report that information to supraspinal centers. The fact they are intermingle with other interneurons is very interesting with respect to previous knowledge. The C3-C4 population was indeed considered as a singular neuronal population, divided only in excitatory and inhibitory neurons, ipsilaterally located and restricted to C3-C4 spinal segments (Alstermark et al. 2011). Our findings demonstrate that different spinal subpopulations encompass double projecting neurons broadly distributed across several segments and are both ipsilaterally and contralaterally located. It is interesting to note that the lateral reticular nucleus neurons do not only receive input from premotor populations, but receive information also from non-premotor neurons that are distributed all along the spinal cord. These interneurons are likely part of polysynaptic pathways to motor neurons since it have been shown that LRN activity is not affected by elimination of sensory feedback information (Arshavsky, Gelfand, Orlovsky, and Pavlova 1978a). Why might the LRN receive information that is coincidently delivered to motor neurons and in addition other information that is not? The most obvious explanation might be that the LRN needs to receive a faithful copy of spinal neuronal network activity that comprises both mono and polysynaptic pathways to motor neurons. Along the the same line or arguments, the input to the

LRN cannot be simply integrated but needs to be organize to maintain the faithful representation of spinal activity. Indeed, we demonstrate that the input is organized according to neuronal subpopulation identity, spinal position (cervical and lumbar) and not by the identity of the targeted motor neuron pool.

Besides the input the LRN receives from the spinal cord in form of efference copy of the ongoing spinal activity, it is worth to mention the strong input from the red nucleus as a potential copy of descending motor commands to the spinal cord. It will be interesting in the future to evaluate the potential overlap and organization of the red nucleus input with the spinal one to the LRN.

7.2 Functional implications of genetically diverse spinal channels to the LRN

Previous studies on spinal neuronal subpopulations addressed their functionality in the locomotor CPGs [reviewed by(Grillner and Jessell 2009; Arber 2012)] mainly through extracellular electrophysiological recordings. Although informative, for a definitive answer on the functional role of defined spinal subpopulations in movement, *in vitro* assays have to be combined with *in vivo* assays. A first attempt towards this direction came from a recent study (Bui et al. 2013) showing that dI3 INs are part of a disynaptic cutaneous motor reflex circuit critical for normal regulation of grasping in response to a specific changes in environment. This study, as the previous ones, regarded spinal subpopulations as affecting local spinal circuits. We have shown a more broad view where potentially each of these subpopulations (with the exclusion of the ones purely descending) have a influence on supraspinal circuits. This will have to be taken into account when manipulating single populations *in vivo* since the behavioral outcome of such manipulations could be at

least partially affected also by the supraspinal impact of these populations. It will be technically challenging to differentiate the pure spinal activity from reporting supraspinal activity of each of these subpopulations in future work.

7.3 Influence of LRN-cerebellar loop on descending motor pathways

Circuit information flows from the lateral reticular nucleus to the cerebellum in form of mossy fibers, excitatory input. After being combined with other mossy fiber inputs, granule cells excite Purkinje cells that in turn have potent inhibitory effects on deep cerebellar neurons. Besides influencing cerebellar cortex activity, mossy fibers exert a parallel simultaneous effect on deep cerebellar nuclei. How the organized input described in my PhD thesis is further transmited to the cerebellum will be an interesting avenue to pursue. For example, different LRN neurons may communicate to specific termination zones in the cerebellum. Moreover, it will be interesting to assess in the future, which mossy fiber inputs are combined at the level of a single granule cell in particular if the input from the LRN is combined with sensory pathway channels signaling forelimb related sensory information, and relayed to the cerebellum by the external cuneate nucleus (ECN) mossy fiber system. A previous study (Huang et al. 2013) has already shown that the pontine nucleus, carrying efference copy information of the motor cortex to the cerebellum is combined at a level of single granule cells with information carried by ECN. It is therefore feasible that similar principles also apply to other mossy fiber sources.

As mentioned in previous chapters of the present thesis, the LRN/ECN pathway has its lumbar counterpart in the VSCT/CC, also projecting as mossy fibers to the cerebellum. It was shown (Jankowska, Krutki, and Hammar 2010) that premotor

lumbar information is relayed to the cerebellum through the VSCT. Although still to be investigated, in case this pathway undergoes similar genetic organization, it will be interesting to evaluate in the future whether the mossy fiber input from the LRN (forelimb) and the one from VSCT (hindlimb) is combined at the level of single granule cell. All this ascending information is likely to also exert an influence on descending pathways through the deep cerebellar nuclei. The deep cerebellar nuclei affect different descending pathways such as the red nucleus, the vestibular nuclei and the reticular formation (Orlovsky et al. 1999). One important question will be to investigate the relative contribution of these ascending pathways on the descending spinal pathways and how this information is modified through information exiting the cerebellar loop. Are all descending pathways equally affected by ascending spinal information or are some descending pathways specifically recruited by ascending information? These questions fall into the more general problem of the role of cerebellar computation in motor behavior. New genetic tools and optogenetic approaches will be necessary to manipulate selectively parts of these circuits and evaluate the specific weight of each component on the overall behavioral outcome.

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