Organization and connectivity of premotor interneurons in the mouse spinal cord.

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Chapter 1

Summary

Movement is the final behavioral 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 interneurons receive a variety of inputs from the brain, cerebellum, and sensory afferents, process this information and as the final outcome, the information reaches the motor neurons that control the activation of the innervated muscles. For generation of movement, precise activation of distinct motor neuron pools at the right moment in time is crucial and this precision is possible due to the cohorts of spinal interneurons, connected with specificity to distinct motor neuron pools that regulate motor neuronal activity. How premotor circuits connect to distinct motor neuron pools with specificity is poorly understood and represented a main question of my PhD thesis work. In my thesis, I will present the results of my studies on connectivity of premotor interneuron populations to specific motor neuron pools in two layers - as general distribution patterns specific to control the regulation of particular muscles and by closer examination of the connection specificity of one class of the spinal pre-motor interneurons, the cholinergic partition cells. One significant part of this project was to develop a tool that allowed studying the pre-motor interneurons innervating defined motor neuron pools. For this purpose, I have adapted a novel rabies virus based tool (Wickersham et al. (2007b)) for mono- transsynaptic tracing of neuronal circuits in the spinal cord in vivo. I was successful in establishing an anatomical rabies-virus based connectivity assay in early postnatal mice in order to study the connectivity scheme of premotor neurons, the neuronal cohorts monosynaptically connected to motor neurons. The main parts of my thesis focus on: 1) motor neuron pools connectivity with premotor interneurons that appear to be widely-distributed when analysed at the segmental level, yet group into stereotypic

populations, and differing for pools innervating functionally-distinct muscles; 2) local or segmental distribution of interneurons depending on their molecular identity; 3) specificity of the connectivity of cholinergic partition cells involved in the regulation of motor neuron excitability - this subpopulation of premotor interneurons segregate into ipsilaterally and bilaterally projecting populations, the latter exhibiting preferential connections to equivalent motor neuron pools bilaterally. A minor part of my thesis is devoted to the connectivity of the spinal pre-motor interneurons in α2-chimaerin mutant mice. Data presented in this part are preliminary and this project needs continuation, but the results begin to provide insight into the function of the α2-chimaerin molecule in the axon guidance and perhaps connectivity process of the bilaterally projecting subclass of partition cells and a dorsal subgroup of premotor interneurons. I demonstrate that the distribution of cholinergic partition cells connected to a particular motor neuron pool is different in α2-chimaerin mutant mice than in the wild-type mice. I also show that the distribution pattern of ectopic bilaterally projecting premotor interneurons in α2-chimaerin mutant mice what concerns the dorsal population of premotor interneurons. These studies of premotor interneurons visualize the widespread but precise nature of connectivity with motor neuron pools, reveal exquisite synaptic specificity for bilaterally projecting cholinergic partition cells and show the importance of the α2-chimaerin molecule in axon guidance and connectivity processes for the establishment of the appropriate premotor circuits in the spinal cord.

Chapter 2

Introduction

One of the most important features of all animals is their ability to move. Some of the movements have to be planned in detail and may need learning experience, others have to be an immediate reaction to an external cue coming from the environment like reflexes pulling our hands away after touching very hot objects. In principle, any kind of behavior depends on muscle activity. But one of the most important motor behaviors for all animals is locomotion. Different sequences of motor behaviors are needed for different purposes: food searching, escaping from dangerous environments or finding a partner and for all of these activities, ability to move through the surrounding environment is a must. Which kind of locomotion needs to be selected is defined by the environment that the animals live in. Animals living on a ground-like terrestrial environment move in a different way than aquatic or flying animals, but all of them need a nervous system that can support their body by a series of well executed decisions to activate the appropriate muscles at the right moment in time. Coordination of muscle activities is extremely important for successful locomotion. From an evolutionary point of view, terrestrial animals evolved from aquatic animals and as a consequence, some of the principal mechanisms of locomotion control are shared or underwent further sophistication.

In any kind of environment, forces required for locomotion are generated by muscles and transmitted by the skeleton to the external environment. What is different in the terrestrial environment from the aquatic? It is the density of the medium the animals live in. Aquatic animals body density is usually similar to the density of water, and as a result the buoyant force counterbalances gravity force, and therefore, there is no need for body support during locomotion. Terrestrial animals on the other hand are exposed to gravity force that needs to be counterbalanced by their muscles and this means that these

animals needed to develop a body support system during evolution. Although there are several possible ways to move in a terrestrial environment, many animals living on the surface of the ground developed limbs. Limbs allow to walk over obstacles, climb trees, jump, dig in the ground. In parallel to the development of limbs, systems controlling their movements had to evolve.

Locomotion of most terrestrial vertebrates depends on the movement of limbs, and muscles can produce only pulling forces. For locomotion, limbed animals need to produce reciprocal movements of the appendages (flexion and extension of a joint) requiring muscles with opposing or antagonistic function. This way, groups of agonistic muscles extend the limbs from the body and antagonistic muscle groups pull the body to the limb. Thus properly coordinated activation of the muscles is necessary to produce locomotion. First observations of locomotion of the terrestrial animals were carried out by picturing animals and humans during different moments of locomotion. Through description of the limb and body position, artists and scientists tried to understand the mechanisms underlying locomotor control. It was noticed that there are two phases of locomotion- a stance phase, which is defined as the time during which a foot is touching the ground and a swing phase when the limb is in the air. Nowadays one can use high-speed cameras and reflectors attached to limbs at the joints to monitor their movements during locomotion, and these methods provide quantitative insight into parameters of movement. At the same time, recordings from muscles (EMG) can provide information about the sequence of muscle activation during particular phases of movements. Nevertheless, despite all this progress, there is no technique available to observe the activity of the motor control circuits in the spinal cord during locomotion and understand how precision in motor control is achieved through the temporally-precise activation of these circuits. Although connectivity of spinal networks has been studied extensively for the past decades, it is still not clear how the nervous system controls locomotion at the circuit level. The general architecture of the motor control system is similar in all vertebrates. Muscles are innervated by motor neurons, the cell bodies of which reside in the ventral spinal cord. Due to intrinsic genetic programs of the motor neurons and molecules secreted by developing limbs, axons of motor neurons target appropriate muscles in the periphery during development. As a result, individual muscles are innervated by a group of motor neurons forming so-called motor neuron pools in the ventral spinal cord, tight and stereotypically positioned clusters of motor neurons. Motor neurons receive input from many different sources: proprioceptive afferents, descending tracts from the brain and brainstem, and spinal interneurons. The main topic of this thesis is the connectivity matrix between premotor interneurons and

specific motor neuron pools. Proper activation of motor neurons depends on the activity of the spinal networks and the precision with which motor circuits are assembled during development. Since spinal interneurons mediate inputs from many different sources to motor neurons and are responsible for specific activation of distinct motor neuron pools it is very interesting to understand how they connect with specificity to distinct motor neuron pools. Studies on the precision of connectivity of pre-motor interneurons are very challenging and so far only two subpopulations of spinal interneurons have been studied extensively from the point of view of specificity of connections with motor neurons. Very recent progress in defining populations of spinal interneurons by molecular markers delivered tools for interneuronal identification, but so far there were no studies on the functional groups of premotor interneuronal populations connected to motor neuron pools innervating particular muscles. In the following chapters, I will present the data on distribution and connectivity of premotor interneurons as cohorts innervating distinct motor neuron pools.

2.1 Assembly of spinal motor circuits

2.1.1 Development

The vertebrate spinal cord develops from the neural tube as a result of an involution of the neural plate after neural induction of the ectoderm germ layer. Multipotent cells in the ventricular zone form progenitor domains characterized by different genetic codes along the dorso-ventral axis of the spinal cord. Progenitor cells undergo proliferation and give rise to many types of neurons. During the process of proliferation, differential gene expression is regulated externally by the influence of factors released by non-neuronal tissues. The epidermal ectoderm and the roof plate influence mainly the differentiation of the dorsal neuronal progenitors while notochord and the ventral plate influence development of the ventral progenitor domains.

The dorsal fate patterning is mediated by bone morphogen proteins (BMPs), which belong to the family of TGF-β proteins (Lee and Jessell (1999)). The patterning of the ventral fates is achieved by an inductive signaling interaction involving sonic hedgehog protein (Shh). Shh and BMPs repress and activate expression of different transcription factors leading controlled regulation of the genetic programs of the progenitor cells and generation of specific neuronal cell types according to a morphogen gradient. Both Shh and BMPs are released from the outside of the neural tube and diffuse forming concen-

tration gradients. Cells in the spinal cord at early developmental stages react in different ways to different concentration values of these proteins and the time point of their exposure to Shh or BMPs as well as the concentration of those proteins is important for the choice of their genetic programs. There are two classes of transcription factors active during early development of the spinal cord. Class I, which consists of Pax6, Pax7, Dbx1, Dbx2 and Irx3 is present in the dorsal spinal cord and this class is repressed by the Shh, class II that is induced by Shh and present in the ventral spinal cord consists of Nkx2.2, Nkx2.9, Nkx 6.2, Nkx6.1, Olig2 and most likely one more yet unidentified transcription factor (Jessell (2000); Shirasaki and Pfaff (2002)). Concentration gradient of Shh protein controls the expression of homeodomaine (HD) patterning genes in the ventral progenitor cells. These transcription factors in turn activate later expression of downstream genes that could act in a similar way or lead to the final specialization of a neuronal type. In the spinal cord, there are 5 ventral progenitor domains (Ericson et al. (1997); Pierani et al. (1999)) and 6 dorsal progenitor domains characterized by the combinatorial expression of different transcription factors giving rise to motor neurons and the different populations of spinal interneurons (see Figure 2.1).

2.1.2 Motor neuron pools - characteristic feature of tetrapodal motor control system

A fundamental feature of the tetrapodal spinal motor circuit formation is the ability to organize motor neurons innervating the same muscle into motor pools. Several studies have shown that motor neurons innervating precisely distinct muscles are clustered in the spinal cord (Romanes (1964) in cat; Landmesser (1978) in chick; Lance-Jones and Landmesser (1981a); Lance-Jones and Landmesser (1981b)) and develop distinct molecular identities that determine their ability to form selective connections with target muscles in the limbs. This is possible due to the motor neuronal intrinsic genetic programs and the control of external factors. All motor neurons develop from one ventral progenitor domain positioned between the p3 and p2 domain. The early generic MN identity is defined by the expression of the LIM homeodomain transcription factor Isl1 and the gene Hb9 (Arber et al. (1999) and Thaler et al. (1999)). Motor neurons undergo different genetic programs and finally segregate into subclasses that can be specified by the expression of Isl1, Isl2, Lim1 and Lhx3 (Tsuchida et al. (1994)) into distinct motor columns. There are 3 main classes of motor neurons. Motor neurons innervating trunk muscles, motor neurons innervating limb muscles and preganglionic neurons innervating sympathetic and

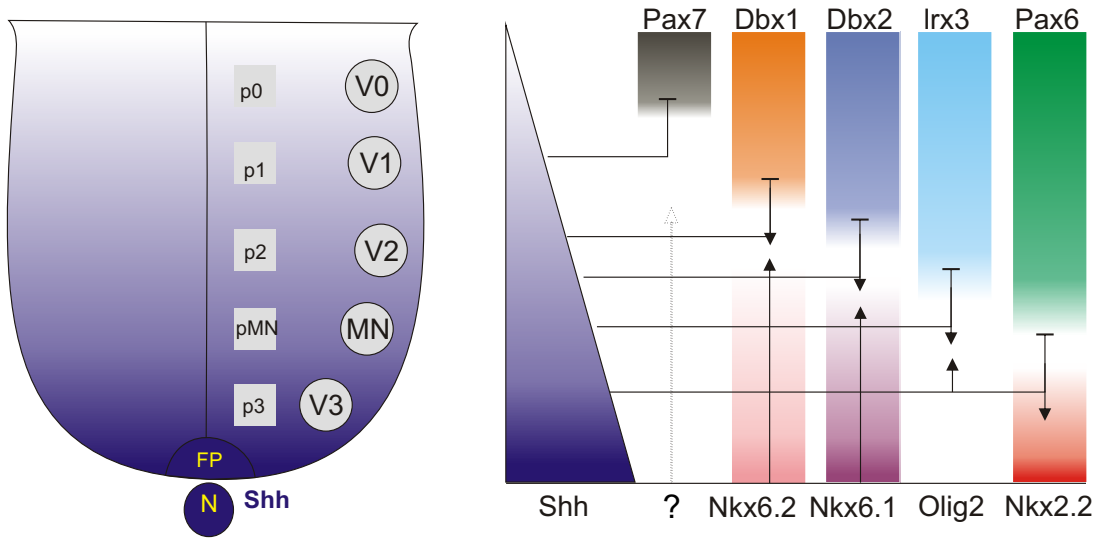


Figure 2.1: **Patterning of the ventral spinal cord.** The identity of the neuronal progenitor cells in the ventral neural tube is specified by a combinatorial code of homeodomain and basic helix-loop-helix transcription factors. A gradient of Sonic Hedgehog (Shh) induces expression of class II and represses expression of class I transcription factors what influences the specification of different progenitors. Individual progenitor domains, termed p3 p0, are further established by crossrepressive interactions between class I and class II transcription factors. Each progenitor domain gives rise to a specific class of postmitotic neurons. Activity of Pax7 is counteracted by so far unknown factor. (Adapted from Jessell (2000)).

parasympathetic ganglia. Preganglionic motor neurons are anatomically separated from the other groups and exist in the thoracic and sacral spinal cord (and parasympathetic also in the brainstem). The other two groups - innervating skeletal muscles, are positioned in the ventral spinal cord in different mediolateral and dorso-ventral positions. Motor neurons innervating trunk muscles are located in an extreme medial position and form the so-called medial motor column (MMC) and subgroups of this column accordingly medial and lateral (MMCm and MMCl) innervate dorsal axial muscles and ventral body wall muscles. The laterally positioned motor neurons in the ventral horn form the lateral motor column (LMC) and its lateral subdivision LMCl -innervates dorsal limb muscles while the medial subdivision LMCm innervates ventral limb muscles. Thus, columnar organization of motor neurons links the cell body position to neuronal function and in this way contributes to the establishment of topographic organization of neuronal maps (see Figure 2.2). Further subdivision of motor neurons to the ones innervating particular muscles is partially influenced by the intrinsic factors in the spinal cord and target-delivered molecules acting retrogradely.

To the spinal cord intrinsic factors critical for appropriate development and settling of motor neurons belongs expression of the LIM family (Tsuchida et al. (1994)) of transcription factors, which in turn regulate motor neuron settling pattern and axonal projection pattern (Pfaff et al. (1996); Sharma et al. (1998); Kania et al. (2000); Kania and Jessel (2003)), the expression of the ETS transcription factors regulating the clustering of MNs into coherent pools (Livet et al. (2002); Price et al. (2002)) and the Hox transcriptional regulatory network that specifies motor neuron pool identity and connectivity by assigning rostro-caudal motor neuron pool position and directing motor neuron pool diversity at a single segmental level. Tsuchida et al. (1994) cloned a family of LIM homeobox genes in chick and demonstrated that combinatorial expression of four of these genes (Islet-1, Islet-2, Lim-1, and Lim-3), contribute to the spatial segregation of motor neurons to diversify motor neuron pools with different topographic organizations of their axonal projections. It has ben also shown that Lim1 controls the development of the dorsal axonal trajectory of LMCl motor neurons (Kania et al. (2000)) probably due to regulation of the distribution of the Eph receptors family (Helmbacher et al. (1998); Eberhart et al. (2002); Kania and Jessel (2003)). Studies done by Kania of the topographic motor neuron projections in the developing limb showed a functional linkage between LIM homeodomain proteins and Ephrin-A:EphA effectors in the control of motor neuronal projections (Kania and Jessel (2003)). In a series of in vivo molecular and genetic manipulation in chick and mouse embryos, they provided evidence that Lim1 and Isl1 repress each other within LMC neurons,

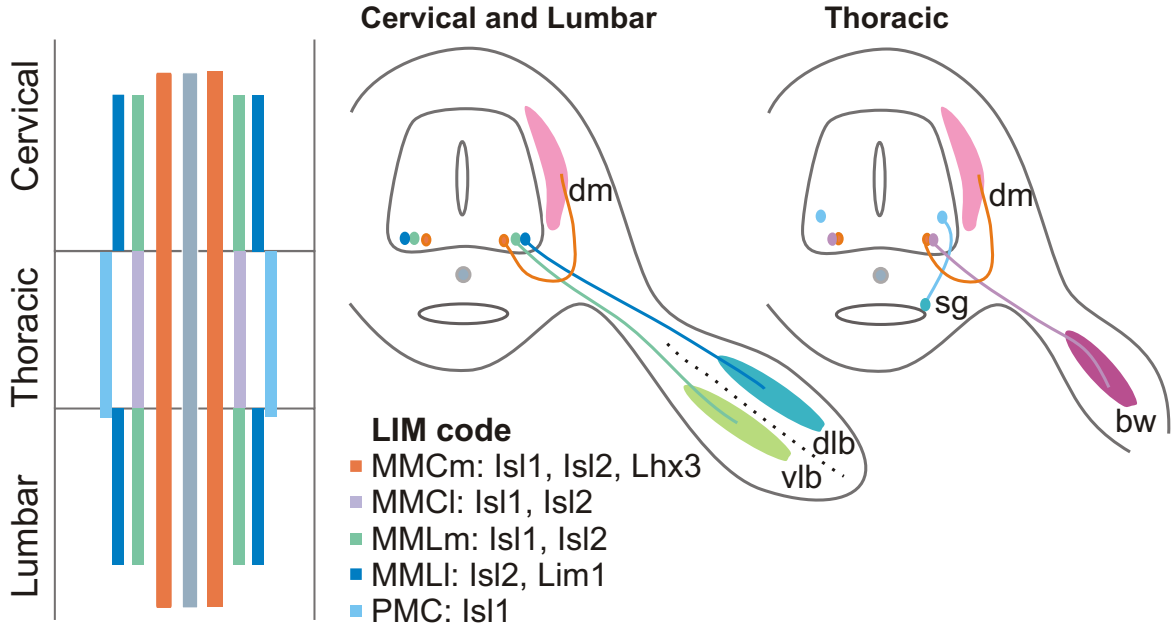


Figure 2.2: **Columnar organization of motor neurons.** Combinatorial LIM-HD transcription factor code defines the columnar organization and target specificity of motor neuron subtypes in the chick spinal cord. MNs in the spinal cord are grouped into MN columns along the anterio - posterior axis of the spinal cord (left). The floor plate at the ventral midline is indicated in grey (left). On the transverse planes for cervical or lumbar and thoracic levels are shown the axonal projection pathways of distinct motor neuron columns (coded in colors) Abbreviations: bw = body wall musculature; dlb = dorsal limb bud; dm = dermomyotome; sg = sympathetic ganglia; vlb = ventral limb bud; LMCl = lateral lateral motor column (blue); LMCm = medial lateral motor column (green); MMCl= lateral medial motor column (yellow); MMCm = medial medial motor column (orange); PMC = preganglionic motor column (purple). (Adapted from Shirasaki and Pfaff (2002))

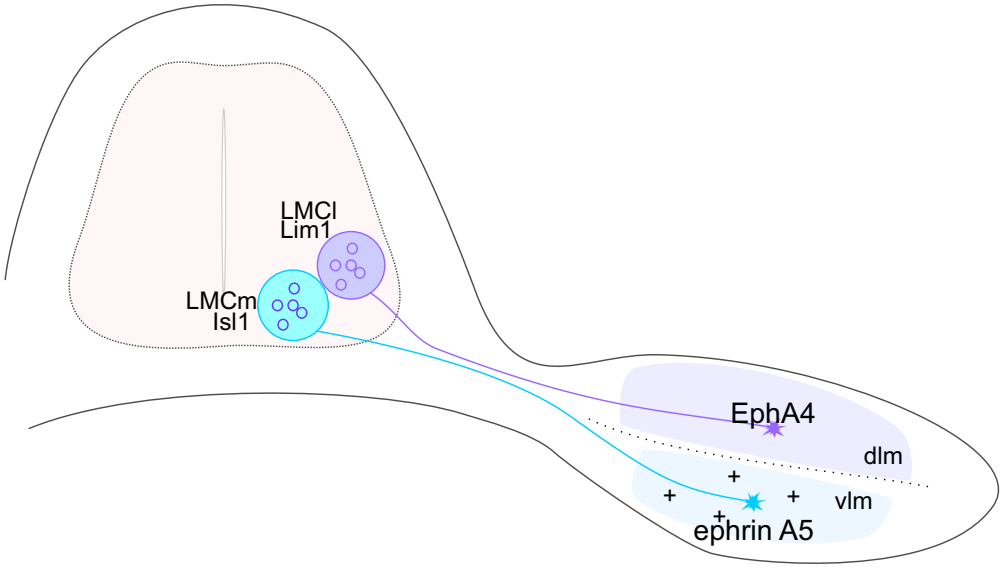


Figure 2.3: **EphrinA5/EphA4 signaling in the periphery.** Innervation of the dorsal and ventral limb bud musculature depends on the expression by outgrowing axons of the EphA4 and the expression by limb muscles of the ephrin A5. High concentration of ephrin A5 in the ventral limb musculature (vlm) repels EphA4 expressing Lim1+ motor axons which innervate the dorsal limb musculature (dlm). Isl1 + axons do not express EphA4 and innervate the vlm.

and as a result, the segregation of the medio-laterally settled motor neurons expressing either Lim1 (lateral) or Isl1 (medial) specifies also the dorso-ventral axonal trajectory of LMC axons within the limb mesenchyme. While Lim1 expression promotes the selection of a dorsal limb pathway by the axons of LMC neurons and high-level EphA4 expression, Isl1 expression results in a ventral bias in the trajectory of LMC axons and low levels of EphA4 expression. Important to mention is the fact that ephrin-A5 was detected to be enriched in the ventral limb mesenchyme. In this system, ephrin-A5 acting as a repellent guides the EphA4 expressing motor neuron axons to the dorsal limb mesenchyme in periphery. LIM homeodomain proteins therefore specify the trajectory of LMC axons in the limb mesenchyme by controlling the pattern of EphA4 receptor expression by LMC motor neurons rendering them sensitive to the distribution of ephrin-A ligands at the dorso-ventral position in the limb mesenchyme (see Figure 2.3).

Also expression of Er81 and Pea3, belonging to the ETS gene family of transcription factors, regulates the allocation of motor neurons to particular motor neuron pools (Lin

et al. (1998); Arber et al. (2000); Sharrocks (2001)). Onset of these transcription factors is regulated by signals from the developing limb bud in the periphery. This was first demonstrated in experiments where developing limb buds were removed from embryos at stages before motor axon invasion to the limb (Lin et al. (1998)). Experiments on spinal cord explants have also shown that lack of limb bud-derived signals prevents motor neurons from expression of the ETS proteins although many different homeodomain transcription factors were expressed intrinsically (Haase et al. (2002)). Livet et al. have further demonstrated that lack of Pea3 protein in cervical motor neurons of mice results in inappropriate clustering and misplaced LMC motor neuron position of this motor neuron pool. In addition, it has also been shown that Pea3 is necessary for expression of molecules like cadherin 8 and semaphorin3e in Pea3 positive motor neurons and exclusion of cadherin 7 (Livet et al. (2002)). Price et al. have demonstrated that in chick embryos distinct motor neuron pools are characterized by expression of different cadherins type II and that the combination of the expressed cadherins was unique for each of the motor neuron pools analyzed (Price et al. (2002)). It was also shown that expression of MN-cadherin regulates the segregation process of distinct motor neuron pools that differ selectively in the expression of this gene in the lumbar spinal cord. In further experiments it was shown that cadherin expression in motor neuron pools is regulated by ETS protein Er81.

2.1.3 Hox genes

It has been shown that Hox transcription factors shape the body plans of animals and determine the morphological and cellular diversity along the rostro-caudal axis (McGinnis and Krumlauf (1992)).
Also in the nervous system, it is now known that Hox genes play a critical role in the neuronal organization and diversification - in the hindbrain and the spinal cord, Hox genes are essential to regulate synaptic specificity of neurons required for respiration and locomotion (Dasen and Jessell (2009); Trainor and Krumlauf (2000)). During early development, Hox expression is controlled by gradients of several molecules: retinoic acid (RA), fibroblast growth factors (FGFs), and Wnts. These factors determine the early spatial profile of Hox transcription in neural progenitors along the rostrocaudal axis (Bel-Vialar et al. (2002); Liu et al. (2001); Nordstrom et al. (2006)). The Hox genes are activated sequentially by signaling gradients and at posterior regions many Hox genes are initially co-expressed in neuronal progenitors (Bel-Vialar et al. (2002); Deschamps

et al. (1999)), only later when cells differentiate they start to display exclusive domains of expression by mechanisms of mutual cross-repression (Dasen et al. (2003)). Hox genes have a dual character in motor neuronal differentiation: Hox genes regulate the emergence of columnar organization of motor neurons and the formation of distinct motor neuron pools. For example, Hox6 and Hox10 proteins initiate the molecular programs that specify the LMC fates at brachial and lumbar levels (Dasen et al. (2003); Shah et al. (2004); Tarchini et al. (2005); Wu et al. (2008)). Within the LMC, more than 20 Hox genes are necessary to generate the needed motor neuron pool subtypes targeting about fifty specific muscles in the limbs, and the combinatorial expression of Hox genes induces the expression of downstream transcription factors such as Runx1, Pea3 or Scip within particular motor neuron pools and together with the motor neuron type specific LIM code separate motor neurons into distinct motor neuron pools (Dasen et al. (2005)). Hox genes are particularly involved in specialization of motor neurons that control motor behavior of muscles of extremities. The mechanisms of how this process happens are not well understood but Jung et al., have shown that already lack of one gene - Hox9 is enough to transform thoracic motor neurons (that do not innervate limb mucles) to an LMC motor neuron fate, normally innervating limbs. This action has been shown to depend on the global repressive activity of Hox9 (Heekyung Jung and Dasen (2010)). Another interesting discovery came from studies of Dasen et al. and Rousso et al. where they have demonstrated that the FoxP1 - a forkhead family cofactor - is responsible for the deployment of the Hox programs in spinal motor neurons and in the absence of FoxP1, Hox controlled molecular programs of LMC motor neurons are lost, transforming these motor neurons into an evolutionarily predecessor type, the HMC motor neuron type. (Dasen et al. (2008); Rousso et al. (2008)). Mice mutant for FoxP1 show phenotypes of properly developed generic motor neurons but these motor neurons project to the muscles in a random pattern and lose genetic subtype identities (Dasen et al. (2008)). These findings suggest that Hox code activity is not enough to generate distinct motor neuron pools meant here as the distinct groups of clustered motor neurons with the same topographic peripheral connectivity. Dasen et al., suggest that the FoxP1 cofactor engages the pre-existing Hox gene programs to selectively activate downstream columnar and motor neuron pool specific programs and that the evolution of this mechanism evolved together with the tetrapodal body construction enabeling the control over many different muscle innervation types and function.

Regulation of the settling of motor neurons in defined rostro-caudal and medio-lateral positions in the spinal cord together with their axonal pathfinding allows the control over

distinct limb muscles. Sequential activity of defined limb muscles is necessary for motor behavior and although the selective innervation of the muscles by distinct motor neuron pools is necessary for it, it is not enough. Another important issue is the connectivity between neurons within the spinal cord and the connectivity between the sensory fibers and spinal neurons. Although the intraspinal circuitry is still not resolved, studies on sensory-motor connectivity have brought some insight into how the information flow from the periphery reaches motor neurons. Below I will focus on the few known aspects of the motor-related intraspinal organization - the connectivity between proprioceptive afferents and the motor neuron pools and circuits of two specific populations of motor control related spinal interneurons.

2.1.4 Ia afferents - motor neuron connectivity

The information about the activity state of the muscles reaches spinal interneurons and motor neurons through the proprioceptive afferents which innervate the sensory end organs embedded within muscles. Proprioceptive afferents are divided into two populations. Group Ia afferents innervate intrafusal muscle fibers which are integral part of so-called muscle spindles and in the spinal cord these afferents terminate in the intermediate zone and on α motor neurons (Eccles and Pritchard (1937); Renshaw (1940); Lloyd (1946)). Group Ib afferents innervate a distal part of each muscle at the transition to tendons, forming so-called Golgi tendon organs. In the spinal cord, group Ib afferents do not terminate on motor neurons, but instead, their termination zone is restricted to the intermediate zone of the spinal cord. Such detailed characteristic of group Ia and Ib afferents comes from early electrophysiological studies on cats. It has been demonstrated that Ia afferents react to the muscle stretch and that their discharge frequency is related to muscle length in a linear fashion (Eldred et al. (1953)). The activation of group Ia afferents in an experimental setup can be obtained by providing mechanistic vibrating stimuli to a muscle (Kuffler et al. (1951); Granit and Henantsch (1956)) because these afferents are very sensitive to changes in muscle length. Group Ib afferents also react to change of muscle length but they have a higher mechanical threshold than group Ia afferents (Matthews (1933); Brown et al. (1967)). Group Ib afferents are considered to have rather regulatory than and emergency function for Golgi tendon organ. An interesting aspect for the topic of this thesis is the specificity with which proprioceptive afferents innervate interneurons and motor neurons in the spinal cord. Ib circuitry is not so well understood as Ia circuitry, but it has been shown that group Ib afferents provide disynaptic autogenetic inhibition

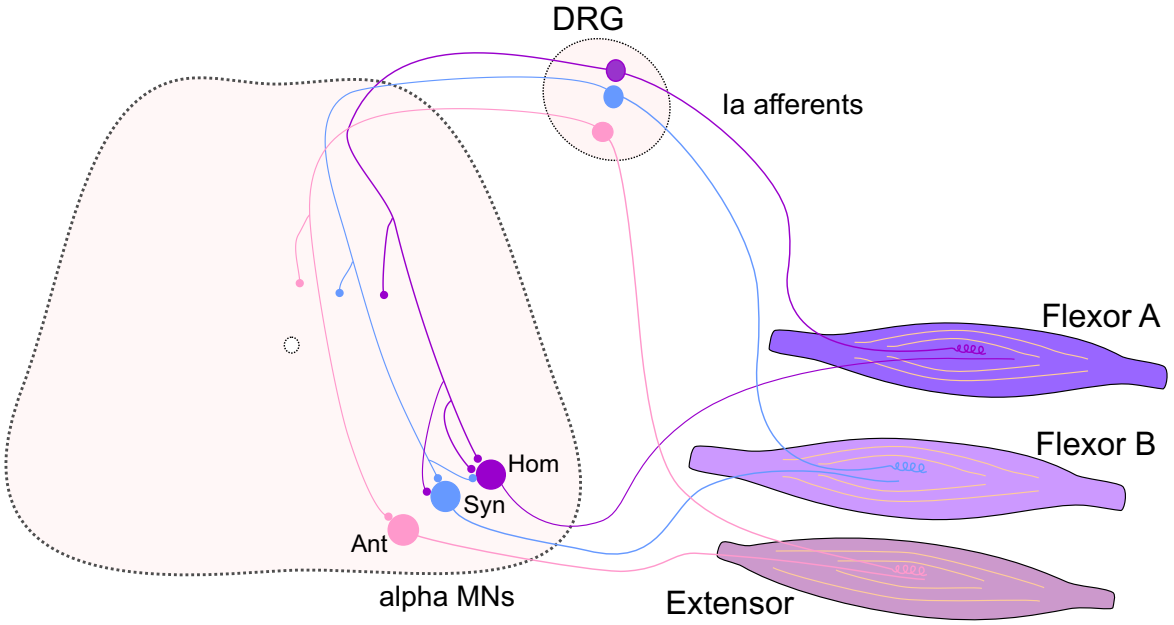


Figure 2.4: **Sensory-motor connectivity in the spinal cord.** Ia proprioceptive afferents innervate peripherally selective muscles, centrally terminating in the intermediate spinal cord and in the ventral spinal cord where they terminate monosynaptically on α-MNs projecting to homonymous and / or synergistic muscles (Hom, Syn in relation to Flexor A) but not antagonistic (ant)

to motor neurons (Granit (1950); Hunt (1952); Laporte and Lloyd (1952); Eccles and Lundberg (1959)). This means that they activate spinal interneurons that in turn inhibit motor neurons innervating the same muscle as the initiating group Ib afferents in case of a strong stretch (see also Figure 2.6). Candidate inhibitory interneurons which could mediate this action probably do not form one population because they are spatially distributed in the spinal cord, but a type of such interneurons has been found to receive also inputs from the cortico-spinal tract, rubro-spinal tract and reticular formation (Hongo et al. (1969); Illert and Tanaka (1976); Andén et al. (1966); Engberg et al. (1968)). Circuitry of group Ia afferents has been studied in more detail and it has been shown that Ia afferents innervating particular muscle, in the spinal cord do not only terminate on the motor neurons innervating the same muscle but also on motor neurons innervating synergistic muscles (Lundberg and Winsbury (1960)), but not the antagonistic (see Figure 2.4). Other studies have shown that group Ia afferents terminate also in the intermediate zone of the spinal cord (lamina VI), lamina XI and on so-called Ia interneurons (IaINs) (lamina VII) (Hultborn et al. (1976); Jankowska and Roberts (1972); Jankowska and Lindstrom (1972)). The connectivity between group Ia afferents and IaINs will be discussed below.

How connectivity between group Ia afferents and motor neurons is controlled is not clear but some aspects of this question have recently been studied. Several discoveries suggest that it is the interaction of a combination of molecules expressed by the innervated motor neurons and group Ia afferents that regulate the ingrowth of the afferents into the motor neuron pools in general and at a more specific level to control fine-grained connectivity.

2.1.5 Factors influencing the sensory-motor connectivity

It has been shown that the ETS transcription factors Er81 and Pea3 are expressed during development by lumbar motor neurons and group Ia proprioceptive afferents (Lin et al. (1998)). The role of these factors in formation of connectivity between group Ia afferents and motor neurons has been studied recently. First, it was shown that in Er81 mutant mice, proprioceptive neurons fail to develop the full innervation pattern in the spinal cord. They terminate in the intermediate zone and avoid the ingrowth into the motor neuron pool area in the ventral spinal cord (Arber et al. (2000)). This phenotype is observed for group Ia afferents in general and is not specific for a particular spinal level. At the same time, motor neuron pools were properly developed and targeted the proper muscles. Changes of group Ia afferent projections were observed at an anatomical level as well as using electrophysiological assays. In particular, the monosynaptic input of group Ia afferents to motor neurons was significantly reduced and the velocity of action potentials is decreased in Er81 mutant mice. Thus, Er81 is necessary for the ingrowth of Ia afferent axons into the ventral spinal cord and its lack prevents the innervation of motor neuron pools by Ia afferents. The role of the Pea3 in proprioceptive innervation of some cervical motor pool has been demonstrated by a recent study that has demonstrated that dendritic pattern and motor neuronal proprioceptive innervation depends on the presence of Pea3 in particular motor neuron pools (Vrieseling and Arber (2006)). It was shown that the dendritic shape of cervical motor neuron pools predicts whether they receive monosynaptic proprioceptive input or not. By reconstructing dendritic trees of cutaneous maximus (CM), latissimus dorsi (LD), Triceps (Tri) and pectoralis major (Pec maj) motor neuron pools and electrophysiological recordings it was shown that motor neurons with radial dendrites (Tri, Pec maj) receive direct input from proprioceptive afferents while motor neurons with dendrites not reaching the central grey matter (CM and LD) receive only di- or poly-synaptic input by Ia afferents. These findings demonstrate that the dendritic shape and connectivity between Ia afferents and CM and Triceps motor neuron pools is

regulated by the induction of the ETS transcription factor Pea3 through peripheral signals. In an elegant set of backfills and electrophysiological recordings it was demonstrated that in Pea3 mutant mice, the position of motor neurons belonging to the CM and Triceps motor pools is swapped, and the morphology of the dendritic tree typical for CM motor neurons is changed to the radial one typical for Triceps motor neurons. In wild type mice, CM motor neurons do not get any direct input from group Ia proprioceptive afferents while Triceps motor neurons receive direct proprioceptive input from Triceps-Ia afferents and polysynaptic input from the CM afferents. In Pea3 mutant mice, CM motor neurons receive direct input from Triceps Ia proprioceptive afferents while Triceps motor neurons received less monosynaptic and more polysynaptic input from Triceps-Ia afferents. A follow-up study investigated the role of semaphorin-plexin interaction (Pecho-Vrieseling et al. (2009)) in synaptic specificity. It was shown that semaphorin3e is expressed by subsets of cervical motor neurons and its high affinity receptor - plexinD1 by proprioceptive afferents and repulsive interaction of these two proteins is the basis for the establishment of the proper proprioceptive innervation of CM motor neurons. Lack of expression of semaphorin3e in motor neurons or plexinD1 in proprioceptive neurons resulted in formation of direct proprioceptive connections to CM motor neurons but did not influence Triceps motor neurons connections and the position of motor neuron pools was also unaltered. Overexpression of semaphorin3e in turn reduced the numbers of monosynaptic inputs of triceps proprioceptive afferents on Triceps motor neurons. Also cell adhesion molecules like cadherins may play a role in the proper targeting of motor neuron pools by group Ia afferents. It was observed that several cadherins are expressed by both motor neurons and Ia proprioceptive neurons and very often, the same combination of different cadherins is expressed by motor neuron pool and group Ia neurons innervating the same muscle peripherally (Price et al. (2002)). However, a possible functional role in controlling sensory-motor connections by this signaling system has not been addressed yet. In conclusion, many different factors are necessary for the establishment of appropriate connectivity between proprioceptive afferents and motor neuron pools. The next intriguing question concerns the specificity of connections between spinal interneurons and motor neuron pools.

2.1.6 Circuitry of Renshaw Cells and Ia Inhibitory Interneurons

Spinal interneurons regulate the activity of the motor neurons in motor neuron pools, both through direct and indirect connections. In turn, interneurons receive inputs from

many areas in the brain and brainstem. They also receive proprioceptive information from muscles and one defined class of interneurons, which has been studied extensively, also receives direct input from motor neurons. Spinal circuitry is very complicated and many investigations from different angles have tried to unravel principles of connectivity and function. Although in the last decades, many studies on spinal interneurons focused on the molecular identification of particular classes based on their genetic ontogeny and connectivity, many of the most informative and functional studies on spinal interneuronal connectivity were performed already before molecular techniques were available.

Electrophysiological properties of mature spinal interneurons have been studied for many years in cats. These studies were initiated by Renshaw and Lloyd (Lloyd (1951); Renshaw (1941)), and presently despite of development of molecular techniques that enable the labeling of different interneuron classes and their synaptic terminals, connectivity of many types of spinal interneurons apart from a few defined well-studied types is still an unresolved and fascinating issue. The next chapters will focus on the studies of motor-related circuitry of two spinal interneuronal classes. Strikingly, so far not much information was accumulated about specific connectivity of other spinal motor-related interneurons, even though their molecular identification and division into subclasses based on molecular markers progresses very fast. Two classes of spinal interneurons were particularly well studied in classical electrophysiological preparations of the spinal cord: Renshaw Cells (RCs) and Ia inhibitory interneurons - IaINs (Eccles et al. (1956); Hultborn et al. (1971); Hultborn and Udo (1972)). Later studies on these two classes provided also their molecular characteristics. Both of these spinal interneuronal classes provide direct inhibition to motor neurons - RCs mediate recurrent inhibition and IaINs mediate reciprocal inhibition. Due to their complete nature, these studies will be outlined in more detail below.

2.1.6.1 Renshaw Cells

In 1941, Birdsey Renshaw initiated studies on recurrent inhibition of motor neurons. In his work Influence of discharge of motor neurons upon excitation of neighboring motoneurons, he described an inhibitory effect on motor neuron activity after antidromic activation of motor neurons of the same or close motor neuron pool. In his experiments, the dorsal roots were cut. This intervention would prevent that the observed inhibitory effect could be a result of the activation of the sensory fibers. In his studies, Renshaw proposed that the inhibitory effect can occur through axonal collaterals of motor neurons

and proposed a model (Figure 2.5) in which an interneuron excited by motor neuron collaterals provides recurrent inhibition to another motor neuron. Such axonal collaterals were indeed observed by neuroanatomists Koelliker,1891;Lenhossek,1893;Cajal,1909 on Golgi staining preparations and were described as terminating in the ventral horn. In his studies, Renshaw was not certain whether the inhibitory effect is mediated by interneurons or directly by motor neuron collaterals but further studies confirmed his hypothesis concerning the interneurons being the mediator of the observed effects. In 1954, Eccles provided evidence that motor neuron collaterals contain acetylcholine and that strychnine depresses the inhibitory effect after antidromic activation of motor neurons. Strychnine was already known to be a blocker of the inhibitory transmission. Therefore the evidence that the inhibitory effects on motor neurons after the antidromic activation are mediated through inhibitory interneurons was provided. This interneuron population was subsequently studied extensively for many years and received its name - Renshaw Cells in honor of the first observations by Renshaw (Eccles (1964)). These findings, based largely on studies of the cat spinal cord, were confirmed also for rodents. Nowadays, much more is known about the role of RCs not only in the adult cat spinal cord but also in postnatal spinal cords or rodents, birds and human (Mazzocchio and Rossi (2010)). The present view is that the recurrent inhibition mediated by RCs influences motor neuron recruitment/de-recruitment and modulates the activity in synergist and antagonist motor neuron pools. This way, it can influence production of synchronous motor output (Hultborn et al. (1979); Windhorst (1990); Jankowska (1992); Maltenfort et al. (1998); Mattei et al. (2003)). Manipulations on RCs and investigation of their role in the generation of locomotor pattern have been difficult due to a lack of a tool that would allow their specific manipulation or deletion. Recordings from single RCs where a dye was deposited after recordings (Jankowska and Lindstrom (1972)) allowed to define the RCs area and study the morphology and contacts of the RCs. Other groups focused on finding molecular markers for RCs. Immunolabelling on filled RCs has shown their glycinergic and GABA-ergic character (Fyffe (1991); Schneider and Fyffe (1992); Cullheim and Kellerth (1981)). In addition (Alvarez and Fyffe (2007)) have demonstrated that RCs are positive for gephyrin, a postsynaptic density protein at inhibitory synapses. It is very characteristic that the postsynaptic densities positive for gephyrin are uncommonly large in RCs. As a molecular marker, the calcium binding protein Calbindin was demonstrated to be expressed by RCs (Carr et al. (1998); Geiman et al. (2000)). Although Calbindin is also expressed by other spinal interneurons, together with the morphology criteria and the position in the spinal cord in the Renshaw area it provides an entry point for unambiguous identification of

RCs on spinal cord sections. Development of molecular and genetic techniques allowed studying RC connectivity in detail also at early developmental stages. It is known today that RCs are encompassed within the V1 embryonic interneurons expressing engrailed-1 at early developmental stages (Sapir et al. (2004); Alvarez et al. (2005)). Sapir et al. observed lack of RCs and no recurrent inhibition of motor neurons in mutant mice lacking Pax6, a homeodomain-protein encoding gene essential for V1 interneurons genesis. Lack of engrailed-1 on the other hand does not result in lack of RCs (Saueressig et al. (1999)) but in wrong axonal pathfinding of V1 interneurons and less synaptic terminals of RCs on motor neurons (Sapir et al. (2004)), but the specificity of these connections had not been studied to date. A summary of current knowledge about the circuitry of RCs is presented in 2.5. RCs receive strong excitatory input from restricted groups of MNs and in turn inhibit the homonymous and synergistic motor neurons (reviewed in Alvarez and Fyffe (2007)). RCs also synapse directly on IaINs, ventral spinocerebellar neurons and other RCs. In early development, RCs also receive input from group Ia afferent proprioceptive neurons, but they seem to lose this input with maturation of the system (Mentis et al. (2006)). RCs display a striking proximo-distal segregation of inhibitory versus excitatory inputs - excitatory synapses identified by vGlut1, vGlut2 and vAChT preferentially target dendrites of RCs (Alvarez et al. (1999); Mentis et al. (2006)), while inhibitory synapses from other interneurons cover the cell body and proximal dendrites. The significance of this input segregation is currently not known. There is no specific mouse mutant lacking RCs or in which these cells are silenced but it seems to be just a question of time as the molecular identification of particular spinal interneurons progresses on a fast trail. However, so far RCs are the only spinal interneurons easy to identify with molecular techniques and for which circuitry in relation to motor neurons is well described and understood.

2.1.6.2 Ia Interneurons

The second class of interneurons which motor neuron related circuitry is known consists of inhibitory interneurons called Ia inhibitory interneurons (IaINs). It was noticed in experiments on cat spinal cords that stimulation of group Ia afferents can cause inhibition of motor neurons (Lloyd, 1941). Previously, it was considered to be direct inhibition but further studies suggested presence of inhibitory interneurons between group Ia afferents and motor neurons (Eccles et al. (1956); Eide et al. (1961); Eccles et al. (1961); Eccles (1964)). Eccles et al. found such interneurons - monosynaptically activated by group Ia afferents, located in the intermediate region of the spinal cord and providing inhibition to motor

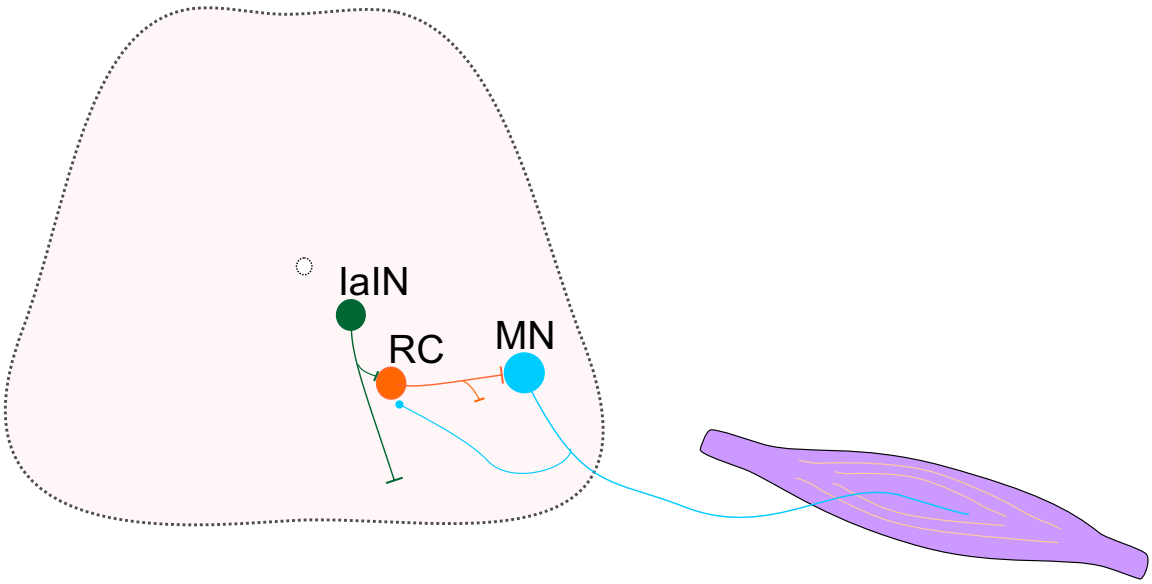


Figure 2.5: **Renshaw Cell circuitry.** RCs (orange) are located in the ventral spinal cord in the proximity of MNs (blue). They provide recurrent inhibition to MNs after beeing excited by them. RCs receive also inhibitory input from IaINs (green).

neurons. Hultborn et al. (1968) performed studies on such interneurons in cats where they stimulated a number of peripheral nerves from ipsilateral and contralateral hindlimbs. Using different stimulation parameters, they stimulated group Ia afferents or antidromically activated motor neurons, at the same time recording from single interneurons and motor neurons. They found inhibitory interneurons in the ventral horn, dorso-medially to motor neurons, that were monosynaptically excited by group Ia afferents from one muscle or from synergistic muscles. At the same time, these neurons were poly(di-)synaptically inhibited after antidromical motor neuron activation and sometimes inhibited by antagonistic group Ia afferents. Such electrophysiological measures were used as a definition for IaIN populations for a long time. More detailed studies on the connectivity of IaINs revealed that they specifically mediate inhibition between antagonistic motor neuron pools (Eccles (1964); Jankowska and Roberts (1972); Jankowska and Lindstrom (1972); Rastad et al. (1990)) and receive strong inhibitory input from RCs (Hultborn et al. (1971); Wang et al. (2008)). The current knowledge about IaINs circuitry is presented in Figure 2.6.

The developmental origin of IaINs remains unclear but some of them derive from the V1 progenitor domain as do RCs (Alvarez et al. (2005)). A molecular marker or

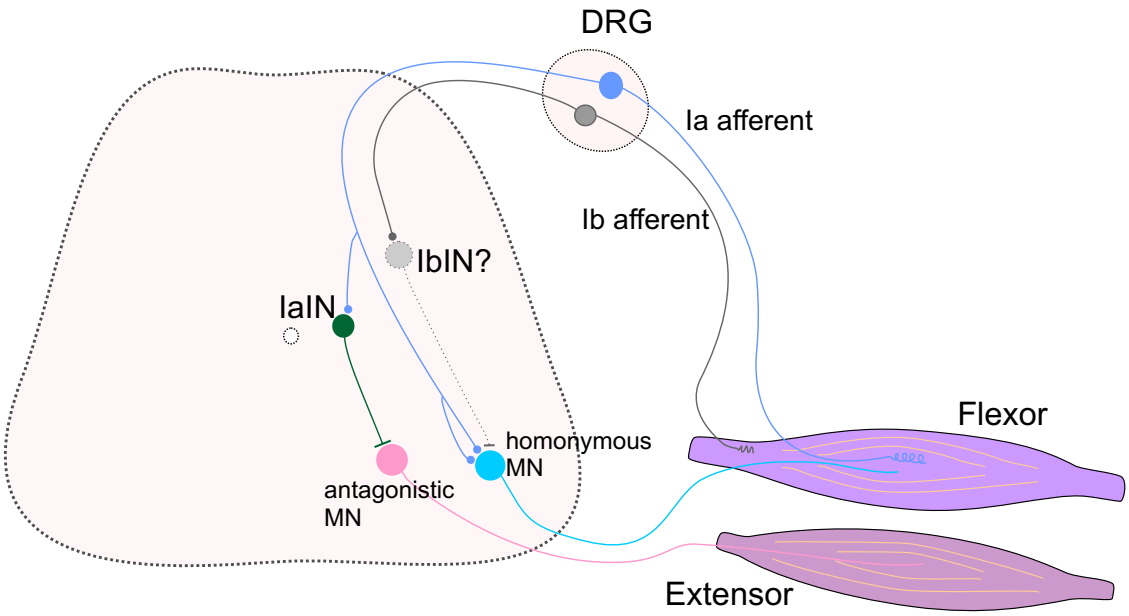


Figure 2.6: **Circuitry of Ia and Ib inhibitory interneurons.** Ia proprioceptive afferents innervate homonymous motor neurons in the spinal cord (blue, in relation to Flexor) and IaINs projecting to the antagonistic motor neurons in the spinal cord (pink, in relation to Flexor) providing disynaptic reciprocal inhibition. Similar circuit may exist for the Ib proprioceptive afferents (grey) that do not terminate on the motor neurons but in the intermediate zone and on inhibitory interneurons providing autogenetic inhibition to homonymous motor neurons (blue, in relation to Flexor)

a combination of markers exclusive for IaINs has not been found yet and the criterion of their position in the spinal cord is also not as well defined as for RCs. Therefore, to unambiguously identify a Ia interneuron, electrophysiological measurements are currently still necessary. Nevertheless, some researchers try to simplify the Ia interneuron definition and define them as ventral engrailed-1 positive interneurons, positive for the calcium binding protein parvalbumin and receiving input from RCs in form of calbindin-positive boutons (Siembab et al. (2010)). The primary molecular definition of the IaINs comes from the study of Alvarez et al., in 2005. They have crossed an engrailed-1-Cre mouse strain with conditional loxP reporter mice and shown that some of the engrailed-1 positive interneurons receive calbindin positive synaptic terminals (as expected from RCs) and glutamatergic (vGlut1) input from group Ia afferents. As no other interneurons have been found to receive RCs input it was concluded that IaINs are enclosed within the V1 neuron populations. This way a definition given to these cells as interneurons mediating reciprocal inhibition between antagonistic motor neuron pools transforms slightly through molecular criteria that does not necessarily have to be the same for all of the IaINs and such redefinitions can also be dangerous in the age of molecular definitions of neuronal populations. Later studies (Wang et al. (2008)) have indeed shown that in Pax6 mutant mice - that lack the entire V1 derived interneuron population - reciprocal inhibition of motor neurons is still present. These results suggest that either Pax6 is not required for the phenotypic development of IaINs or that not all IaINs are enclosed within the V1 population.

Classical experimental paradigms used to study spinal circuitry were typically carried out in anesthetized cats and were based on stimulation of particular nerves, sensory afferents or single motor neurons and recordings from particular motor neuron pools, nerves or interneurons. This classical electrophysiological approach used to study the connectivity issue in adult cats is difficult to apply to mice or chick that are suitable for developmental and molecular studies. As a consequence, there is currently still a gap in spinal cord research that could be filled by a technique combining the study of specificity of connections in the spinal cord and molecular or developmental identification of spinal interneuron subpopulations. It is especially interesting to understand how different interneurons connect to particular motor neuron pools, which of those exhibit and follow rules of specificity, and which other ones may be connected in less specific ways. The examples of RC and IaINs studies summarized above show that understanding the specificity underlying connections of particular interneurons with defined motor neuron pools is crucial for our understanding of motor control. Yet, these examples also demonstrate that very little is known so

far about the organization of connectivity of interneuronal classes that establish direct connections to motor neurons.

In the next part I will summarize the knowledge about other spinal interneurons that are involved in the processes of locomotor control. The first part of the following text is derived from a preview written for the journal of Neuron to accompany the publication of two articles in the same issue (Crone et al. (2008) and Zhang et al. (2008)). In the second part, there will be an update of studies on spinal interneurons appearing after the year 2008.

Chapter 3

Probing the Locomotor Conundrum: Descending the V Interneuron Ladder

Anna Stepien and Silvia Arber

(Neuron 60, October 9, 2008)

The assembly of neuronal circuits involved in locomotor control in the mammalian spinal cord is influenced by genetic programs specifying four ventral (V) interneuron populations (V0-V3). In this issue of Neuron, Crone et al. and Zhang et al. make use of genetic tools to map connectivity patterns and to abolish the function of V2a and V3 interneurons. The absence of V2a interneurons reveals defects in left-right alternation during locomotion, whereas ablation of either V2a or V3 interneurons leads to disturbances in the precision and reliability of the motor output.

Walking and similar rhythmic locomotor behaviors are among the best-studied repetitive animal behaviors. The seemingly simple question of how the coordinated contraction and relaxation of muscles is guided by the activation of different motor neuron subpopulations in the spinal cord through precise input from upstream neuronal networks has been a challenge for many years. Connectivity of locomotor circuits is rather well understood in lower organisms, such as lamprey (Grillner (2003)). In contrast, solving the puzzle of functionality and connectivity of the more complex mammalian locomotor circuits is a more challenging enterprise. Indeed, despite major progress on the physiological understanding of the mammalian central pattern generator (CPG) network over many years (Barbeau et al. (1999)), pairing of this information with the developmental origin of defined neuronal populations has only become possible in recent years. The discovery of important organizational principles in the generation of implicated interneuron classes and the use of sophisticated mouse genetics have helped to pave the way (Briscoe et al. (2000); Goulding and Pfaff (2005); Jessell (2000); Kiehn (2006)).

In the ventral spinal cord, four cardinal classes of interneurons (V0, V1, V2, and V3) can be distinguished on the basis of their developmental origin and combinatorial transcription factor expression (Briscoe et al. (2000); Jessell (2000)). Each of these four classes can be further subdivided into several functionally and genetically distinct subclasses of interneurons (Al-Mosawie et al. (2007); Lanuza et al. (2004); Lundfald et al. (2007)). To understand the contribution of the V0-V3 interneurons to locomotion, an important entry point has been to remove each one of them from spinal circuits either by selective genetic cell ablation technologies or by decreasing excitability and blocking output through genetic means. Whereas previous work has addressed the contribution of the dorsally located V0 and V1 interneurons to locomotion (Gosgnach et al. (2006); Lanuza et al. (2004)), two papers in this issue of Neuron descend the V ladder to assess the role of the more ventral V2a and V3 interneurons in spinal locomotor activity in mice (Crone et al. (2008); Zhang et al. (2008)).

To determine the contribution of individual interneuron subclasses to locomotion, let us

start by asking which parameters are important to assess in these studies. The physiological output is assayed by measuring the rhythmic motor bursting from ventral roots at different segments (Figure3.1 A). So-called fictive locomotion can be induced in neonatal spinal cord preparations in vitro by the application of 5-HT and NMDA to mimic descending input, or alternatively by electrophysiological stimulation of dorsal root ganglion sensory afferents or descending tracts (Kiehn (2006); Kudo et al. (1987)). These treatments result in bursting episodes interspaced by silent periods at individual ventral roots, representing the net output activity of motor neurons at the respective segmental level. Motor bursting episodes in the wild-type are highly reproducible and hence exhibit constant burst duration, interburst periods, and burst amplitudes (Figure 3.1 A). In addition, as would be expected from the mouse walking behavior with alternating left-right movement of extremities, recording from left and right roots at the same spinal level shows left-to-right alternation of motor bursts (Figure 3.1 A). Moreover, phase shifts of motor bursts can also be detected in recordings simultaneously assessing motor burst patterns from lumbar ventral roots L2 and L5, and this asynchrony is thought to reflect activity driving flexion and extension of extremities (Figure 3.1 A).

The fictive locomotion assay can therefore determine the contribution of identified interneuron populations to (1) general rhythmic bursting parameters, (2) neuronal networks involved in left-right alternation, and (3) neuronal networks steering extensor-flexor alternation. To interpret information gained from recording motor burst patterns, it is equally important to understand anatomy and connectivity of interneurons. Which neurons do the studied interneurons connect to? Do they act through excitation or inhibition? And finally, from where do they get their input? Resolving these issues relies heavily on mouse genetics to identify interneuronal projections and connections in conjunction with tools to determine their respective neurotransmitters. Both papers in this issue of Neuron provide a composite physiological and anatomical analysis of the contribution of two distinct excitatory interneuronal classes (V2a and V3) to the puzzle f the mammalian spinal locomotor network (Crone et al., 2008; Zhang et al., 2008).

Crone et al. address the role of V2a interneurons in locomotion (Crone et al. (2008)). The V2 interneuronal class is derived from $Lhx3^+$ progenitor cells (Jessell (2000)) and splits into a glutamatergic $Chx10^+$ V2a and an inhibitory $GATA2/3^+$ V2b class, both of which exhibit mainly ipsilateral projection patterns (Al-Mosawie et al. (2007); Lundfald et al. (2007)) (Figure 3.1 B). Using an inducible diphtheria toxin A (DTA)-based genetic cell ablation system from the Chx10 locus (Chx10-DTA), the authors generate mice in which V2a spinal interneurons are eliminated selectively without affecting the generation

and maintenance of other interneuron classes (Crone et al., 2008). What are the functional consequences of V2a interneuron elimination? Surprisingly, analysis of the general motor burst parameters such as the mean locomotor cycle period and normalized burst amplitude did not differ between Chx10-DTA and wild-type preparations. However, the analysis of individual motor bursts revealed an increased variability in individual burst amplitudes and cycle periods. In addition, the authors analyzed the sequences of ipsilateral flexor (L2) -extensor (L5) motor bursts and left-right motor bursts at L2 ventral roots. They found that Chx10- DTA mice exhibit disrupted left-right alternations but maintained normal flexor-extensor activity. Together, these findings suggest that V2a interneurons contribute to the stabilization and precision of locomotor patterns but are not involved in the generation of intrinsic rhythmicity. In addition, V2a interneurons tie into the circuits required for the functional coupling of left-right alternation of motor bursts. These findings raise the question of how V2a interneurons interact with previously studied neuronal populations and whether V2a ablation may indirectly affect the differentiation of those neurons. Since Chx10-DTA mice exhibit defects in left- right alternation, commissural inhibitory interneurons (CINs) are a key neuronal population to analyze. Previous work has demonstrated that Dbx1$^+$ V0 interneurons project mainly contralaterally (Pierani et al. (2001)), and their genetic elimination or general blockade of inhibitory neurotransmission in wild-type mice lead to defects in left-right alternation (Lanuza et al. (2004)). Using three independent genetic ways to label the projections of V2a interneurons in combination with retrograde tracing of commissural interneurons, Crone et al. demonstrate that V2a neurons contact V0-derived CINs directly. In addition, the authors provide anatomical as well as electrophysiological evidence that differentiation of CINs does not seem to be affected in Chx10-DTA mice. Together, these findings lead the authors to propose a model in which the main intersection of V2a neurons with circuits involved in the regulation of left-right alternation occurs by direct connections between V2as and CINs. A final important question in understanding the workings of V2a interneurons is how they are activated by upstream inputs. Stimulation of either brainstem or dorsal root ganglia sensory afferents reliably induces locomotor-like activity in wild-type spinal cord preparations. However, both of these neurally evoked stimuli elicit only asynchronous and uncoordinated activity in Chx10-DTA mice, while application of NMDA and 5-HT initiated normal motor bursting (Crone et al. (2008)). These findings suggest that V2a interneurons mediate neurally evoked activation of locomotion, and in their absence, normal initiation of locomotor patterns fails to occur. Early postnatal death of Chx10-DTA mice unfortunately precluded behavioral studies.

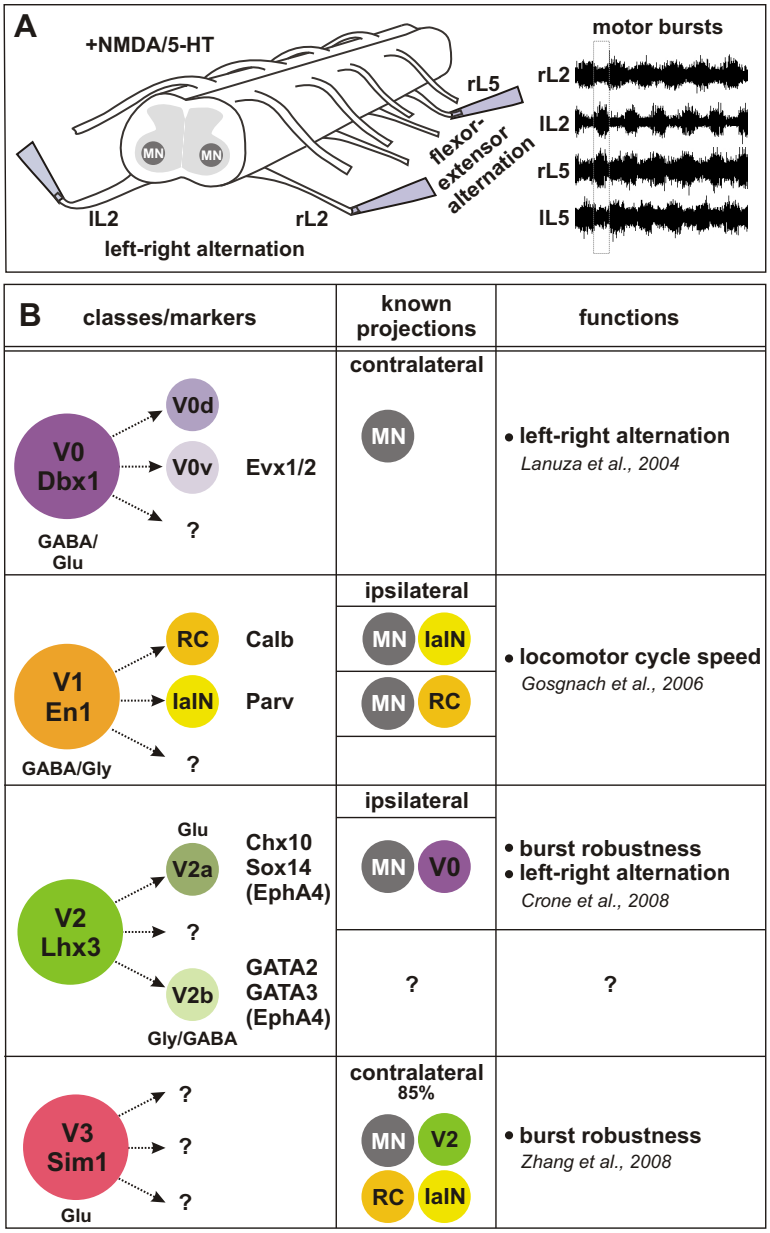


Figure 3.1: **Interneuron Classes in the Ventral and Spinal Locomotor Network.** (A) (Left) Schematic illustration of a neonatal lumbar spinal cord preparation used to assay left-right and flexor-extensor motor burst patterns. Suction electrodes for recordings are placed at left L2 (lL2), right L2 (rL2), left L5 (lL5), and right L5 (rL5). Motor neurons (MN) in the ventral spinal cord are indicated in gray. (Right) Example of recorded traces at the four indicated ventral roots to show the alternation of motor bursts (picture courtesy of Ole Kiehn). (B) Table illustrating the expression of known markers, neurotransmitters, projections, and functions of the four cardinal ventral interneuron classes (V0, V1, V2, V3). Where known, fractionation of these classes in subpopulations is also shown. Note that depicted projections are limited to currently assessed partners, not excluding additional connections. MN, motor neurons; RC, Renshaw cells; IaIN, Ia inhibitory interneurons; GABA, GABAergic; Gly, glycinergic; Glu, glutamatergic; Calb, calbindin; Parv, parvalbumin.

Zhang et al. investigate the role of the glutamatergic Sim1$^+$ V3 interneuron population (Zhang et al., 2008), which is derived from the most ventral Nkx2.2$^+$ p3 progenitor cell domain (Jessell, 2000). Generating a Sim1- Cre mouse strain, the authors first determine the projection pattern of V3 interneurons by crossing it to a reporter mouse strain expressing membrane-linked eGFP. These studies show that85 % of V3 interneurons are commissural and only a minority projects ipsilaterally (Figure 3.1 B). V3 interneurons contact a broad array of different neurons in the ventral spinal cord. These include (1) motor neurons, (2) the two premotor inhibitory interneuron typesRenshaw cells and Ia-inhibitory interneurons, both part of the V1 interneuron cohortand (3) Lhx3-derived V2 interneurons (Figure 3.1 B). Transsynaptic retrograde tracing experiments using pseudorabies virus injections into several limb muscles consolidate the findings for V3 connections to motor neurons and show that 80 % of V3 interneurons labeled shortly after viral infection of motor neurons project contralaterally. From these anatomical studies, it appears that V3 interneurons as a whole population are wide-tuned with respect to their target specificity, although it remains to be seen whether an individual V3 interneuron indeed makes synaptic connections with all possible partners or whether this picture arises only in a whole population analysis. To remove V3 interneurons from ventral spinal cord locomotor circuits, Zhang et al. use two related approaches to attenuate synaptic transmission of Sim1$^+$ neurons. First, by crossing Sim1-Cre mice to a mouse strain conditionally expressing tetanus light chain (TeNT) from a ubiquitous promoter, the authors permanently block synaptic release from Sim1$^+$ neurons. Second, employment of a previously developed allostatin receptor based system (Gosgnach et al. (2006)) reduces activity of Sim1$^+$ V3 interneurons in the adult animal acutely and allows monitoring of motor behavior. Both approaches of inactivating V3 interneurons resulted in disruption of locomotor activity. In vitro analysis of Sim1::TeNT spinal cords revealed defects in the regularity of motor burst activity when compared to wild-type mice. In particular, individual bursts showed high variability in duration and overall step cycle period. This decrease in locomotor robustness was also underscored by the fact that sensory afferent stimulation or lower doses of 5-HT/NMDA often failed to induce locomotor activity in Sim1::TeNT mice. Analysis of left-right alternation of motor bursts however did not reveal any major defects in Sim1::TeNT spinal cords. Do these defects in robustness also manifest themselves in adult animals and upon acute V3 blockade? Adult animals expressing allostatin receptor in V3 interneurons showed irregularity in walking behavior when allostatin was applied to the spinal cord in vivo. These experiments demonstrate that inactivation of V3 interneurons or a subpopulation thereof disrupts reliable rhythmicity of walking behavior in vivo. Taken together,

while V3 interneurons only play a minor role in setting the left-right alternation pattern, their major role is in supporting the precision and regularity of the overall motor bursting pattern. Collectively, the two papers in this issue of Neuron address the role of distinct interneuron populations in shaping locomotor output of the spinal cord. Whereas V2a interneurons show predominantly ipsilateral projections, V3 interneurons project mainly contralaterally. Nevertheless, striking similarities in several aspects of the phenotype arise upon elimination of V2a or V3 neurons from the network. Elimination of either of them results in greater variability of individual motor bursts induced by 5-HT/NMDA, and naturally evoked activation by sensory afferent stimulation fails to induce robust motor bursting. It appears that, in the absence of V2a or V3 neurons, the striking precision normally observed in motor output is lost. It is currently unclear, however, how to explain this phenotype at the circuit level. Since V2a and V3 neurons both provide excitatory drive to the locomotor network, albeit through different routes, it is feasible to speculate that a general reduction in overall excitatory drive activates motor neurons through the remaining circuitry only unreliably and through variable pathways, ultimately resulting in a less robust motor bursting pattern. Intriguingly, even studies on stable network performance of rhythmic motor bursting behavior in the much simpler stomatogastric nervous system of lobsters or crabs revealed that individual neurons can switch between different functional circuits (Marder and Bucher (2007)). Similar principles are likely to apply also to mammalian locomotor circuitry, especially given the high degree of observed interconnectivity. Disturbing the network by unplugging an entire excitatory interneuron class may therefore interfere with stable network performance and be revealed by unreliable motor bursting. How do these new results tie in with previous studies on the role of other molecularly defined interneuronal classes? Defects in the robustness of motor burst output patterns have not previously been described in other interneuron-ablated mutants. In contrast, circuits controlling left-right alternations have been approached from several different angles, and V2a neuron-ablated mice also show defects in left-right alternation. Dbx1 mutant analysis has shown that V0 interneurons contribute to left-right alternation (Lanuza et al. (2004)) and as described above, V2a interneurons most likely channel their contribution to the left-right program toward V0 interneurons. The most dramatic left-right coordination phenotype has been observed in EphA4 mutant mice (Kullander et al. (2003b)), essentially mimicking the phenotype observed in complete absence of inhibition. Some EphA4$^+$ interneurons are glutamatergic, and a fraction of these interneurons aberrantly projects contralaterally in EphA4 mutant mice, most likely resulting in the loss of left-right asynchrony (Kullander et al. (2003b)). Nevertheless, a recent study demon-

strates that while EphA4 is also expressed by V2a interneurons, no aberrant crossing of V2a interneurons can be observed in EphA4 mutant mice (Lundfald et al. (2007)). To complete the quartet in functional analysis, V1 interneurons are required to set the speed of locomotor bursts but do not appear to contribute to the locomotor pattern otherwise (Gosgnach et al. (2006)) (Figure 3.1 B). The new papers also highlight a series of interesting questions which remain to be addressed in the future. None of the ventral interneuron ablation experiments so far has revealed a contribution to the control of flexor-extensor alternations, a prominent signature of the rhythmic motor bursting, raising the question of how these patterns are generated. Moreover, an emerging principle from several recent papers including the two highlighted in this preview is that V0-V3 interneuron classes fractionate into finer subcategories. Understanding motor circuits will require a profound know-how of the connections between functionally unique neuronal classes and how these circuits channel toward individual motor neuron pools to steer the contraction of a particular muscle. Analysis of connectivity of more functionally uniform neuronal populations than the cardinal V classes should hopefully provide deeper insight into the connectivity map of motor circuits in the spinal cord. Let the exciting puzzle of assembling locomotor circuits for motor behaviors continue.

3.1 Latest progress in spinal interneuronal identification

There is still a great interest to understand the role of different classes of spinal interneurons in the motor pattern generation. Several recent studies brought more insight to the spinal circuitry understanding and the roles played by distinct interneuronal classes. Studies of the V2a interneurons activity in vivo in adult V2a mutants have shown that left-right coordination is only altered when mice lacking V2a interneurons step quickly or trot, which suggests that the V2a interneurons are primarily active at higher stepping speeds (Crone et al. (2009); Dougherty and Kiehn (2010); Kiehn et al. (2010)). Zhong et al. have demonstrated that recruitment of V2a interneurons depends on the speed of locomotor cycle and the faster the cycle is the more V2a interneurons are recruited by other pattern regulating interneurons. However there is a group of V2a interneurons that are not recruited during locomotor activity at all (Zhong et al. (2011)). Another class of the V2 interneurons has been added to the classification by Panayi et al. In their genetic fate mapping and loss-of-function experiments they have demonstrated that transcription

factor Sox1 is expressed and required for a third type of V2 interneuron classes - V2c (Panayi et al. (2010)). In the absence of Sox1, V2c interneurons switch to the V2b fate. In addition, the same study has shown that late-born V2a and V2b interneurons are heterogeneous, and subsets of these cells express the transcription factor Pax6. What is more, some of the neurons generated from the p2 domaine primarily express Pax6 but do not express Chx10, Gata3 or Sox1 what suggest existence of some more subtypes of V2 interneurons. Fine aspects of motor control by spinal interneurons have been revealed by the analysis of the V0c function in adult mice. In their studies, Zagoraiou et al. specifically knock-out choline acetyl transferase (ChAT) in Pitx2^{+} V0c cells what prevented synaptic transmission from this particular neuronal population. EMG recordings from gastrocnemius and tibialis anterior muscles, respectively ankle extensor and flexor, during walking and swimming tasks revealed that in the absence of V0c interneuron activity, the task-dependent modulation of the gastrocnemius muscle was impaired (Zagoraiou et al. (2009)). Also recently it was demonstrated that Netrin-1 mutant spinal cords display a switch from alternating left-right to synchronous left-right locomotor activity (Rabe et al. (2009)). In the same study it was noticed that the axons of all dorsal classes known to consist of commissural interneurons (dI1 dI3, dI5, dI6) and V0d were affected by the lack of netrin-1 and did not cross the midline while glutamatergic V3 commissural neurons were still able to cross the ventral midline when netrin-1 was absent. This demonstrated that V3 commissural interneurons are not enough to rescue the alternating locomotor pattern. What proves the hypothesis from (Zhang et al. (2008)) that the contribution of V3 commissural axons to left-right stepping is minor.

Chapter 4

Tracing of the premotor circuits in the spinal cord

A fundamental question regarding the organization of circuitry involved in motor control is how the different interneuron cohorts connect to particular motor neuron pools. Such connectivity questions are not easy to resolve for the spinal cord circuitry using traditional retrograde tracers. There are few commonly used retrograde neuronal tracers (HRP, CTB, Dextrans) which in principle work similarly - after being injected into a region of interest in the nervous system, they are taken up by axonal terminals of neurons that project to the injection site and render these neurons visible. Such tracing studies are not very specific at a single cell and connectivity level and depending on the excellence and accuracy of the injection technique more or less reliable. It is even more difficult to apply it to the spinal cord. To find the spinal interneurons connected to a particular motor neuron pool, retrograde dyes would have to be injected into the one particular motor neuron pool and this is technically very difficult. In addition, most inputs to motor neurons are made with dendrites, further complicating the problem. Howewer, the anatomy of the motor control system allows a very precise tracing of motor neurons projecting to individual muscles. A retrograde tracer injected into a muscle is taken up only by motor neurons innervating this particular muscle. But to trace the interneurons connecting to this particular motor neuron pool, one needs a marker that can cross a synapse and in the perfect case not dilute. Instead of using substances that are actively transported from the axon to the cell body, we decided to adapt virus based tools developed by I. Wickersham in the Callaway lab (Wickersham et al. (2007a)). The idea to use neurotrophic viruses for neuronal circuit tracing studies is not new. For the purpose of specific circuit tracing

mainly two neurotrophic viruses have been used - the pseudorabies virus and rabies virus. Modifications in their genome allowed to produce strains carrying marker genes like green fluorescent protein (GFP) or a version of red fluorescent protein - mCherry (Card and Enquist (2001); Wickersham et al. (2007a)). Injecting such virus into a nucleus in the brain or a muscle, it is possible to visualize the neuronal circuits through several synaptic steps in a retrograde manner. The limitation of this technique is the lack of control over the transsynaptic spread. The most challenging aspect of the neuronal circuitry tracing is to obtain a tool that would allow a controlled transsynaptic labeling of the given neuronal circuit. Progress in studies of the rabies virus features (Ugolini (1995)) and rabies virus genetic manipulation mainly from the labs of Conzelmann and Callaway led to the development of several tools allowing above. The lab of Conzelmann published in Cell (Mebatsion et al. (1996)) a version of the rabies virus with a deletion of the envelope glycoprotein gene (G) - deltaG rabies. They have shown that the G protein is necessary for the viral invasion of the cells and lack of this protein prevents the virus from entering the cell in the culture conditions. This virus was grown in complementing cells and as the result, the G protein was incorporated into the viral membrane. It could infect the contacting neurons, express its remaining genes and replicate, but the newly replicated viruses could not spread due to the lack of the glycoprotein. Similar studies in in vivo conditions (Etessami et al. (2000)) have proved the ability of the deltaG rabies to infect neurons but no spread of the rabies was observed. This version of the rabies virus was next modified again and the GFP gene was introduced into the viral genome in the place of lacking G protein gene (Wickersham et al. (2007a)). This way, infected neurons could be visualized by GFP expression but again, this virus was not able to spread through synaptic contacts.

In the same study (Wickersham et al. (2007a)), a system was presented that controls the monosynaptic - transsynaptic spread of rabies virus from defined cells on cortical slices in culture. The rabies virus, in which the gene encoding G protein was substituted by the enhanced GFP gene, with incorporated glycoprotein into its membrane was additionally pseudotyped with the glycoprotein from the avian sarcoma and leukosis virus termed EnvA. The EnvA can restrict the virus infection to cells that express the viral receptor - TVA. This receptor is expressed only in birds and never in mammals in nature (Barnard et al. (2006); Bates et al. (1993); Federspiel et al. (1994); Young et al. (1993)). Genetically modified neurons by electroporation of the TVA, dsRED (a red marker) and rabies glycoprotein were primarily infected by the pseudotyped rabies virus and at the same time, they were a source of rabies spread to their presynaptic partners. Addition-

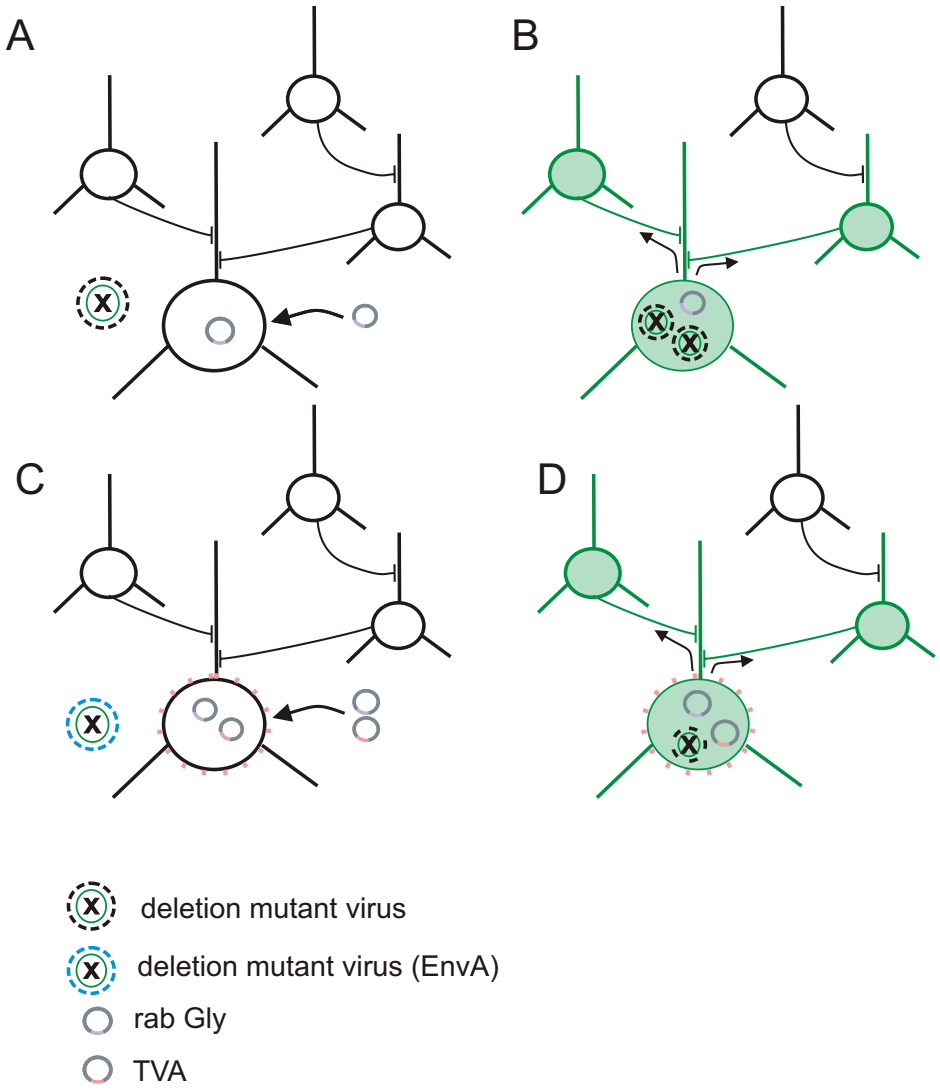


Figure 4.1: **Restricted spread of the rabies virus.** (A) A deletion mutant rabies virus carrying a GFP gene but missing glycoprotein G gene required for transsynaptic spread infects a neuron. Missing glycoprotein G gene is delivered by electroporation to the neuron allowing the rabies virus for spread to the presynaptic partners of the primary infected neuron (B) Since the missing viral genes are not present in the presynaptic neurons the virus cannot spread further and all infected neurons express GFP - (B). (C) Selective infection. EnvA pseudotyped rabies virus cant infect mammalian neurons unless they express the receptor for EnvA - the TVA. Electroporation of the TVA gene into cell of interest enables viral infection. At the same time electroporation of glycoprotein G gene is necessary. From cells containing both genes (TVA and glycoprotein G) monosynaptically restricted rabies spread is possible (D). Adapted from Wickersham et al. (2007a).

ally coupled recordings from primarily infected cells and transsynaptically infected ones confirmed their monosynaptic connections. A feature that makes this tool very useful is that all the infected neurons express GFP which makes it possible to study even fine details of neuronal morphology (Wickersham et al. (2007b)). This way, Wickersham and colleagues delivered a tool for controlled neural circuit tracing. The principles of this system are summarized on the Figure 4.1. This system however is restricted by the choice of the primary neurons and the electroporation efficiency. In in vivo studies of the nervous system this strategy is hard to apply due to the technical problems in electroporation of specified cells. Modifications of this technique are possible and several labs decided to use herpes or adeno-associated viruses to complement the G-protein or advanced mouse genetic manipulation to adapt this tool for studies of different parts of the nervous system (Yonehara et al. (2010); Haubensak et al. (2010); Miyamichi et al. (2010)). The advantage of the motor circuit organization is the possibility to infect particular motor neuron pools from the periphery in a very specific and reliable manner and additional selectivity is not necessary. In our studies, by injecting single muscles with rabies virus, we were able to infect specific motor neurons. To deliver the glycoprotein gene to the same motor neurons we chose to use another virus - the adeno-associated virus (AAV) that was modified to carry the glycoprotein gene (G). The AAV is able to infect neuronal axons and incorporates its genes to the genome of the cell but it does not replicate nor spread, thus the G-protein gene can be expressed only in the primarily infected cells after intramuscular injection, but not by interneurons. The principles of our monosynaptic restricted transsynaptic rabies virus spread are presented in the Figure 4.2.

In the following chapter, our study on motor related circuit tracing using rabies virus will be presented.

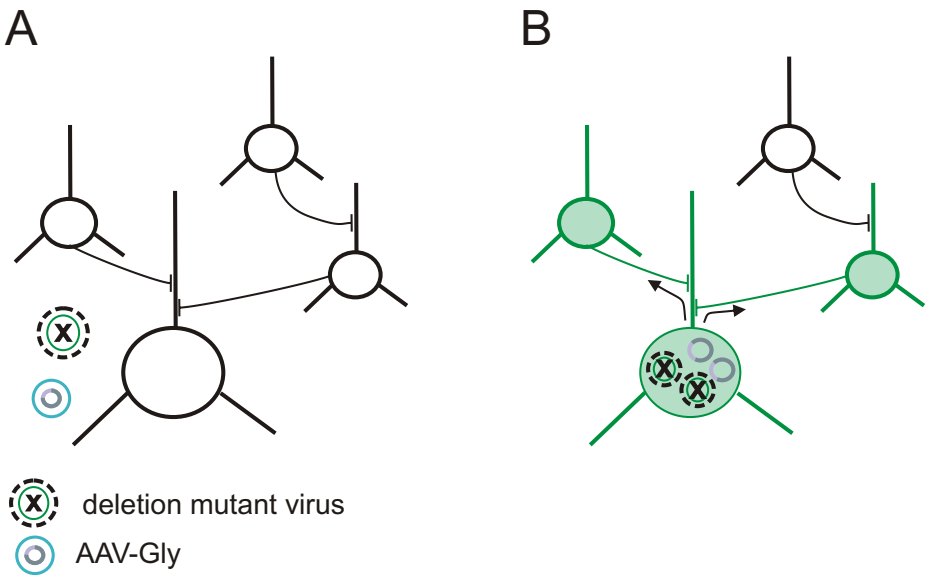


Figure 4.2: **In vivo monosynaptically restricted rabies virus tracing of the motor control system.** A deletion mutant rabies virus carrying a GFP gene but missing glycoprotein G gene required for transsynaptic spread infects a motor neuron. Missing glycoprotein G gene is delivered by coinfection of the same motor neuron by AAV-Gly (A). Monosynaptically restricted rabies virus spread is possible from double infected motor neurons (B) and no specificity in targeting is needed due to their anatomical isolation

Chapter 5

Monosynaptic rabies virus reveals premotor network organization and synaptic specificity of cholinergic partition cells.

Anna Stepien, Marco Tripodi and Silvia Arber

(Neuron 68, November, 2010)

5.1 Summary

Movement is the behavioral output of neuronal activity in the spinal cord. Motor neurons are grouped into motor neuron pools, the functional units innervating individual muscles. Here we establish an anatomical rabies-virus based connectivity assay in early postnatal mice. We employ it to study the connectivity scheme of premotor neurons, the neuronal cohorts monosynaptically connected to motor neurons, unveiling three aspects of organization. First, motor neuron pools are connected to segmentally widely-distributed yet stereotypic interneuron populations, differing for pools innervating functionally-distinct muscles. Second, depending on subpopulation identity, interneurons take on local or segmentally-distributed positions. Third, cholinergic partition cells involved in the regulation of motor neuron excitability segregate into ipsilaterally and bilaterally projecting populations, the latter exhibiting preferential connections to functionally equivalent motor neuron pools bilaterally. Our study visualizes the widespread yet precise nature of the connectivity matrix for premotor interneurons and reveals exquisite synaptic specificity for bilaterally projecting cholinergic partition cells

5.2 Introduction

The activity of a neuronal network is governed by synaptic connections between neurons encompassing the circuit. The firing of an individual neuron within a neural circuit is dictated by the timed activity of its presynaptic neuronal population. Much progress has been made investigating the rules and properties of connections between identified neuronal pairs using electrophysiological and anatomical approaches (Klausberger and Somogyi (2008); Sakmann (2006)), advancing our understanding of how specific synaptic connections contribute to circuit activity and function. However, the enormous diversity in distinct presynaptic neuronal subtypes as well as the fact that cell body location of interconnected neurons is often at a far distance make the comprehension of the overall connectivity scheme of large cohorts of neurons a challenging task.

In the motor system, muscle contractions are controlled by the timed activation of motor neurons in the spinal cord. Coherent locomotor sequences are achieved by temporally appropriate recruitment of motor neurons through the activity of presynaptic neurons. In vertebrates, individual muscles are innervated by motor neuron pools, the cell bodies of which are clustered in stereotypic positions in the ventral horn of the spinal

cord (McHanwell and Biscoe (1981); Romanes (1964)). The motor neuron pool is therefore a critical functional unit in the organization of the motor output system. Since its activity is controlled by the cohort of premotor neurons, the last-order neurons establishing direct synaptic connections, understanding the connectivity rules of defined premotor interneurons to functionally distinct motor neuron pools is essential to understand the organization of the motor output system.
Many different spinal interneuron populations with direct connections to motor neurons have been described, using electrophysiological, anatomical and genetic approaches. Electrophysiological studies have categorized premotor interneurons on the basis of excitatory or inhibitory neurotransmitter identity, ipsi- versus contralateral cell body position, as well as presynaptic input from sensory, descending or other sources, leading to models of connectivity diagrams encompassing the core circuits driving motor output (Grillner (2006); Jankowska (1992); Jankowska et al. (2003); Kiehn (2006); McCrea and Rybak (2008); Windhorst (1990); Windhorst (2007)). Two prime examples of interneuron populations, for which a high degree of synaptic specificity has been observed, are the Renshaw cells establishing an inhibitory feedback loop to motor neurons (Alvarez and Fyffe (2007); Nishimaru et al. (2006); Renshaw (1941); Windhorst (1990)) and group Ia inhibitory interneurons relaying sensory reflex inputs to motor neurons (Hultborn et al. (1971); Jankowska (1992); Wang et al. (2008); Windhorst (2007)). In recent years, these studies were complemented by a systematic analysis of distinct spinal interneuron classes derived from four transcriptionally-defined progenitor domains in the ventral spinal cord (Jessell (2000)). These studies have begun to assign functional roles to interneuron classes in locomotor output circuitry based on developmental ontogeny (Goulding (2009); Jessell (2000); Kiehn (2006); Stepien and Arber (2008)). Nevertheless, and in part due to the extensive three-dimensional nature of the connectivity matrix, overall distribution patterns and connection specificity of premotor neurons to defined motor neuron pools are known for remarkably few of these either electrophysiologically or genetically defined premotor populations, prompting us to develop a connectivity-based anatomical assay to begin to address these questions.

The use of a variety of retrogradely and anterogradely spreading transsynaptic viruses expressing fluorescent marker proteins or other transneuronally transported tracers has permitted anatomical visualization of connectivity within neuronal circuits (Ekstrand et al. (2008); Jankowska and Skoog (1986); Ugolini (2010)). Since these viruses cross multiple synapses, unambiguous identification of monosynaptically connected presynaptic

neurons has remained difficult. To overcome this problem, a rabies-virus based transsynaptic system with exclusive retrograde direction has been developed recently allowing the visualization of monosynaptically connected presynaptic neurons in the cortex (Marshel et al. (2010); Wickersham et al. (2007b)). The system makes use of a modified rabies virus with a genomic substitution in the gene encoding the envelope glycoprotein (G-protein) essential for transsynaptic spreading by a fluorescent marker protein. G-protein deletion does not interfere with virus production in primarily infected neurons, but subsequent infection of presynaptic neurons can only be achieved by complementation with G-protein expression (Marshel et al. (2010); Wickersham et al. (2007b)), thus introducing selectivity to the system to visualize solely neurons presynaptic to the neuronal population co-expressing G-protein. One main goal of this study was to adapt this transsynaptic rabies virus technology to its application in the motor system, by targeting primary infection and thus the source of retrograde spread to the motor neuron pool as a defined functional unit.

In this study, we develop a method to visualize premotor interneuron populations connected to motor neuron pools using retrograde double-virus infection from defined target muscles in early postnatal mice. We employ this method (1) to determine and probe the reproducibility of three-dimensional premotor interneuron distributions, (2) to visualize the local or distributed nature of defined premotor interneuron subpopulations, and (3) to determine distribution and connectivity rules of cholinergic premotor partition cells, known to regulate motor neuron excitability through C-bouton synapses with motor neurons (Conradi and Skoglund (1969); Hellstrom et al. (2003); Miles et al. (2007); Zagoraiou et al. (2009)). We find that virally labeled premotor spinal interneurons exhibit highly reproducible and segmentally widespread distribution patterns, but these patterns can be distinct for premotor interneurons connecting to motor neuron pools with different functions. Within the cohort of premotor interneurons, defined populations (Renshaw cells, Lhx3- or Isl1-derived interneurons) display cell-type specific distribution patterns. Analysis of connectivity between cholinergic partition cells and motor neurons reveals the existence of ipsi- and bilaterally projecting populations, both with widespread rostrocaudal segmental origin and the latter with preferential connectivity to equivalent motor neuron pools bilaterally. Our study establishes the use of monosynaptically restricted rabies viruses in the motor system, determines the connectivity matrix in the motor output system at high resolution and makes use of this method to reveal rules of synaptic specificity of one defined premotor interneuron population.

5.3 Results

5.3.1 G-protein expression in motor neurons allows rabies virus spread to spinal interneurons

To determine whether glycoprotein-deficient rabies virus expressing mCherry (Rab-mCherry) can infect spinal motor neurons retrogradely, we targeted viral injections to identified limb muscles of mice and analyzed mCherry expression in the spinal cord several days after muscle injections (Figure 5.0 A). We focused our initial analysis on injections of the Quadriceps (Q) muscle, a dorsal thigh muscle of the hindlimb. 2-3 days after injection, we observed retrogradely labeled motor neurons at lumbar (L) levels L3-L4 (Figure 5.0 B, C) in the spinal cord, in agreement with the described Q motor neuron pool position (**??**). Expression of mCherry remained confined to $ChAT^{on}$ motor neurons and no interneurons were detected upon Q intramuscular Rab-mCherry injections (n=6), but motoneuronal dendritic morphology was readily detected (Figure 5.0 C). We found that spinal motor neurons persisted for at least 12 days after muscle injections, but thereafter started to degenerate, a time course similar to previous observations in cortical slice experiments (Wickersham et al. (2007b)). We only observed successful retrograde infection of motor neurons by intramuscular rabies injection from postnatal day (p) p0 to approximately p10, after which the efficiency of infection declined dramatically (data not shown). We therefore carried out further injections at p7, a stage 10 days after cessation of neuronal birth in the spinal cord and at which many local functional connections to motor neurons are established (Goulding (2009); Kiehn (2006); Ladle et al. (2007); Mears and Frank (1997); Nishimaru et al. (2006); Wang et al. (2008)).

We next developed a strategy permitting us to restrict expression of glycoprotein (G-protein) to motor neurons infected by Rab-mCherry, but avoiding G-protein expression in spinal interneurons. To confine G-protein expression to motor neurons innervating muscles injected by Rab-mCherry, we made use of the observation that the replication-incompetent adeno-associated virus (AAV) serotype 6 efficiently delivers transgenes to motor neurons through retrograde infection (Towne et al. (2010)). Using the same approach, we produced an AAV expressing G-protein (AAV-G-protein), co-injected it with Rab-mCherry virus into the same muscle (Figure 5.0 D) and analyzed mCherry expression in the spinal cord 8 days after co-injection of the two viruses. We found that in addition to $ChAT^{on}$ Q motor neurons, many interneurons were now fluorescently labeled, also several spinal segments away from the source motor neuron pool (Figure 5.0 E-G; n=6).

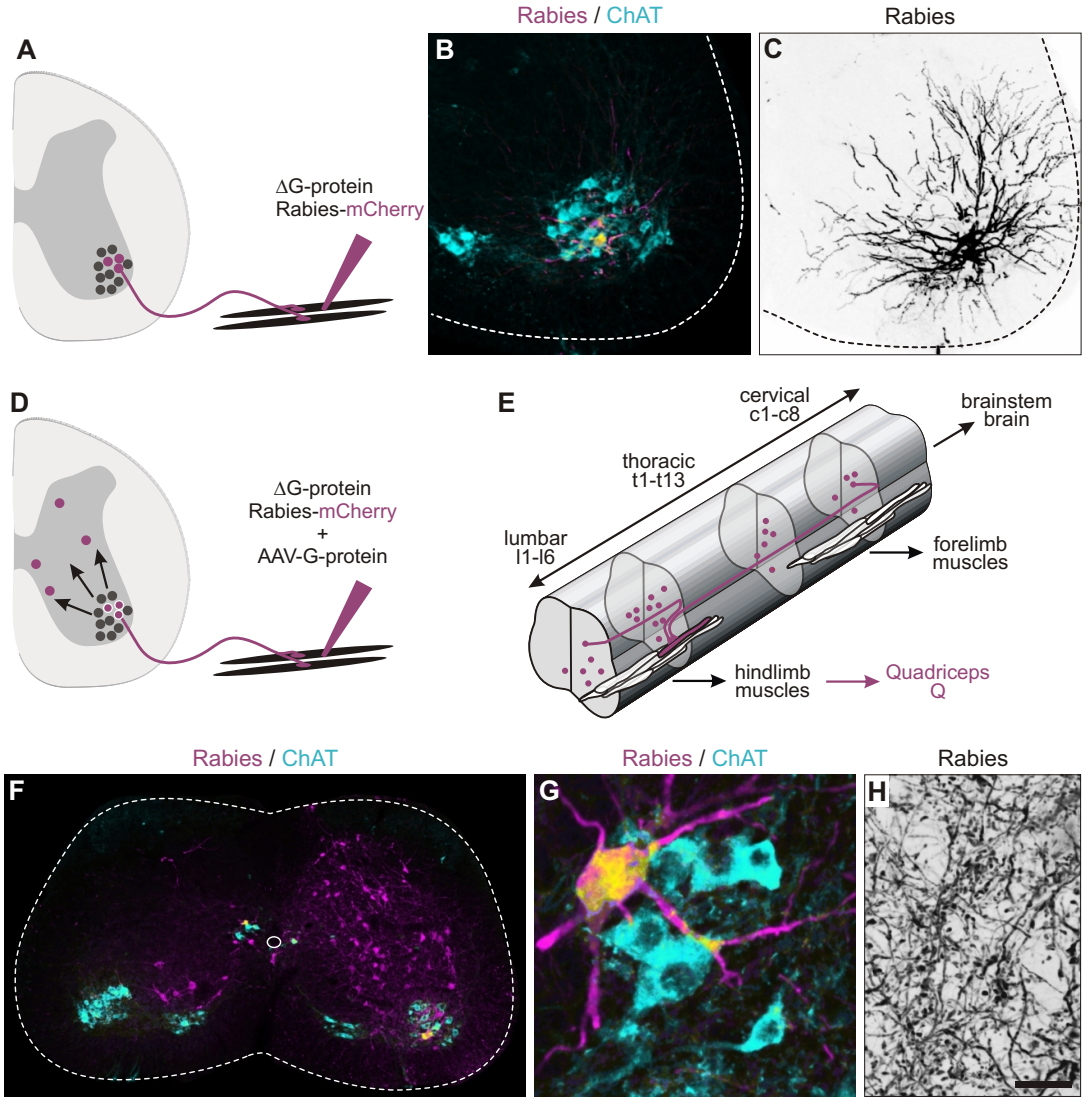

A
ΔG-protein
Rabies-mCherry
Rabies / ChAT
B
Rabies
C
D
ΔG-protein
Rabies-mCherry
+
AAV-G-protein
E
cervical
c1-c8
brainstem
brain
thoracic
t1-t13
lumbar
l1-l6
forelimb
muscles
hindlimb
muscles
Quadriceps
Q
Rabies / ChAT
F
Rabies / ChAT
G
Rabies
H

Figure 5.0: **Viral labeling of premotor interneurons from an identified motor neuron pool.** (A) Diagram illustrating viral infection strategy used in this study. Intramuscular injection of G-protein deficient rabies virus expressing mCherry (Δ G-protein Rab-mCherry) leads to retrograde infection of motor neuron pool innervating injected peripheral muscle. (B) Rab-mCherry injection into Q muscle results in $Rab-mCherry^{on}$ Q motor neurons (purple) in the spinal ventral horn, surrounded by non-infected $ChAT^{on}$ motor neurons (turquoise). Note absence of labeled interneurons. (C) Cell bodies and dendrites of $Rab-mCherry^{on}$ Q motor neurons are labeled. (D) Diagram illustrating monosynaptic transsynaptic viral infection strategy used in this study. Intramuscular injection of Δ G-protein Rab-mCherry in combination with AAV-G-protein leads to retrograde infection of motor neuron pool and associated premotor neurons (arrows illustrate transsynaptic spreading in transverse plane). (E) Diagram depicting three-dimensional arrangement of motor neuron pools in the ventral spinal cord at limb levels (cervical C1-C8; thoracic: T1-T13; lumbar L1-L6) and premotor neurons connected to the Q motor neuron pool (purple), innervating one hindlimb muscle. (F, G) Transverse spinal cord section analyzed at lumbar level L3 shows labeling of premotor interneurons by Rab-mCherry (purple) connected to infected $ChAT^{on}$ Q motor neurons (yellow; G: high resolution of infected motor neuron), using the injection strategy described in (D). (H) High resolution analysis of $Rab-mCherry^{on}$ labeled axonal ramifications in close vicinity to infected Q motor neuron pool. Scale bar, 130 μm in (B) and (C), 200 μm in (F), 15 μm in (G) and (H).

These findings indicate that the Rab-mCherry virus can be complemented successfully by the AAV-delivered G-protein to acquire transsynaptic spreading activity. In animals co-injected with AAV-G-protein and Rab-mCherry, the intensity of fluorescent protein expression in interneurons was similar to motor neurons in animals injected only with Rab-mCherry (Figure 5.0 F), underscoring the transcriptional independence of the rabies virus system from host cells (Wickersham et al. (2007a); Wickersham et al. (2007b)). Transsynaptically marked premotor neurons form a dense network of axonal ramifications in the vicinity of labeled motor neurons (Figure 5.0 H), consistent with their connectivity with motor neurons (Figure 5.0 E). In a time course analysis, we found that first interneurons were visible 6 days after co-injection and peak numbers of labeled neurons were observed after 8 days. To avoid neuronal degeneration due to long-term rabies virus infection, we therefore carried out further experiments 8 days after intramuscular injections.

To begin to probe the robustness and reliability of the method, we injected Q muscles bilaterally in individual mice with rabies viruses expressing fluorescent markers of different colors (Figure 5.1 A). Using Rab-mCherry (right side) and Rab-GFP (left side) viruses, each in combination with AAV-G-protein injection, we found that in these doubly rabies virus injected mice, spinal interneurons labeled by Rab-mCherry and Rab-eGFP were distributed in a bilaterally symmetrical pattern (Figure 5.1 A, B). Artificial super-

imposition of images acquired from Rab-mCherry injections and mirrored images from Rab-eGFP injections displayed in distinct colors showed clustering of neurons in similar domains within the spinal cord (Figure 5.1 C). In particular, the majority of ipsilateral interneurons was found in Rexed laminae VI, VII and X, while contralateral interneurons were mainly confined to Rexed lamina VIII and ventro-medial lamina VII (Figure 5.1 B, C). This contralateral population included both excitatory (vGlut2^{on}) and inhibitory (Gad67^{on}) interneurons (Figure 5.1 L), consistent with previous observations (Bannatyne et al. (2003); Quinlan and Kiehn (2007)). Unilateral omission of AAV-G-protein during injection resulted in spreading of the rabies virus exclusively from the co-injected side (Figure 5.1 D, E), further demonstrating the selective requirement of G-protein expression for the initiation of transsynaptic virus spreading in motor neurons, even within one animal.

Together, these results show that intramuscular injection of G-protein deficient Rab-mCherry or Rab-eGFP viruses in combination with AAV-G-protein is well suited to uncover the distribution and identity of premotor interneurons. The establishment of this dual virus-based method to visualize neurons monosynaptically connected to defined motor neuron pools lays the ground for a detailed quantitative analysis of premotor interneurons and their connections in the spinal cord.

5.3.2 Monosynaptically connected interneurons are revealed by transsynaptic rabies virus

To determine whether neuronal subpopulations with previously described monosynaptic connections to motor neurons can be observed by intramuscular virus injections and to analyze their distributions, we next chose six such neuronal populations: Four spinal interneuron subtypes, group Ia proprioceptive afferents, and one supra-spinal source. For each of them, specific tools for molecular identification exist, allowing evaluation of co-localization between these markers and transsynaptically transported rabies viruses.
Renshaw cells receive excitatory input by cholinergic motor axon collaterals in the ventral spinal cord and in turn provide feedback inhibition to motor neurons (Figure 5.1 C) (Alvarez and Fyffe (2007); Windhorst (1990)). The cell bodies of Renshaw cells are located in the ventralmost domain of Rexed lamina VII and IX in close proximity to motor neurons and express the Calcium binding protein Calbindin (Figure 5.1 A) (Alvarez et al. (2005)). We evaluated whether co-injection of Rab-mCherry and AAV-G-protein into limb mus-

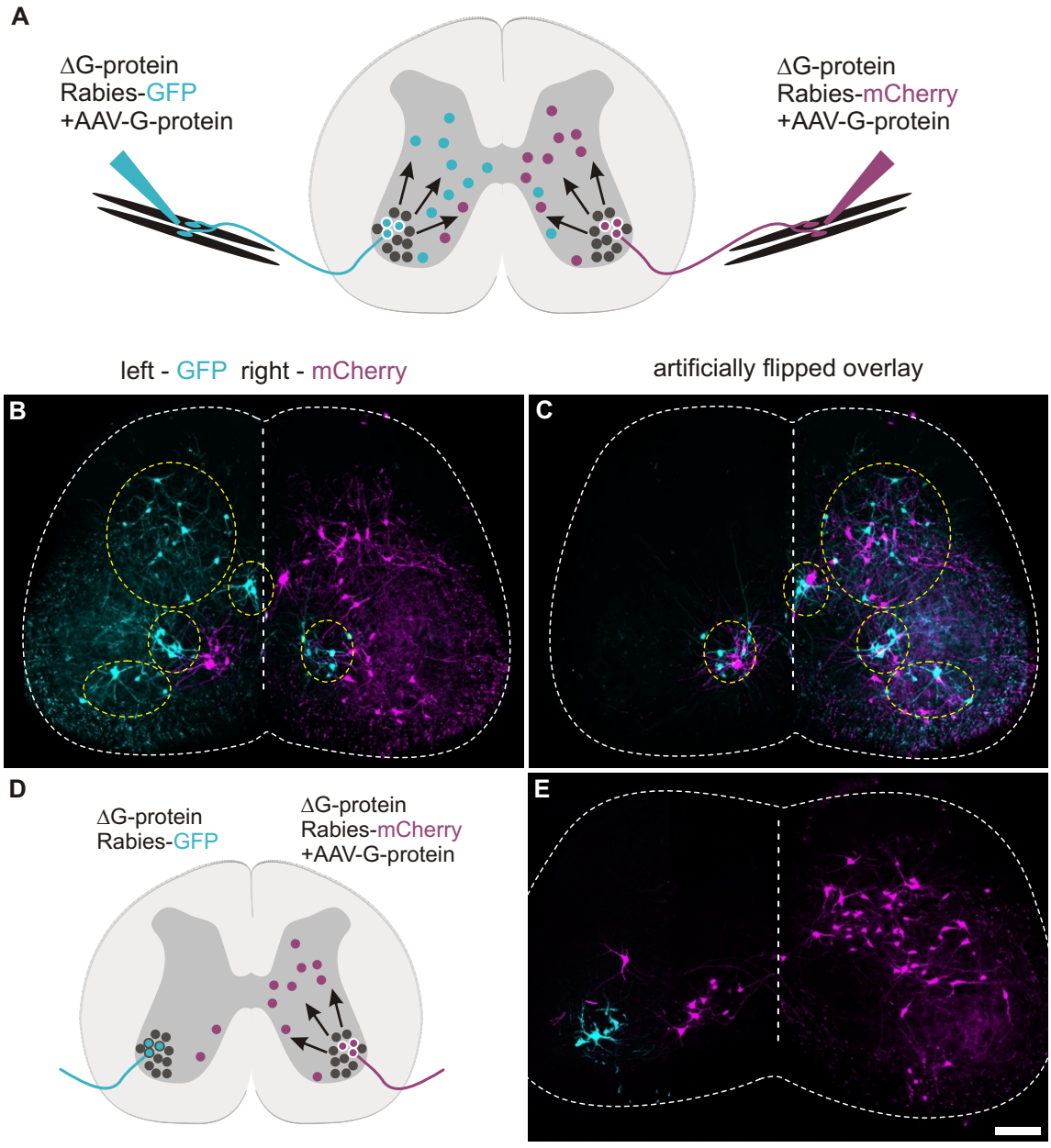


Figure 5.1: **Bilateral virus injections reveal symmetrical premotor interneuron distribution.** (A-C) Bilateral intramuscular injections of ΔG-protein Rabies-GFP and AAV-G-protein into left Q muscle (turquoise) and ΔG-protein Rabies-mCherry and AAV-G-protein into right Q muscle (purple). (A) Diagram illustrating experimental strategy; (B) transverse spinal cord section revealing a symmetrical distribution of premotor interneurons (yellow dashed circles visualize analogous areas in the spinal cord in which premotor interneurons are grouped); (C) artificial flipped overlay of images shown in (B). (D, E) Bilateral intramuscular injections as in (A) but omission of AAV-G-protein injection into left Q muscle. Note absence of spread to premotor interneurons from the left side, but labeled premotor interneurons derived from spread initiated from the right side (purple). Scale bar, 150 μm.

cles labels Renshaw cells transsynaptically based on Calbindin expression and cell body position. Of all mCherry^{on} spinal interneurons analyzed at segmental levels around the source motor neuron pool (Tri: C2-T8), 3.4% were Calbindin^{on} Renshaw cells (Figure 5.1 F). In addition, all mCherry/Calbindin^{on} Renshaw cells were located ipsilaterally and in close segmental proximity to the source motor neuron pool, with a minority of them positioned slightly caudally to the pool (Figure 5.1 F). These findings are in agreement with a wealth of electrophysiological studies demonstrating that Renshaw cells establish exclusively ipsilateral connections to local motor neuron pools (Alvarez and Fyffe (2007); Windhorst (1990)).

A subset of V2 interneurons has previously been shown to establish direct connections to motor neurons using mouse genetic tools (Al-Mosawie et al. (2007); Crone et al. (2008); Stepien and Arber (2008)). We therefore evaluated whether V2 interneurons derived from Lhx3^{on} progenitor cells (Figure 5.1 G, H) are part of the premotor neuronal class revealed by monosynaptic virus tracing. We employed a Cre-based developmental lineage-tracing strategy, targeting nls-LacZ expression to V2 interneurons at postnatal stages by crossing $Lhx3^{Cre}$ mice with a conditional reporter strain ($Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$) (Hippenmeyer et al. (2005); Sharma et al. (1998)), and co-injected Rab-mCherry and AAV-G-protein into the Q muscle. Excluding labeled motor neurons identified by ChAT expression in Rexed lamina IX, we found that 3% of all mCherry^{on} spinal interneurons at L1-L6 with solely ipsilateral distribution were derived from Lhx3 progenitor cells (Figure 5.1 H), and consistent with previous observations (Crone et al. (2008)), at least a fraction of these neurons encompasses the excitatory V2a subpopulation marked by Chx10 expression (Figure 5.5). In addition, and unlike Renshaw cells, V2 interneurons directly connected to Q motor neurons were not restricted to these segmental levels, but were also found several segments rostrally and caudally to the Q motor neuron pool (L1-L2: 18%; Q pool L3: 30%; L4-L6: 52%), reminiscent of projection patterns described for V2a interneurons (Dougherty and Kiehn (2010)). We next investigated whether also interneuron classes derived from progenitor cells in the dorsal spinal cord establish direct connections to motor neurons. Interneurons emerging from the dorsal progenitor domain dI3 marked by the expression of the Lim-homeodomain transcription factor Isl1 (Goulding (2009); Liem et al. (1997)) represent an excitatory interneuron subpopulation for which monosynaptic connectivity to motor neurons has been proposed (Carlin et al. (2009)). We therefore induced expression of nls-LacZ in Isl1^{on} dI3 interneurons by crossing $Isl1^{Cre}$ mice with $Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ mice (Hippenmeyer et al. (2005); Srinivas et al. (2001)), and co-injected Rab-mCherry and AAV-G-protein into the Q muscle. We found

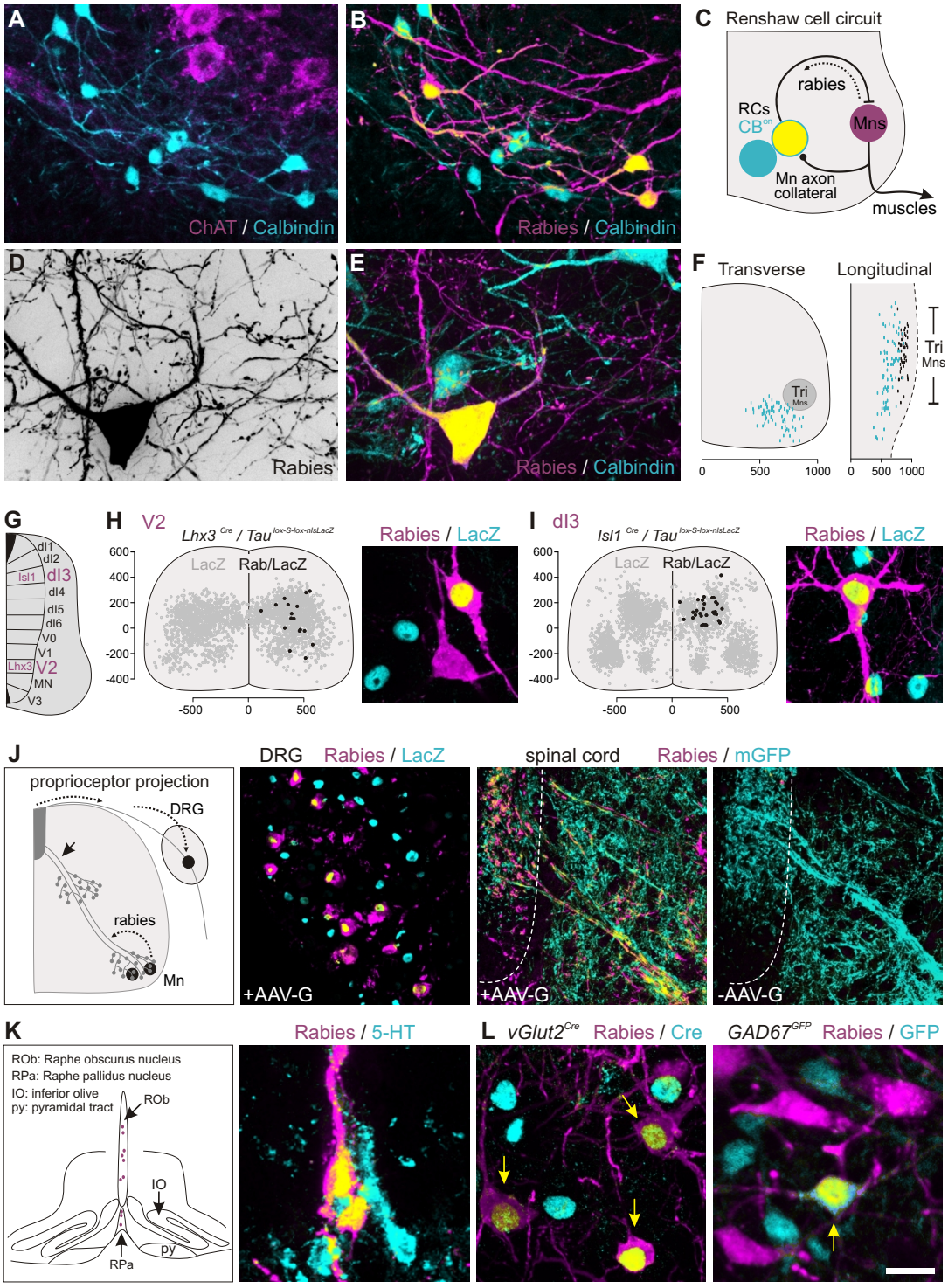
that all Isl1^{on} interneurons marked by Rab-mCherry expression were located ipsilaterally, not exclusively confined to the segmental levels of the Q motor neuron pool (L1-L2: 55%; Q pool L3: 6%; L4-L6: 39%) and that they represented 2.6% of all mCherry^{on} spinal interneurons at L1-L6 (Figure 5.1 I). These findings provide evidence for direct connectivity of dI3 interneurons to motor neurons and reveal the exclusive ipsilateral nature of these connections.

Group Ia proprioceptive sensory neurons provide an important source of premotor input to motor neurons and have been estimated to account for 1-2% of all synapses on motor neurons (Burke and Glenn (1996); Eccles et al. (1957); Ladle et al. (2007); Wang et al. (2007); Windhorst (2007)). These observations prompted us to determine whether proprioceptors genetically marked in $PV^{Cre}\ Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ mice (Hippenmeyer et al. (2005)) can be labeled retrogradely from spinal motor neurons upon co-injection of Rab-mCherry and AAV-G-protein into the Q muscle. mGFP^{on} proprioceptive afferents enter the spinal cord in a position lateral to the dorsal funiculus and many display colocalization with mCherry^{on} labeling, a pattern not observed with solely Rab-mCherry injections (Figure 5.1 J). In addition, analysis of proprioceptor cell bodies in dorsal root ganglia (DRG) revealed many mCherry^{on} LacZ^{on} sensory neurons in double virus injected mice (Figure 5.1J), whereas only very few were observed in control experiments, most likely due to peripheral infection.

Lastly, mCherry^{on} neurons were not restricted solely to spinal levels. Upon intramuscular virus injection, we also detected labeled neurons in the serotonergic ($5-HT^{on}$) Raphe nucleus in the brainstem (Figure 5.1 K), a nucleus well-known to establish descending and direct connections to motor neurons in the spinal cord (Holstege and Kuypers (1987)). In summary, our experiments show that several neuronal populations for which monosynaptic connectivity had previously been demonstrated using a variety of different methods can be reliably detected using monosynaptically-restricted rabies virus based technology.

5.3.3 Premotor interneuron distribution of Quadriceps motor neurons

We next developed quantitative methods to assess the three-dimensional distribution of premotor interneurons labeled retrogradely from the source motor neuron pool. Comparison of positional coordinates in three dimensions yields information about the rostrocaudal, medio-lateral and dorso-ventral distribution of Rab-mCherry and Rab-eGFP labeled spinal interneurons. In addition, assignment of such three-dimensional coordinates


A
ChAT / Calbindin
B
Rabies / Calbindin
C
Renshaw cell circuit
rabies
RCs
CB
Mns
Mn axon collateral
muscles
D
Rabies
E
Rabies / Calbindin
F
Transverse
Longitudinal
Tri
Mns
0
500
1000
G
dI1
dI2
Isl1
dI3
dI4
dI5
dI6
V0
V1
Lhx3
V2
MN
V3
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V2
Lhx3 Cre / Tau lox-S-lox-nlsLacZ
LacZ
Rab/LacZ
600
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-400
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dI3
Isl1 Cre / Tau lox-S-lox-nlsLacZ
LacZ
Rab/LacZ
Rabies / LacZ
J
proprioceptor projection
DRG
rabies
Mn
DRG
Rabies / LacZ
+AAV-G
spinal cord
Rabies / mGFP
+AAV-G
-AAV-G
K
ROb: Raphe obscurus nucleus
RPa: Raphe pallidus nucleus
IO: inferior olive
py: pyramidal tract
ROb
IO
py
RPa
Rabies / 5-HT
L
vGlut2Cre
Rabies / Cre
GAD67GFP
Rabies / GFP

Figure 5.1: **Identification of functionally and molecularly distinct premotor neurons.** Analysis of monosynaptic connectivity between specific motor neuron pool and defined premotor neuron populations identified by molecular tools and monosynaptically restricted transsynaptic viruses. (A-F) Calbindin^{on} Renshaw cells (turquoise; A, B, E) are positioned ventro-medially to lateral motor column ChAT^{on} motor neurons (purple in A) and can be identified using the monosynaptic rabies virus approach (purple in B, E; black in D). (C) Diagram depicting Renshaw cell circuit (Renshaw (1941)). Motor axon collaterals make monosynaptic connections to Calbindin^{on} Renshaw cells which in turn inhibit motor neurons (purple). Due to monosynaptic connections between Renshaw cells (RC) and motor neurons, Renshaw cells are labeled by monosynaptic rabies virus tracing technology (retrograde dashed arrow, double labeled Renshaw cell: yellow). (F) Analysis of transverse and longitudinal distribution of Renshaw cells (turquoise), labeled transsynaptically from Tri motor neurons (black). Note that labeled Renshaw cells are found in close vicinity to the source motor neuron pool.(G) Diagram depicting developmental origin of interneuron classes (V0-V3; dI1-dI6) and motor neurons (Mn) (Goulding (2009); Jessell (2000); Stepien and Arber (2008)). V2 interneurons are marked by expression of Lhx3, and dI3 interneurons by Isl1 expression.(H, I) Analysis of Lhx3-derived V2 (H) and Isl1-derived dI3 (I) interneurons premotor to Q motor neurons assayed in $Lhx3^{on}$ $Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ (H) or $Isl1^{Cre}$ $Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ (I) mice. Overall distribution of LacZ^{on} neurons (grey) and mCherry^{on}/LacZ^{on} neurons (black) in L1-L4 spinal cords visualized as transverse z-projection. LacZ (turquoise) and mCherry (purple) immunohistochemistry to visualize double labeled premotor interneurons. (J) Schematic diagram illustrates the central route of retrograde infection of proprioceptive afferents from Q motor neurons. Co-injection of Rabies-mCherry and AAV-G-protein into Q muscle (+AAV-G) results in labeling of PVon group Ia afferents as visualized by expression of mCherry in DRG cell bodies (left) or centrally projecting axons (middle). In contrast, injection of solely Rabies-mCherry into Q (-AAV-G) does not result in pronounced labeling of proprioceptive axons (right). Proprioceptive afferents are marked by expression of mGFP (membrane-tethered GFP) and nls-LacZ in PV^{Cre} $Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ mice. (K) $5-HT^{on}$ premotor neuron in the Raphe Nucleus of the brainstem marked by immunohistochemistry to 5-HT (turquoise) and mCherry (purple). Overview diagram illustrates 5-HT neuron positions in the brainstem marked by retrograde virus infection. (L) Contralateral (commissural) interneurons in contact with Q motor neurons can be either excitatory, as visualized by Cre antibody labeling in $vGlut2^{Cre}$ mice (left) or inhibitory, as visualized in $GAD67^{GFP}$ mice (right). Scale bar, 30 μm in (A) and (B), 10 μm in (D) and (E), 15 μm in (H, I, K, L), 100 μm in (J, left), 80 μm in (J, right). See also Figure 5.5

allows an unbiased evaluation of the reliability and reproducibility of the method upon viral injections into a defined muscle in different mice.

To reconstruct three-dimensional digital coordinates of premotor spinal interneurons and quantitatively assess their distribution, we acquired images of transverse spinal cord sections of defined segments around the Q source motor neuron pool (T11 S1; Q pool L3/4; Figure 5.1 A, B). On individual transverse sections (x-y dimension), we set the central canal as the 0-0 coordinate for the medio-lateral (x-axis) and dorso-ventral axis (y-axis), with the y-axis parallel to the midline of the spinal cord and the x-axis orthogonal to it (Figure 5.1 C). The 0-coordinate for the rostro-caudal (longitudinal; z-axis) dimension always marked the rostral-most section of a reconstruction.

Using this method, we assigned three-dimensional coordinates to all interneurons labeled transsynaptically upon viral injections into Q muscles (n=6 mice). To visualize longitudinal and transverse distributions of labeled interneurons, we plotted projections of these datasets derived from individual injections (Figure 5.1 A, B). Ipsilateral interneurons made up the majority of all premotor interneurons (Figure 5.1 D) and they were much more broadly distributed along the medio-lateral and dorso-ventral axis than contralateral premotor neurons. These were mainly confined to Rexed lamina VIII and ventro-medial lamina VII (Figure 5.1 A, C), although the rostro-caudal distribution with respect to the source pool between ipsi- and contralateral interneurons was comparable (rostral to Q [ipsi: 196%; contra: 225%]; Q level [ipsi: 354%; contra: 277%]; caudal to Q [ipsi: 49±11%; contra: 48±12%]). To quantitatively assess variability in overall premotor interneuron distributions, we compared individual experiments using correlation analysis, a method suitable for extraction of common features from pairs of multivariate data (Venables and Ripley (2002)). We found that individual Q premotor interneuron distributions were highly correlated ($r \leq 0.9$; Figure 5.1 G). The high overall reproducibility of the method is further underscored by the visualization of cell densities using density distributions along one axis or contour plots at the transverse level (Figure 5.1 E, F). In summary, our experiments demonstrate that co-injection of G-protein deficient rabies viruses and AAV-G-protein into defined muscles results in reliable and reproducible tracing of premotor interneurons in the spinal cord.

5.3.4 Premotor interneuron distribution of Cutaneous Maximus motor neurons

The establishment of a reliable method to map premotor interneurons opens the possibility to compare the distribution of interneurons controlling the activity of motor neuron pools innervating muscles with distinct function. Not all motor neuron pools receive synaptic input from functionally analogous premotor sources and this variegation in input specificity may contribute to the observed differences in the activation of these motor neuron pools (Eccles et al. (1957); Hultborn et al. (1971); Ladle et al. (2007); Nishimaru et al. (2006); Wang et al. (2008); Windhorst (1990); Windhorst (2007)). For example, recent experiments show that while Tri motor neurons receive direct input from proprioceptive sensory afferents, cutaneous maximus (Cm) motor neurons lack direct proprioceptive connections and instead only receive sensory feedback through di- or polysynaptic pathways (Vrieseling and Arber (2006)). These findings raise the question of whether premotor input to Tri and Cm motor neuron pools may differ more fundamentally than only in proprioceptive input. We therefore analyzed and compared the distribution of Tri and Cm premotor interneurons by injection of Rab-mCherry and AAV-G-protein into Tri or Cm muscles.

Tri and Cm motor neuron pools share similar rostro-caudal positions (C7/8), and at these segmental levels, Cm motor neurons are found in an extreme ventro-lateral position while Tri motor neurons are located more dorsally (Figure 5.2 A, B) (Holstege et al. (1987); Theriault and Diamond (1988b); Vrieseling and Arber (2006)). Despite similar rostro-caudal motor neuron pool positions, the distribution of premotor interneurons was dramatically different between Tri and Cm muscle injections (Figure 5.2 A, B). Tri premotor interneurons were positioned over many segments both rostrally and caudally to the Tri motor neuron pool with an extended distribution ipsilaterally and with the peak of contralateral density confined to lamina VIII and ventro-medial lamina VII (Figure 5.2 A; analysis C1-T8). In contrast, Cm premotor interneurons were located almost exclusively at segmental levels overlapping with and caudally to the Cm motor neuron pool while spinal cord segments rostrally to the motor pool were devoid of Cm premotor interneurons (Figure 5.2 B). Further analysis showed that the majority of all Cm premotor interneurons was positioned in the ipsilateral dorsal spinal cord, as also quantitatively confirmed on contour plots and by cross-correlation analysis between Tri and Cm (Figure 5.2 A-C). The observation that Cm premotor interneurons are preferentially located in the dorsal spinal

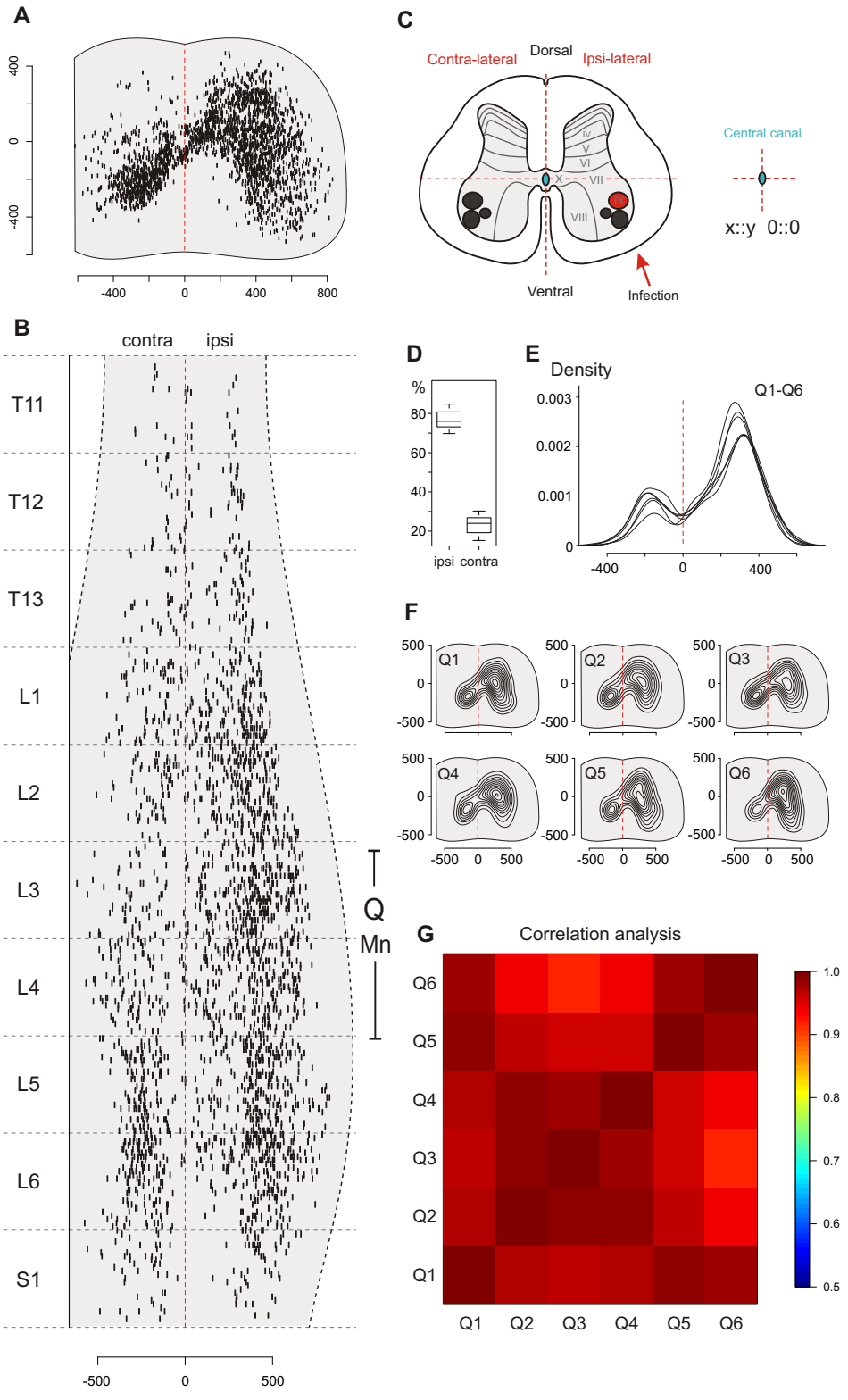
A
400
0
-400
-400
0
400
800
C
Contra-lateral
Dorsal
Ipsi-lateral
IV
V
VI
X
VII
VIII
Ventral
Infection
Central canal
x::y 0::0
B
contra
ipsi
T11
T12
T13
L1
L2
L3
L4
L5
L6
S1
Q
Mn
-500
0
500
D
%
80
60
40
20
ipsi contra
E
Density
Q1-Q6
0.003
0.002
0.001
0
-400
0
400
F
Q1
Q2
Q3
Q4
Q5
Q6
500
0
-500
G
Correlation analysis
Q6
Q5
Q4
Q3
Q2
Q1
1.0
0.9
0.8
0.7
0.6
0.5

Figure 5.1: **Reproducibility of Quadriceps premotor interneuron distributions** (A, B) Digitally reconstructed three-dimensional distribution of spinal interneurons premotor to Q motor neurons shown as transverse (A) and longitudinal (B) projection at levels T11-S1. Rostro-caudal extension of source motor neuron pool (Q) is indicated in (B). (C) Diagram illustrating Rexed lamina position in the adult spinal cord to illustrate assignment of three-dimensional coordinates. Infection of motor neurons occurs in ipsilateral spinal cord (red arrow) and positions in contralateral spinal cord opposite to source motor neuron pool are assigned negative values. Central canal (blue circle) is assigned 0::0 coordinates at the x::y level and rostral-most section of a reconstruction is 0 at the z-level. (D-F) Boxplot showing percentage of ipsilateral and contralateral rabies virus infected premotor interneurons (D), medio-lateral distribution densities (E), and 2D transverse contour density analysis (F) for n=6 Q three-dimensional reconstructions. (G) Cross-correlation analysis of n=6 Q premotor interneuron distributions. Color scale to the right indicates correlation values (all 0.9 for individual Q-Q comparisons).

cord (Figure 5.6) and at segmental levels C7-T3 is in agreement with a proposed model of how sensory feedback derived from the skin overlying the Cm muscle drives activation of Cm motor output based on functional mapping experiments (Theriault and Diamond (1988a)) (Figure 5.2 D).

5.3.5 Cholinergic partition cells segregate in ipsi- and bilaterally projecting subpopulations

The transsynaptic virus tracing method established in this study allows the visualization of the three-dimensional nature of premotor interneuron distributions and their connections in great detail. We therefore next used this approach to determine the spatial distribution and connectivity profiles of one defined spinal interneuron population involved in the modulation of motor output. Cholinergic partition cells in Rexed lamina X are known to establish direct connections to motor neurons through C-bouton synapses, and regulate motor neuron excitability by reducing after-hyper-polarization (Conradi and Skoglund (1969); Hellstrom et al. (2003); Miles et al. (2007)) (Figure 5.3 A). Recent work revealed a role of this neuronal population in modulation of locomotor behavior (Zagoraiou et al. (2009)), yet important aspects to understand the function of cholinergic partition cells including segmental distribution with respect to innervated motor neurons, as well as the specificity of synaptic connections to identified motor neurons, remain uncharacterized.
To visualize partition cells monosynaptically connected to defined motor neuron pools, we co-injected Rab-mCherry and AAV-G-protein into Q or Tri muscles, and evaluated the distribution of mCherry^{on} neurons in Rexed lamina X marked by co-expression of choline acetyl transferase (ChAT) in these animals (Figure 5.3 B). We found that only a small frac-

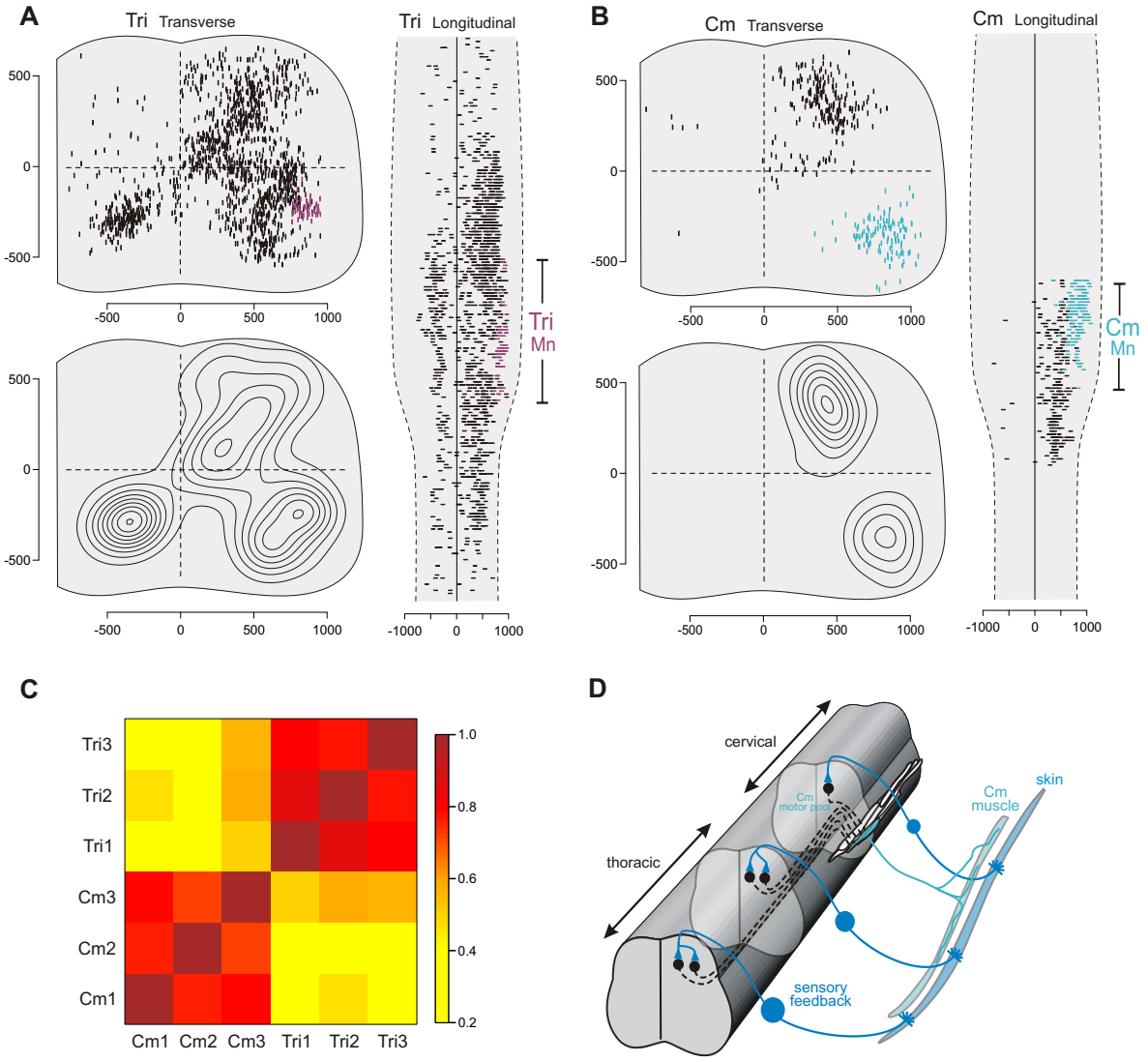

Figure 5.2: **Cm premotor interneurons are found in dorsal and caudal position to the pool.** (A, B) Transverse, longitudinal and transverse contour plot analysis of Tri (A) and Cm (B) premotor interneurons. Rostro-caudal extent of source motor neuron pools (Tri and Cm) is indicated on longitudinal projection plots. (C) Cross-correlation analysis for Tri and Cm premotor interneuron distributions. Color scale to the right indicates correlation values. (D) Proposed circuit diagram for skin reflexes controlled by Cm muscle contraction, based on monosynaptic rabies virus tracing experiments in this study and previous functional studies (Theriault and Diamond (1988a)). See also Figure 5.6.

tion of all mCherry^{on} spinal interneurons fell into this category (Figure 5.3 E, F; Q: 2.1%; n=4 mice; Tri: 1.5%; n=4 mice). Strikingly however, not all of these mCherry^{on} ChAT^{on} neurons were located ipsilaterally, prompting us to quantify their three-dimensional distribution in more detail. We found that 75% of Q mCherry^{on} ChAT^{on} neurons were located ipsilaterally whereas 25% of them were found contralaterally and a similar ratio was observed for partition cells premotor to Tri motor neurons (Figure 5.3 C-F). In addition, Q partition cells were found at levels T11-L6 (Figure 5.3 G), an extent way beyond the rostro-caudal segments at which the Q source motor neuron pool was located. The distribution of cholinergic partition cells in relation to a defined motor neuron pool is thus strikingly different from the local and purely ipsilateral positioning of premotor Renshaw cells as described above (Figure 5.1 F).

The observation that 25% of Q mCherry^{on} ChAT^{on} neurons were found contralateral to the source motor neuron pool raised the question of whether this neuronal population exhibits an exclusively contralateral axonal projection or whether alternatively, it projects bilaterally and makes connections to both ipsi- and contralateral motor neurons. To distinguish between these two possibilities, we carried out two sets of experiments. First, we performed bilateral injections into Q muscles, using G-protein deficient rabies viruses expressing two distinct fluorescent proteins (Rab-mCherry: left; Rab-eGFP: right), both in combination with AAV-G-protein, reasoning that cholinergic neurons expressing both mCherry and eGFP should only be observed if neurons establish bilateral connections. Indeed, we found that mCherry^{on} eGFPon ChAT^{on} neurons were detected in such experiments (Figure 5.3 H), supporting a model of bilateral connectivity. Second, we determined whether unilateral Rab-mCherry injection combined with AAV-G-protein marks contralateral mCherry^{on} terminals derived from cholinergic partition cells in the vicinity of motor neurons. In these experiments, we used the accumulation of vesicular acetylcholine transporter (vAChT) in C-boutons (Hellstrom et al. (2003)) in combination with mCherry expression to track these synaptic terminals. Contralateral to the Q source motor neuron pool, mCherry^{on} vAChT^{on} terminals were readily detected, as assumed to occur for bilaterally projecting neurons (Figure 5.3 I). Together, these experiments provide strong support for the existence of a bilateral connectivity pattern of a subpopulation of cholinergic interneurons. Based on our results, we estimate that this bilaterally projecting population makes up approximately half of all cholinergic partition cells in the spinal cord, while the remaining fraction projects exclusively ipsilaterally.

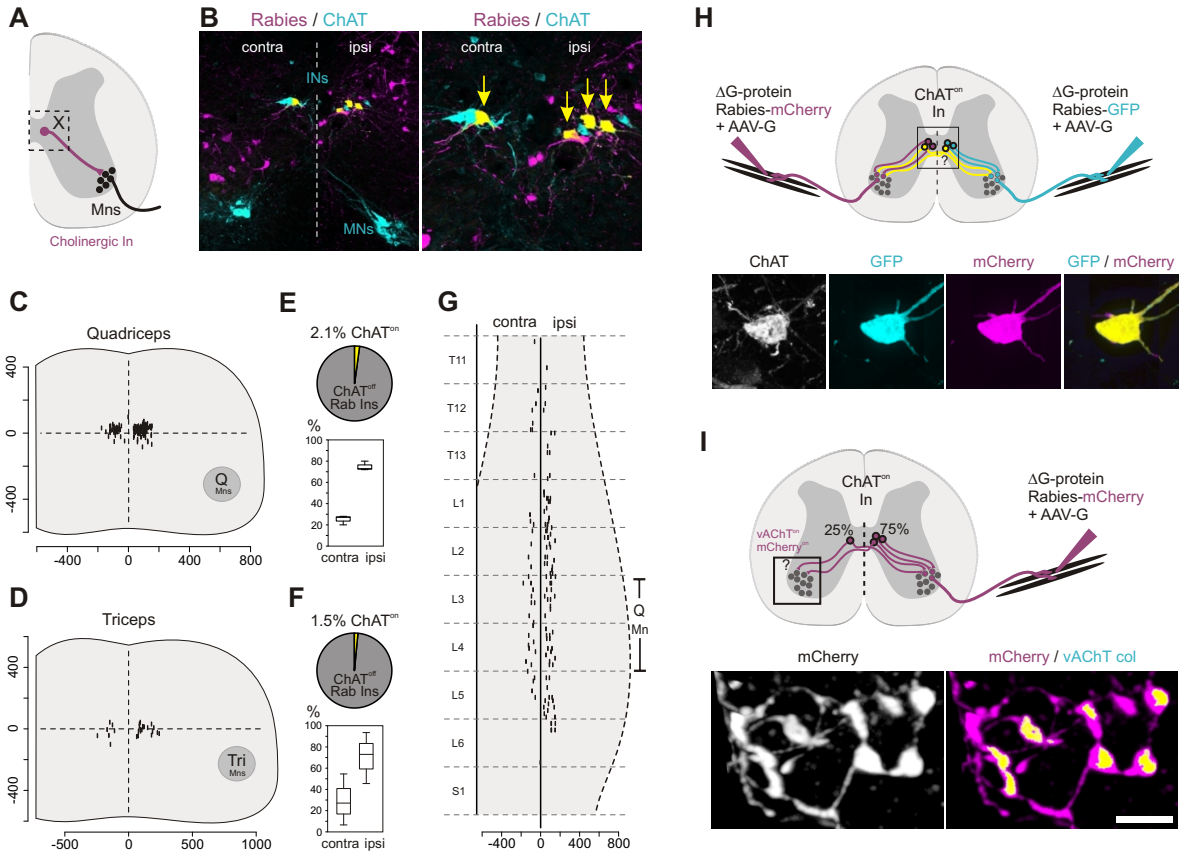

Figure 5.3: **Cholinergic partition cells project across segments and bilaterally.** (A) Diagram illustrating cell body position (Rexed lamina X) and projection trajectory of cholinergic partition cells giving rise to C-bouton synapses on motor neurons (Mns). (B) Analysis of cell body position of cholinergic partition cells (INs) in lamina X marked by ChAT (turquoise) and monosynaptic connectivity to Q motor neurons using transsynaptic Rab-mCherry (purple). Yellow arrows point to ChAT^{on} mCherry^{on} partition cells which are found both ipsi- and contralaterally to the Q source motor neuron pool. Dashed line marks midline. (C-G) Digitally reconstructed three-dimensional distribution of cholinergic partition cells premotor to Q (C, E, G) and Tri (D, F) motor neurons shown as transverse (C, D) and longitudinal (G) projection. Rostro-caudal extension of source motor neuron pool (Q) is indicated in (G). Percentage of all rabies virus infected interneurons (E, F: top), and boxplot of ipsi- and contralateral percentage of rabies virus infected premotor cholinergic partition cells (E, F: bottom) for Q (E) and Tri (F) are shown. (H) Diagram of experimental setup for bilateral intramuscular injection of rabies-GFP and AAV-G-protein into right Q muscle (turquoise) and rabies-mCherry and AAV-G-protein into left Q muscle (purple), and analysis of ChAT^{on} cholinergic partition cells marked by both mCherry and GFP expression (bottom row). (I) Diagram of experimental setup to analyze projections of cholinergic partition cells in the contralateral ventral spinal cord (top; box indicates area analyzed) and immunohistochemistry detecting mCherry^{on} (purple) terminals colocalized with vAChT (bottom) in vicinity of contralateral motor neurons. Scale bar, 220μm in (B, left), 100μm in (B, right), 25μm in (H), 2.5μm in (I).

5.3.6 Bilaterally projecting partition cells exhibit a high degree of connection specificity

To determine the synaptic connection specificity of bilaterally projecting cholinergic partition cells to defined motor neuron pools, we next analyzed the direct synaptic contacts established between mCherry^{on} vAChT^{on} terminals and contralateral motor neuron pools, marked by retrograde tracing from identified muscles using fluorescent dextran dyes (f-dex) (Figure 5.3 A). Q and Adductor (AD) motor neurons in mice are bound by strong rules of synaptic specificity at the level of sensory-motor connectivity, where Q sensory afferents establish strong direct input to Q but not AD motor neurons (Mears and Frank (1997); Wang et al. (2007)), raising the question of whether cholinergic interneurons obey similar specificity rules for these motor neuron pools. Q and AD motor neurons lie at the same segmental level of the spinal cord (L3), with the Q motor neuron pool adjacent and positioned laterally to the AD pool (McHanwell and Biscoe (1981)). In synaptic reconstructions of contralateral f-dex motor neurons, mCherry^{on} vAChT^{on} terminals transsynaptically labeled from Q motor neurons were readily detected in direct contact with Q motor neurons, whereas neighboring AD motor neurons were sparsely contacted by these terminals (Figure 5.3 B, C). To quantitatively assess these differences, we determined a Synaptic Filling Index (SFI), as a measure for the percentage of all vAChT^{on} terminals found in contact with an analyzed motor neuron (Figure 5.7) also marked by mCherry^{on} (SFI 0: no vAChT terminals are mCherry^{on}; SFI=100: all vAChT terminals are mCherry^{on}). We carried out these experiments in animals in which both Q and AD motor neurons were retrogradely labeled by different f-dex dyes, allowing direct comparison of the SFI within individuals and thus assessing the terminals of the same set of cholinergic partition cells. We found a median SFI of 21.1 for Q-Q contacts and only 1/56 Q neurons was not contacted, whereas the median SFI for Q-AD contacts was only 2.6, and 21/48 AD neurons received no Q contact (Figure 5.3 D, E), indicating that a high degree of synaptic specificity exists for these connections. We also assessed whether Gastrocnemius (GS) motor neurons, positioned at the more caudal L5 spinal level but exhibiting functional similarities with Q motor neurons (Eccles et al. (1957)), were contacted by Q cholinergic terminals. We found that the SFI for Q-GS connections was not as high as for Q motor neurons, but much higher than for AD motor neurons (SFI: 15.5; Figure 5.3 F). These findings show that bilaterally projecting partition cells connecting to Q motor neurons exhibit specificity rules with bilateral preference for Q motor neurons and functionally related motor neuron pools.

To assess whether tight synaptic specificity represents a general feature of bilaterally projecting cholinergic partition cells, we set out to analyze cholinergic interneurons connected to the Tibialis Anterior (TA) motor neuron pool, innervating a calf muscle with antagonistic function to the GS. TA and GS motor neuron pools are segmentally aligned (L4/5), in a dorsal position characteristic for calf motor neurons (McHanwell and Biscoe (1981)). While the median SFI for TA cholinergic input to TA motor neurons we observed was 15.0 (n=0/77 TA neurons with 0 inputs), the vast majority of GS motor neurons was only sparsely contacted by TA cholinergic axons with a median SFI of 3.4 (n=31/81 GS neurons with 0 contacts), and similarly low values (SFI=4.6) were observed for AD motor neurons (Figure 5.3 G-I). Taken together, these findings demonstrate that bilaterally projecting cholinergic partition cells show strong preference to establish synaptic connections with functionally equivalent or related motor neuron pools bilaterally, but avoid functionally antagonistic neighboring motor neuron pools (Figure 5.3 J, K).

5.3.7 Synaptic specificity is shaped by terminal arborization sizes of bilateral partition cells

We next assessed whether C-bouton synaptic specificity is determined exclusively by the number of bilaterally projecting partition cells contacting a particular motor neuron, or whether in addition, cholinergic partition cells differ in terminal arborization sizes. For this purpose, we reconstructed individual terminal arborizations in cases where we were able to perform three-dimensional axonal reconstruction (Figure 5.4 A), and determined the number of mCherry^{on} vAChT^{on} presynaptic specializations encompassed within an individual axon terminal arbor in contact with identified motor neurons (Figure 5.4 A, B). We found that Q cholinergic interneurons establish the highest number of synapses per individual axon arborization with Q motor neurons, whereas inputs to AD motor neurons encompass fewer presynaptic terminals per axon, and values to GS motor neurons were at intermediate levels (Figure 5.4 D-F; mean number of contacts / n axons analyzed: Q-Q: 5.53.7, n=44; Q-AD: 2.11.3, n=32; Q-GS: 3.11.2, n=36; nonparametric t-test, Wilcoxon: Q-Q vs Q-AD: ***p0.001; Q-Q vs Q-GS: **p0.01; Q-AD vs Q-GS **p0.01). Similarly, TA cholinergic interneurons contact TA motor neurons with significantly larger terminal arborizations than GS or AD motor neurons (Figure 5.4 G-I; mean number of contacts/ n axons analyzed: TA-TA: 5.82.5, n=35; TA-GS: 2.71.3, n=46; TA-AD: 2.41.2, n=34; nonparametric t-test, Wilcoxon: TA-TA vs TA-GS: ***p0.001; TA-TA vs TA-GS:

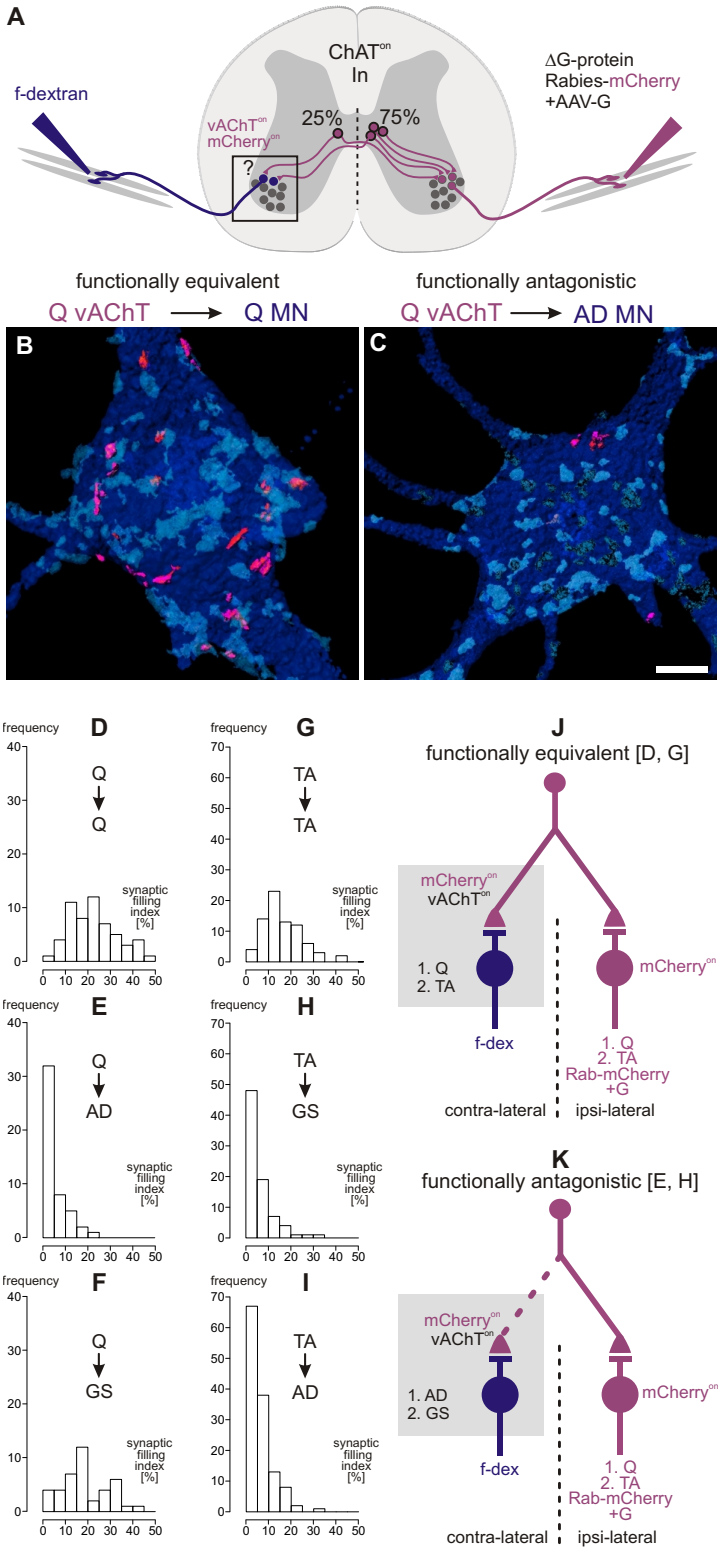
A
ChAT^on In
ΔG-protein Rabies-mCherry +AAV-G
f-dextran
vAChT^on mCherry^on
25%
75%
?
functionally equivalent
Q vAChT → Q MN
functionally antagonistic
Q vAChT → AD MN
B
C
frequency
D
Q → Q
synaptic filling index [%]
G
TA → TA
E
Q → AD
H
TA → GS
F
Q → GS
I
TA → AD
J
functionally equivalent [D, G]
mCherry^on vAChT^on
1. Q
2. TA
f-dex
mCherry^on
1. Q
2. TA
Rab-mCherry +G
contra-lateral
ipsi-lateral
K
functionally antagonistic [E, H]
mCherry^on vAChT^on
1. AD
2. GS
f-dex
mCherry^on
1. Q
2. TA
Rab-mCherry +G
contra-lateral
ipsi-lateral

Figure 5.3: **Bilaterally projecting cholinergic partition cells exhibit synaptic specificity.** (A) Diagram of experimental setup to analyze specificity of synaptic connections between bilaterally projecting cholinergic partition cells and contralateral motor neuron pools. Rabies-mCherry and AAV-G-protein are injected ipsilaterally into defined muscles, and combined with f-dextran injection into defined contralateral muscles. (B, C) IMARIS reconstruction of cholinergic inputs to retrogradely labeled contralateral Q (B) and AD (C) motor neurons (dark blue). Light blue indicates apposition between $vAChT^{on}$ terminals and analyzed motor neuron, pink indicates $vAChT^{on}$ contacts also exhibiting mCherry immunohistochemistry derived from bilaterally projecting cholinergic partition cells marked by their direct connection to Q motor neurons on the opposite side. Note higher number of contacts to Q motor neuron than AD motor neuron shown. (D-I) Quantitative analysis of synaptic filling index (SFI) observed for connections between Q-labeled cholinergic partition cells (D-F) to Q (D), AD (E) and GS (F) contralateral motor neurons, and TA-labeled cholinergic partition cells (G-I) to TA (G), GS (H) and AD (I) contralateral motor neurons. (J, K) Summary of connectivity diagram observed between bilaterally projecting partition cells connected to a particular motor neuron pool ipsilaterally to functionally equivalent (J) and antagonistic (K) contralateral motor neuron pools. A high connectivity index can be observed for Q-Q and TA-TA connections (functionally equivalent; J), but only a low connectivity index (depicted as dashed line in contralateral projection of cholinergic interneuron) is seen for Q-AD or TA-GS connections (antagonist). Scale bar, 6 μm. See also Figure 5.7.

***p0.001; TA-GS vs TA-AD: ns). In addition, average numbers of terminal specializations on preferred motor neuron targets were similar for Q-Q and TA-TA connections and encompassed an average of five contact points (Figure 5.4 C; Q-Q v TA-TA: ns). Our findings therefore support a model in which not only the absolute number of premotor cholinergic interneurons contributes to the observed synaptic specificity between bilaterally projecting neurons and motor neurons, but also the terminal arborization patterns of individual cholinergic axons represent an element in this equation.

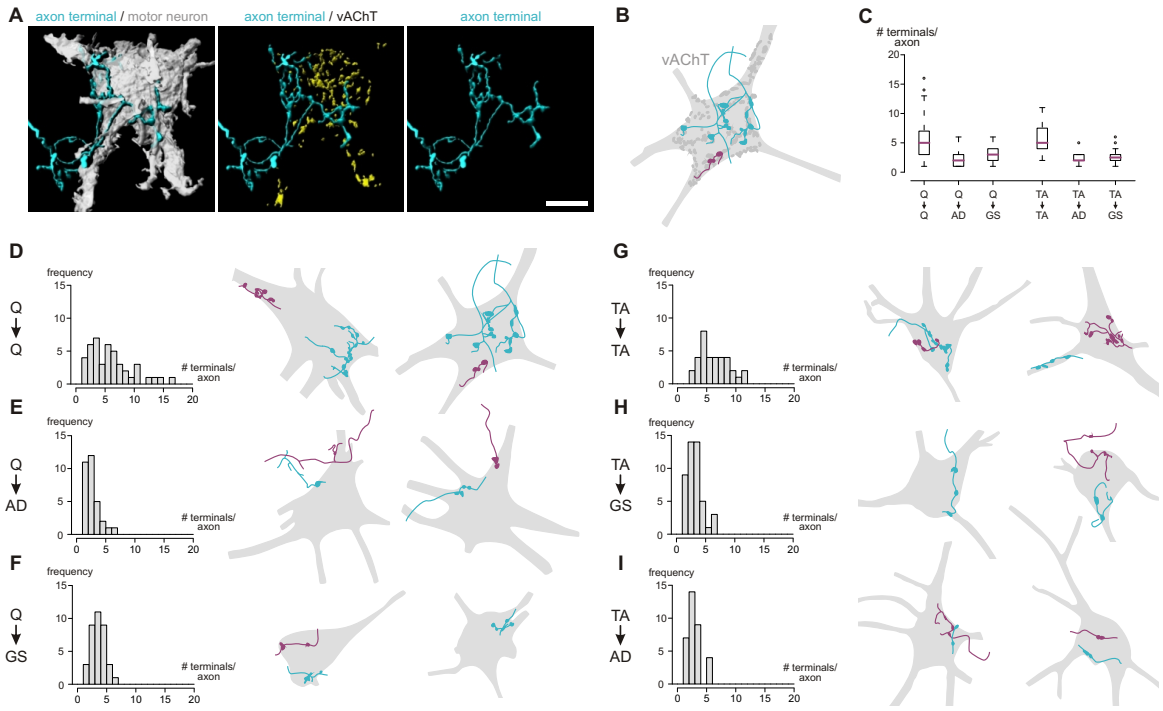

Figure 5.4: **Sizing of terminal arborizations of cholinergic partition cells contributes to bilateral synaptic specificity.** (A) Example of single axonal terminal reconstruction of rabies virus labeled bilaterally projecting cholinergic partition cell (turquoise) in contact with f-dextran labeled contralateral motor neuron (grey). Remaining non-covered vAChT contacts are shown in yellow (middle panel). (B) Example of analysis of number of synaptic contact points of individual axonal terminals of cholinergic partition cells on contralateral motor neurons (purple and turquoise represent different axonal terminals). (C) Boxplot showing analysis of number of synaptic specializations observed per reconstructed individual axon for all pairs analyzed as indicated. (D-I) Frequency distributions of number of vAChT specializations per axon in all pairs analyzed with two reconstructed examples displayed to the right of quantitative analysis. Pairs analyzed are: Q– >Q (D), Q– >AD (E), Q– >GS (F), TA– >TA (G), TA– >GS (H), TA– >AD (I). Scale bar, 10μm.

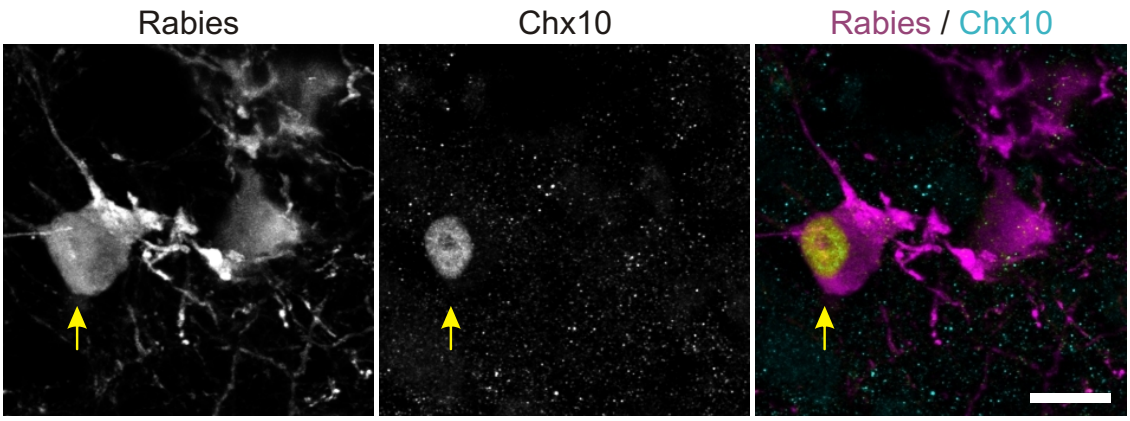

Figure 5.5: **Identification of V2a excitatory interneurons.** Chx10on V2a interneuron identified by monosynaptic rabies virus from Q motor neurons. Scale bar, 15μm.

5.4 Discussion

Movement is the final output of nervous system activity manifested by timed muscle contractions initiated by innervating spinal motor neurons. The activity of a motor neuron is directly controlled by the cohort of associated presynaptic neurons. In this study we establish an anatomical virus-tracing based assay to reveal and study the spatial distributions and connectivity rules between premotor interneurons and functionally identified motor neuron pools. We discuss our findings in the context of principles of general organization in the motor output system and more specifically with a focus on connectivity and roles of neuromodulatory cholinergic interneurons.

5.4.1 Premotor interneuron distributions revealed by transsynaptic tracing

The transsynaptic virus-based method applied here permits a global assessment of the three-dimensional distributions of premotor neurons. It demonstrates that while many premotor interneurons in the spinal cord are positioned in segmental alignment with the motor neuron pool they contact, the overall premotor interneuron network distributes over many segments rostrally and caudally to the pool. Despite this at first sight widespread distribution of the network, parallel analysis of both the global three-dimensional premotor interneuron distribution and subpopulation identity within this framework provides

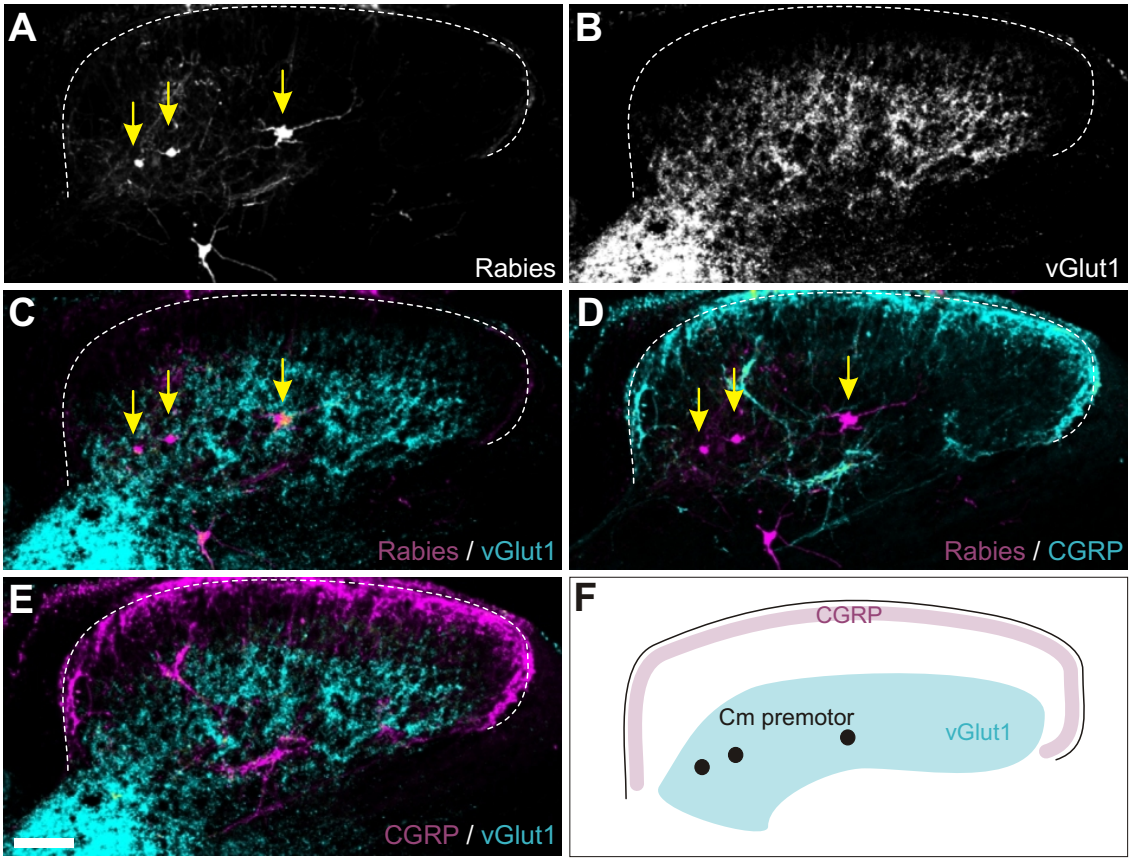

Figure 5.6: **Cm premotor interneurons in the deep dorsal horn of the spinal cord.** Immunohistochemistry of Cm premotor interneurons (A, C, D) in the deep dorsal horn of the spinal cord (dashed lines) shows their preferential location in the vGlut1^{on} area of the deep dorsal horn (B, C, E), but avoidance of the more superficial CGRPon layers (D, E). (F) shows summary diagram of analysis. Scale bar, 80μm.

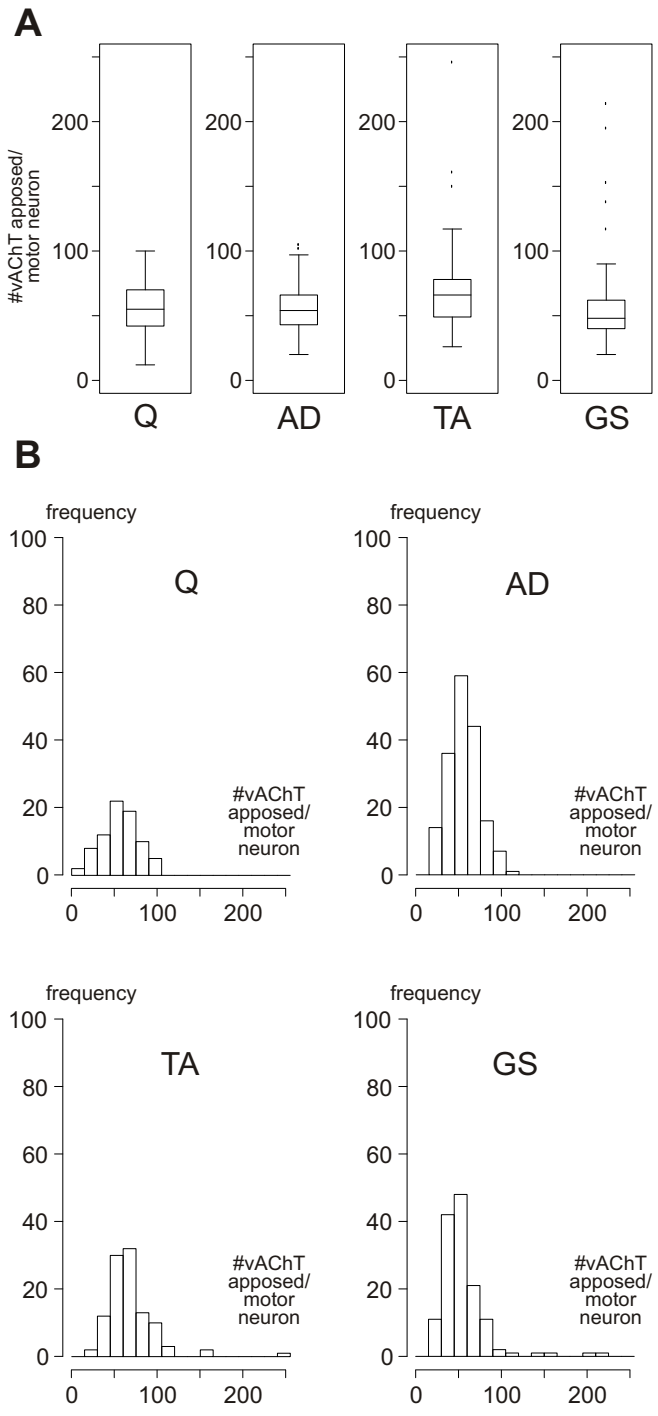

Figure 5.7: **Similar C-bouton input to different motor neuron pools analyzed.** Quantitative analysis of the number of vAChT appositions with motor neuron cell bodies retrogradely labeled with f-dex from Q, AD, TA, GS muscles (A:horizontal lines in box plot show medians; B: frequency histogram of vAChT apposed/motor neuron cell body)

support for a highly specific and spatially precise placement of premotor interneuron subtypes in the motor output circuit.

A striking feature of both Q and Tri premotor interneuron distributions is that 20% of all labeled interneurons are confined to lamina VIII and ventro-medial lamina VII of the contralateral spinal cord, with only few in other scattered positions, an observation consistent with previous studies in neonatal mice (Quinlan and Kiehn (2007)). This area is essentially devoid of labeled premotor interneurons ipsilaterally, suggesting the existence of a spatially delineated domain within the spinal cord, dedicated to the preferential integration of information for direct delivery to contralateral motor neurons, and an involvement in left-right coordination. Neurons in this domain are known to receive input from the reticular formation in the brainstem and contain both excitatory and inhibitory commissural interneurons with direct connections to contralateral motor neurons (Bannatyne et al. (2003); Jankowska et al. (2003); Kiehn (2006); Quinlan and Kiehn (2007)), as also observed in our experiments. Confining specific interneuron subpopulations to restricted spatial domains represents an elegant strategy to channel selective presynaptic input. Similar cellular mechanisms may be exploited by other premotor populations to assimilate dedicated or private presynaptic channels.

Analysis of premotor interneuron populations connected to functionally distinct motor neuron pools can also provide insight into the organization and function of motor circuits. We found that Cm and Tri premotor interneurons show major differences in three-dimensional arrangement. The dominant location for Cm premotor interneurons is the ipsilateral dorsal spinal cord at the level and caudal to the Cm motor neuron pool. Many of these interneurons are located in deep dorsal layers within the domain of vGlut1^{on} terminals (Figure 5.6) suggestive of direct input from mechanoreceptive sensory neurons (Luo et al. (2009)), information which in turn is conveyed directly to Cm motor neurons. Our anatomical virus tracing experiments thus support experiments testing the Cm-skin-reflex functionally (Theriault and Diamond (1988a)), and suggest the existence of a disynaptic circuit involved in the activation of Cm motor neurons (Figure 5.2 D). An important avenue to pursue in the future will be to evaluate whether also premotor interneuron populations connected to motor neuron pools with finer degrees of functional nuances show distinct distribution patterns. This includes in particular a careful comparison of premotor interneurons connected to motor neurons pools innervating extensor and flexor muscles, active in ipsilateral alternation during many movements. As demonstrated for the Tri-Cm analysis in this study, such experiments can also provide important insight into regulatory input to premotor interneuron populations and principles of circuit

organization.

At an analysis level gated towards neuronal subpopulations, while Renshaw cells are found exclusively ipsilaterally in close motor neuron pool proximity, V2- and dI3-derived premotor interneurons are positioned also solely ipsilaterally, but both locally and several segments away from the innervated motor neuron pool. Cholinergic partition cells on the other hand are found both ipsi- and contralaterally, but again are not confined to the level of the pool. In addition, long-distance propriospinal interneurons of characteristic morphologies made up a very small fraction of all labeled interneurons, providing direct connectivity hubs between cervico-lumbar and lumbo-cervical interneuron-motor neuron pairs (data not shown). These findings support a concept of subpopulation-specific distribution patterns within the spinal premotor interneuron cohort associated with a motor neuron pool.

Although the rabies virus technology has been praised for its faithfulness in retrograde and synaptic spreading compared to other transsynaptic virus techniques (Ugolini (2010)), careful evaluation of results and possible future methodological improvement have to be kept in mind as with all newly introduced technologies. In our study, we provide evidence for reliable visualization of several previously known premotor neuron subpopulations, but one important future application of the described method will of course be its exploitation as discovery tool for previously unknown premotor neuron populations. Detailed future studies also using complementary tools on individual populations will no doubt enlarge the know-how on the reliability of rabies virus based circuit tracing technology. While we cannot exclude the possibility that certain premotor neuron populations remain unlabeled in our experiments, we have at present no evidence that neuronal populations without synaptic connections to doubly-infected neurons are labeled. A recent study starting retrograde transsynaptic spread from single cortical neurons observed only sparse labeling of presynaptic neurons, and proposed that future work improving G-protein expression levels and additional mutations in the Rabies genome might overcome these problems (Marshel et al. (2010)). Finally, while connection specificity of several spinal premotor interneurons in mice already appears to be in mature configuration at neonatal stages (Mears and Frank (1997); Nishimaru et al. (2006); Wang et al. (2008)), we cannot exclude the possibility that certain premotor neurons revealed in our experiments are not connected in their mature pattern yet.

5.4.2 Synaptic specificity of a cholinergic neuromodulatory spinal interneuron population

Cholinergic partition cells provide direct neuromodulatory input to motor neurons through C-bouton synapses, regulating motor neuron excitability by reducing afterhyperpolarization (Conradi and Skoglund (1969); Hellstrom et al. (2003); Miles et al. (2007)). Recent studies demonstrate that partition cells represent a subpopulation of V0-derived interneurons marked by the transient expression of the transcription factor Pitx2 (Miles et al. (2007); Zagoraiou et al. (2009)), and that these V0c interneurons are involved in task-dependent motor neuron firing and muscle activation (Zagoraiou et al. (2009)). Yet, the synaptic connectivity scheme in place between cholinergic partition cells and distinct motor neuron pools has remained unaddressed.

Our findings provide evidence that cholinergic partition cells can be subdivided into two approximately equally-sized populations, one with entirely ipsilateral connections to motor neurons and a second population with a bilaterally bifurcating connectivity pattern to motor neurons. Furthermore, we found that the cell bodies of both of these populations are not positioned exclusively at the level of the innervated motor neuron pool, but also several segments rostrally and caudally thereof. The distributed segmental origin of C-bouton synapses as well as the observed divergent connectivity scheme raises intriguing possibilities about the regulation of this neuromodulatory input system by upstream connections to cholinergic partition cells. While no obvious overall differences in the nature of neurotransmitter profiles of the input to cholinergic interneurons were observed (Zagoraiou et al. (2009)), it is entirely conceivable that such differences exist only at more subtle levels of input analysis. For example, distinct sets of cholinergic partition cells could be activated at different times, depending on the segmental origin of input, yet leading to the regulation of the same downstream motor neuron population. The two subpopulations with divergent projection patterns may also be regulated by differential upstream input systems, allowing the activation of distinct motor behavioral output (see below). We found that partition cells do not receive synaptic input from any premotor interneuron population labeled by transsynaptic viruses (data not shown), excluding a regulation by bifurcating premotor interneurons and pointing to synaptically more distant neuronal populations at the core of cholinergic interneuron activation.

Previous evidence for the existence of bilaterally projecting spinal interneurons with direct bilateral connectivity to motor neuron pools is sparse. While bifurcating and bilaterally projecting interneuron populations in the spinal cord are commonly observed, most of

them have been described to connect to interneurons (Bannatyne et al. (2003); Jankowska et al. (2009)). Interestingly however, it has been noted that axonal termination patterns of bilaterally projecting interneurons frequently appear to be symmetric on the two sides of the spinal cord, pointing to a possible coordinator function to access certain interneuron populations on both spinal sides (Jankowska et al. (2009)). A second rather unexpected trait of this neuronal population is the high degree of synaptic specificity observed, with preferential targeting of corresponding motor neuron pools bilaterally. Although a cholinergic interneuron population with local and selective activity has recently been described to exist in the cortex (von Engelhardt et al. (2007)), neuromodulatory systems often act through diffuse pathways and are not generally known to exhibit a high degree of synaptic specificity (Lucas-Meunier et al. (2003)).

What could be the functional role of a bilaterally symmetric and synaptically precise organization of cholinergic modulatory input to motor neurons? By eliminating ChAT expression in all partition cells to mute synaptic output, previous experiments have demonstrated a role for these neurons in modulation of extensor burst amplitude specifically during swimming, implicating these neurons in task-dependent gain control of motor output (Zagoraiou et al. (2009)). These findings left open the exciting possibility that partition cells with divergent projection patterns (ipsi- vs bilateral and targeting functionally different motor neuron pools) may be differentially recruited during alternate tasks to specifically modulate motor neuron pools implicated in appropriate task execution. Bilaterally projecting partition cells with synaptic input to functionally equivalent motor neuron pools might for example be used during bilaterally symmetric movements or control of posture to modulate motor output, to prefigure coordinate and joint regulation of the same muscle groups bilaterally. In support, in addition to precise synaptic targeting of limb-innervating motor neurons, we also observed segmentally-matched collaterals of bilaterally projecting partition cells to motor neurons of the median motor column (data not shown), known to be involved in control of posture. Bi- and ipsilaterally projecting partition cells may also be activated by distinct upstream pathways, with the ipsilateral population more geared towards the regulation of left-right asymmetric movements. Our findings provide a possible synaptic mechanism for how this neuromodulatory system may mediate differential task-dependence of gain control, accessing exquisite sets of motor neurons with high synaptic precision, a mechanism which may also be applicable more generally throughout the CNS.

5.5 Experimental Procedures

5.5.1 Mouse genetics

$Lhx3^{on}$ (Sharma et al. (1998)), $Isl1^{Cre}$ (Srinivas et al. (2001)), PV^{Cre} (Hippenmeyer et al. (2005)), $Tau^{Lox-STOP-Lox-mGFP-IRESnlsLacZ}$ (Hippenmeyer et al. (2005)), $GAD67^{GFP}$ (Tamamaki et al. (2003)) and $vGlut2^{Cre}$ (Borgius et al. (2010)) mouse strains have been described previously. All mouse strains were maintained on a mixed genetic background (129/C57Bl6).

5.5.2 Virus and retrograde tracing experiments

Rab-mCherry and Rab-GFP viruses were produced in BHK21 cells stably expressing G-protein for complementation to produce G-protein pseudotyped viruses as described (Marshel et al. (2010); Wickersham et al. (2007a); Wickersham et al. (2007b); Wickersham et al. (2010)), and viral titers were determined using BHK21 cells and FACS analysis as described (Wickersham et al. (2010)). G cDNA (Wickersham et al. (2007b)) was cloned into pAAV-MCS (Stratagene) and AAV of serotype 6 was produced at a titer of $3e10^{12}$ (Applied Viromics). For muscle injections, rabies viruses were set to titers of $1e10^{8}$ and complemented by equal volumes of AAV-G-protein for transsynaptic labeling experiments just prior to muscle injections. Injections were targeted to defined muscle groups, including all separate muscle heads with anatomical separation (e.g. Q, Tri, GS), with 5μl of mixed virus solution (or where indicated 2.5μl of only rabies virus) used for individual muscle injection experiments at p7. Animals were sacrificed 8 days following virus injection at p15 upon Ketamine (Vetoquinot AG) / Xylazine (Bayer Healthcare) terminal anesthesia, using perfusion fixation (ice cold PBS followed by 4% PFA). Upon dissection, spinal cords were fixed for an additional 4-6 hours on ice and cryoprotected by immersion in 30% Sucrose/PBS overnight. For retrograde labeling of motor neurons using fluorescent dextran dyes (FITC or tetramethylrhodamine-conjugated; Invitrogen), injections were performed as previously described by targeting specific muscles (Pecho-Vrieseling et al. (2009)).

5.5.3 Immunohistochemistry and imaging

Antibodies used in this study were: chicken anti-GFP (Invitrogen), rabbit anti-Chx10 (Arber et al. (1999)), guinea pig anti-fluorescein (Vrieseling and Arber (2006)), guinea pig

anti-vGlut1 (Chemicon), goat anti-ChAT (Chemicon), goat anti-LacZ (Biogenesis), goat anti-vAChT (Chemicon), mouse anti-NeuN (Chemicon), rabbit anti-Calbindin (Swant), rabbit anti-Cre (Abcam), rabbit anti-CGRP (Chemicon), rabbit anti-GFP (Invitrogen), rabbit anti-LacZ (Invitrogen), rabbit anti-RFP (Chemicon), rabbit anti-tetramethylrhodamine (Invitrogen), rabbit anti-vAChT (Sigma). Spinal cords were sectioned using a cryostat at 40μm section thickness unless otherwise indicated. Cryostat sections were processed for immunohistochemistry using primary antibodies listed above and fluorophore-conjugated secondary antibodies (Invitrogen and Jackson Laboratories) as described before (Pecho-Vrieseling et al. (2009)). Images were acquired with an Olympus confocal microscope (FV500) or a custom made dual spinning-disk microscope (Life Imaging Services GmbH, Basel, Switzerland) including the following main components: Olympus BX61 microscope, Yokogawa CSU-X1 confocal scanner, LIS Medusa synchronization unit, LIS LaserBank (405/491/561/640nm), Photometrics Cascade II:1024 EMCCD camera, ASI MS-2000 XYZ-stage, MDS Metamorph Software. Images were deconvolved using Huygens Remote Manager v1.2.3. For quantitative analysis of synaptic input to motor neurons, IMARIS colocalization tool (version 7) and ImageJ with the plugin Cell Counter (version 2009; Kurt De Vos, University of Sheffield, Academic Neurology, UK) was employed to assess cholinergic input to motor neurons, using image stacks acquired at 0.21μm confocal steps and 60x magnification.

5.5.4 Statistical analysis

To compare the spatial distribution of premotor neurons across individual experiments, we computed 2d density estimates (Venables and Ripley (2002)) with a kernel size of 250x250. For each three-dimensional dataset analyzed by comparative analysis, this resulted in a set of matrices representing neuron density sampled at a resolution of 100 times 100. Similarity between two different conditions was measured by the Pearson correlation coefficient, using a total of 100^2 data points. This analysis was performed for all experimental pairs, resulting in a matrix of pairwise correlations. Densities, contour distributions and box plots were calculated and plotted in R project (R Foundation for Statistical Computing, Vienna, Austria, 2005. http://R-project.org). For all box plots shown, the midline indicates the median value, box limits represent the 25^{th} and 75^{th} percentile, whiskers extend to the most extreme data point if less than 1.5x the interquartile range from the box, and outside values are plotted as single points. Kernel densities for

contour plots were estimated using the Kde2D function in the MASS library.

5.6 Acknowledgments

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Chapter 6

Premotor circuits of the α-2-chimaerin mutant mice.

6.1 Introduction

Spinal circuitry allows generating locomotion. The hypothesis of the central pattern generators (CPGs) in the spinal cord assumes that the intra-spinal local networks that generate and coordinate rhythmic muscle activities of the ipsilateral or of the contralateral limbs exist in the spinal cord on both sides and at different segmental levels (Grillner (2003); Kjaerulff and Kiehn (1997)). Spinal CPGs involved in coordination of left - right limb activity, need information flow across the midline of the spinal cord. But at the same time, circuits involved in the alternation of ipsi-lateral limb muscles or flexor-extensor activity need to communicate with the ipsilateral neurons of particular type but not the contralateral ones. Assuming that the spinal circuitry achieves its final shape due to the same processes on the left and right side of the spinal cord, there is a need for a midline border that prevents certain axons from passing through, but is open for selected populations and for those does not allow a second passage. Studies on axon guidance have shown the existence of such a midline border to control selective crossing of only certain axons. One of the first studies in the area of axon guidance was performed on spinal commissural axons. It was noticed that Netrin-1, present at the midline in the spinal cord can act as a chemoattractant for commissural axons and that its source are the floor plate and midline cells (Serafini et al. (1994); Kennedy et al. (1994)). Further studies demonstrated that the midline in the spinal cord is a source of attractants for midline crossing axons being at the same time a repellent source for axons that should not cross the

midline or recross it back after primary crossing (Colamarino and Tessier-Lavigne (1995); Tessier-Lavigne and Goodman (1996); Kaprielian et al. (2001)). Other studies have shown that commissural axons, before crossing the midline respond to the attractants derived from the floor plate: Netrin-1 and Shh (Kennedy et al. (1994); Charron et al. (2003)). Repellents of the Slit and Semaphorin families expel axons from the floor plate after they crossed the midline and prevent recrossing (Zou et al. (2000); Long et al. (2004)). The moment of crossing the midline induces changes in the cell profile since pre-crossing axons are insensitive to midline repellents and attraction to floor plate attractants is silenced in post-crossing axons (Shirasaki et al. (1998); Zou et al. (2000); Stein and Tessier-Lavigne (2001)). There are few particularly well-studied midline border axonal guidance systems.

6.1.1 The ROBO/Slit model

Receptors of the Slit molecules belong to the family of ROBO (named after its founding member roundabout in Drosophila). There are three mammalian Slit homologues (Slit1, Slit2, Slit3) and four ROBO family members (ROBO1-ROBO4). In the developing spinal cord, Slit genes are expressed by the floor plate and motor neurons, whereas Robo1 and Robo2 expression is not restricted to a particular area but its expression level depends on the state of the axons. The expression level is low on axons that are navigating but increases after axons enter the ventrolateral funiculus (Long et al. (2004)). Many interneurons including the commissural ones express Robo1 and Robo2 proteins (Itoh et al. (1998); Brose et al. (1999); Li et al. (1999)), while Robo3 is expressed exclusively in the commissural subpopulation of interneurons. There are two splice forms of ROBO3 that act in opposite manner (Sabatier et al. (2004); Chen et al. (2010)). Robo3.1 prevents from premature responses to Slits and is expressed only on precrossing axons, whereas Robo3.2 is expressed after the moment of midline crossing and in the presence of Slits contributes in the process of repulsion from the midline (Chen et al. (2010)) (see Figure 6.1). Most likely, there are also other receptors for Slits than Robo proteins. In a set of mutant mice for Robos (Jaworski and Tessier-Lavigne (2010)) found that loss of Robo1 and Robo2 does not fully rescue midline crossing in mice lacking Robo3. It is possible that Robo3 plays an additional role as a receptor for a putative midline attractant in this process.

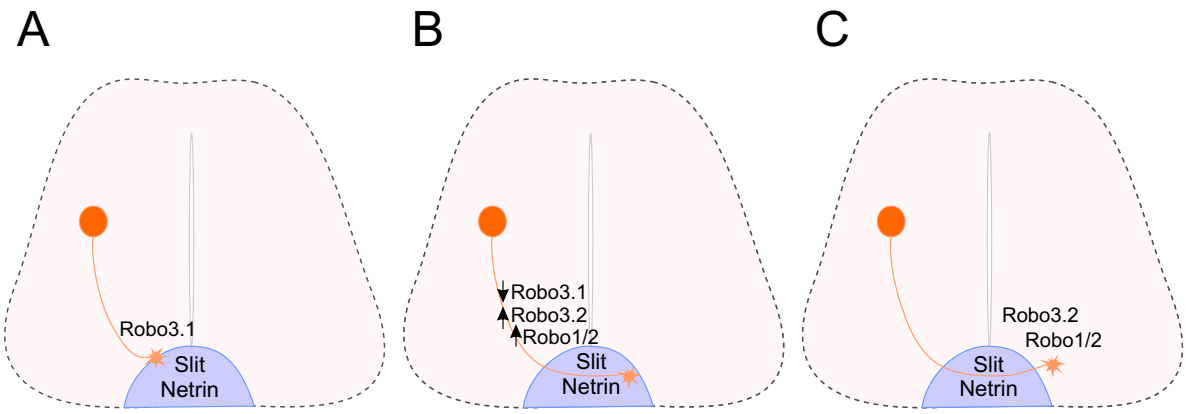

Figure 6.1: **Role of Robo in midline crossing by commissural axons.** Robo3 is expressed exclusively in the commissural neurons responsive to the midline attractant Netrin-1. Robo3.1 is expressed only in the precrossing axons (A) and prevents from premature responses to Slits. Robo3.2 is expressed from the moment of midline crossing(B and C). Robo1 and Robo2 expression starts after the moment of midline crossing and prevents from back-crossing

6.1.2 Ephrin-B3 and EphA4

Another set of molecules forming a midline border regulates axonal ingrowth to the contralateral side of the spinal cord. Ephrins are surface-associated molecules that bind to the big class of receptor tyrosine kinases - Ephs. Binding ephrin molecule by the Ephs induces internal signaling in the neuron leading to axonal growth cone collapse and axon retraction (Egea and Klein (2007)). Based on the sequence similarities of their extracellular domain the Eph family receptors can be divided into two groups, EphA and EphB (Orioli and Klein (1997)). Each EphA receptor is able to bind several Ephrin A ligands and EphB group of receptors binds predominantly to ephrin-B ligands (Gale et al. (1996a); Gale et al. (1996b)). There is one exception from this rule - EphA4 (known also as Sek1) receptor was found to bind to some more ligands than to Ephrins A, it can also bind ephrin-B3 (Gale et al. (1996a); Gale et al. (1996b); Bergemann et al. (1998)). The signaling pathways downstream of Eph activation are not fully understood but roles of the effectors of the Eph receptors - Rac-specific GTPase-activating proteins (Rac-GAPs) have been shown in the regulation of the outgrowth and pathfinding on neuronal axons and dendrites (Wegmeyer et al. (2007); Beg et al. (2007)). Several studies described the role of ephrin-B3/EphA4 forward signaling in the formation of the circuit between neurons in layer 5 of motor cortex and the spinal circuits in the lumbar spinal cord or in the midline crossing process by spinal interneurons (Dottori et al. (1998); Coonan et al. (2001);

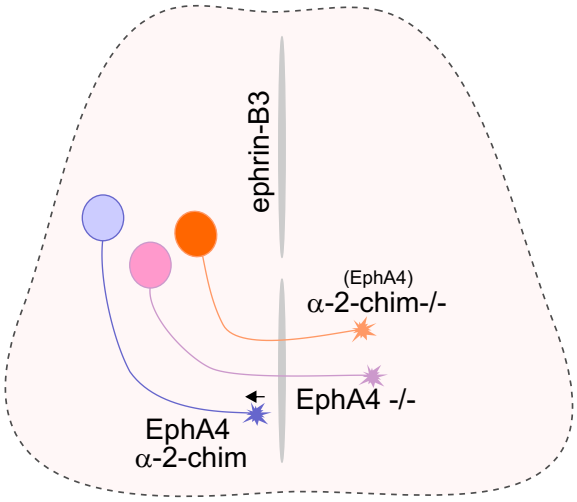

Figure 6.2: **Ephrin-B3, EphA4 and α-2-chimaerin interactions.** Ephrin-B3 is expressed at the midline in the spinal cord, axons expressing EphA4 are repelled by ephrin-B3, neurons lacking EphA4 or α-2-chimaerin do not react to the ephrin-B3 and cross the midline

Kullander et al. (2001); Butt and Kiehn (2003)). Interactions between midline repellent ephrin-B3 and its receptor EphA4 are already functional during early development of the spinal cord, when circuit formation and axonal outgrowth happens. (M Douglas Benson and Parada (2005)) demonstrated that ephrin-B3 is expressed along the midline in the spinal cord by oligodendrocytes and that genetic ablation of ephrin-B3 results in a hopping-gait locomotion and extensive midline crossing of axons descending from the motor cortex. Mice mutant for EphA4 show similar phenotypes as ephrin-B3 mutants - the hopping gait locomotion and aberrant midline crossing by descending tracts and spinal interneurons (Kullander et al. (2001)). Since the hopping gait locomotion is already observed during the very early postnatal period when cortico-spinal neurons only start to invade the spinal cord, the locomotor phenotype develops most likely due to the changes in the spinal cord (Figure 6.2).

Kullander et al., investigated the role of local spinal CPGs in the abnormal locomotive behavior in EphA4 and ephrin-B3 mutant mice. They examined locomotor activity in isolated spinal cords from newborn mice in wild type and in EphA4 mutants. Normally, in a wild type in vitro preparation, rhythmic locomotor activity can be induced by addition of serotonin and N-methyl-D-aspartate (NMDA) to the bath. Rhythmic locomotor activity in this preparation, revealing the alternation between left and right lumbar ventral root bursts (Kjaerulff and Kiehn (1997); Whelan et al. (2000)), is thought to be a reflection of the left and right limb activity. In this preparation, also alternation between

L2 and L5 activity on the same side of the spinal cord can be observed (similar to the flexion and extension in one limb during locomotion). While wild type spinal cords generated alternating activity of the left and right L2 or L5 ventral roots, EphA4 mutants and ephrin-B3 mutant mice generated synchronized activity of left and right L2 and L5 ventral roots (Kullander et al. (2003a)). However after pharmacological manipulation by strengthening the inhibition in spinal cord networks, when blockade of the glycine reuptake was applied by addition to the bath of sarcosine, the activity of the ventral roots in EphA4 mice changed its pattern to alternated at single segmental levels on left and right sides. Thus, chemical reinforcement of the CIN inhibitory component to counteract a stronger aberrant excitatory innervation restored the wild type-like locomotion pattern. The conclusion of this study was that fibers that aberrantly cross the midline and connect to contralateral CPG components in EphA4- and ephrin-B3-null mice are predominantly excitatory. This model would predict that excitatory EphA4 positive interneurons involved in locomotor rhythm generation normally provide excitatory input to ipsilateral CPGs. However, EphA4 is expressed in both inhibitory and excitatory interneurons in the ventral and dorsal spinal cord (Butt et al. (2005)) and it has not been resolved yet due to which types of misguided projections of spinal interneurons the hopping gate phenotype occurs. Butt et al. provided also evidence that although EphA4 positive interneurons are not a homogeneous population, some of the ventral ones fire in a rhythmic manner. Important to mention here is that the CPG neurons are thought to be in the ventral spinal cord, shown by the study of Kiehn and Kjaerluff in neonatal rodent spinal cord preparations. In this study, recordings from pieces of spinal cord have suggested that rhythm generating neuronal networks can be found in the ventral spinal cord. This study influenced the view on the CPG so much that many further studies continued to look for the CPG - involved interneurons in the ventral spinal cord. Kullander and Butt focused their analysis exclusively on ventral interneurons where in a set of in situ hybridization for neurotransmitters and EphA4 expression (Kullander et al. (2003a)) or single cell recordings (Butt et al. (2005)), they tried to characterize EphA4 positive interneurons. Later independent studies identified another factor regulating axonal growth cone collapse. Wegmeyer et al. (2007) and Beg et al. (2007) have shown that the Rac-specific GTPase-activating protein a2-chimaerin binds activated EphA4 and mediates EphA4-triggered axonal growth cone collapse that leads to axonal repulsion. In vivo lack of α-2-chimaerin results in aberrant axonal midline crossing in spinal interneurons and corticospinal neurons. Again, mutant mice exhibit hopping gate locomotion already at early postnatal stages. However expression of α-2-chimaerin is very broad and its role in axonal guidance in interaction

with other transmembrane receptors is to be expected. A fascinating issue here is that although EphA4 and α-2-chimaerin molecules are expressed by a broad spectrum of the spinal interneurons, lack of them does not cause an overall disruption of all locomotor behavior. For example, flexor-extensor alternation seem to be preserved while only left-right alternation transforms to synchronization. I found it interesting to understand how this pattern is formed in terms of premotor interneuronal connectivity and which elements of the spinal locomotor control circuit are affected. Traditional tracings of midline crossing axons, where a dextran or another dye was injected into one side of the spinal cord, marks any type of crossing fibers and their relation to motor neurons in terms of connectivity is not clear. In this context, it can for example be important whether commissural neurons contact another population of neurons or project directly to motor neurons and whether this concerns both flexor and extensor interneurons or just one of these two groups. In this part of my thesis project, I have performed monosynaptically restricted rabies virus tracing experiments from hindlimb muscles in α-2-chimaerin mutant mice. I have found premotor interneuron distribution patterns significantly different from the wild type patterns. In fact, some additional premotor neurons, with midline crossing axons were observed, but the subclass of cholinergic partition cells expressed another phenotype and a smaller fraction of these neurons crossed the midline than in wild type.

6.1.3 Premotor interneurons tracing in α-2-chimaerin mutants

The primary goal of this study was to understand which premotor interneurons in the spinal cord are dependent on EphA4 mediated axon repulsion and connect to the wrong partners in the mutant situation. Since both EphA4 mutants and α-2chimaerin mutants exhibit similar locomotor phenotype - the hopping gate, it is possible that similar phenotypes in the spinal circuitry of these two mutants in terms of mis-wired interneurons can be found. We decided to perform the monosynaptic rabies tracing of premotor neurons in alph α-2-chimaerin and EphA4 mutant mice to reveal possible phenotypes at the cellular level. For technical reasons, the experiments on EphA4 mutant mice had to be postponed but the injections of hindlimb muscles of the α-2-chimaerin mutant mice revealed interesting patterns of premotor distribution patterns.

6.2 Premotor interneuron pattern revealed by monosynaptic rabies virus tracing in α-2-chimaerin mutant mice

6.2.1 Experimental procedures

Two kinds of experiments were performed - specific muscle (Q) injections and broad hindlimb muscle injections, where at least Q, TA, GS and their neighboring muscles were injected. The purpose of the Q muscle injections was to compare the distribution pattern of the premotor neurons in the α-2-chimaerin mutant mice with the one observed in wild type mice and to find the population of premotor interneurons dependent on the EphA4 mediated axon collapse. Using our monosynaptically restricted rabies virus tracing approach, it is possible to restrict the analysis of the commissural neurons to the premotor interneurons and study the connectivity of other premotor interneurons that do not belong to the commissural population. The purpose of the unspecific injection into the hindlimb muscles was to target as many as possible premotor interneurons and among them the ones that project bilaterally to the motor pools of both sides of the spinal cord. This was to understand the influence of the midline barrier on the connectivity of the premotor interneurons and among them those located at the midline or in a very close proximity. For this purpose, rabies tracing experiments were performed simultaneously from both limbs using RFP and GFP rabies viruses. In this setup, I expected to double label bilaterally projecting neurons and among them the cholinergic partition cells to test whether these neurons are also sensitive to the lack of the α-2-chimaerin.

Rabies tracing experiments were performed following the same procedures as described in the previous chapter. Also tissue preparation, cutting, antibody staining and reconstructions were performed like in the previous chapter using the same antibodies and reagents. Injections of the mix of deltaG rabies virus and AAV-Gly into the Q muscle or to the Q, TA, GS and their neighboring muscles were performed at p7 in α-2-chimaerin mutant animals. Reconstructions of the premotor interneuron distributions were done the same way as described in the previous chapter. The distribution patterns of the mutant premotor interneurons were compared to the ones obtained from the wild type animals. In case of bilateral hindlimb injections, the spinal cords were analyzed for the interneurons double positive for RFP and GFP and distributions of these interneurons were reconstructed.

6.3 Results

6.3.1 General patterns of premotor interneuron distribution in α-2-chimaerin mutant mice differ from wild type patterns especially in the dorsal area

After injection of monosynaptically spreading rabies viruses into the Q muscle of α-2-chimaerin mutant mice, observed distribution patterns differed significantly from the wild type patterns in the dorsal half of the spinal cord. General tracing of premotor interneurons revealed ectopic contralateral dorsal interneuron populations (Figure 6.3) and at the same time, the ipsilateral dorsal interneuronal population was shifted towards the midline and many cell bodies were placed directly on the midline. As a result, it was hard to distinguish between ipsi- and contralateral dorsal population of interneurons in α-2-chimaerin mutant mice as their cell bodies were spread about 200 μm from the midline in both directions. This feature can be nicely seen on the density plot (Figure 6.3 F) and on the visualization of the longitudinal distribution of ventral and dorsal premotor subpopulations (Figure 6.4). A less pronounced phenotype concerned the contralateral ventral population of Q premotor interneurons. The general impression is that there are comparatively fewer contralateral ventral premotor interneurons in α-2-chimaerin mutant mice than in wild type mice (Figure 6.4 A), but this observation was not consistent between all experiments so far (n=3, data not shown) and needs additional experiments.

6.3.2 Dorsal Q premotor interneurons project across the midline to contralateral motor neurons and in the dorsal area.

Detailed analysis of the dorsal population of Q premotor interneurons in the α-2-chimaerin mice revealed 2 types of phenotypes. The cell body positions were closer to the midline as mentioned above but, in addition, axons and dendrites of these dorsal interneurons crossed the dorsal midline (Figure 6.5), a phenotype not observed in wild type mice (Figure 6.5 B). It is important to point out here that the interneurons belonging to the dorsal premotor population in wild type mice probably connect to each other within the population. Easy to observe are the dense networks of axons detected around their cell bodies of these dorsal neurons (Figure 6.5 B). However these terminals could also originate from more anterior spinal neurons or from the brainstem. Such dense axonal networks were also observed within the dorsal population of premotor interneurons in α-2-chimaerin mutants, but on

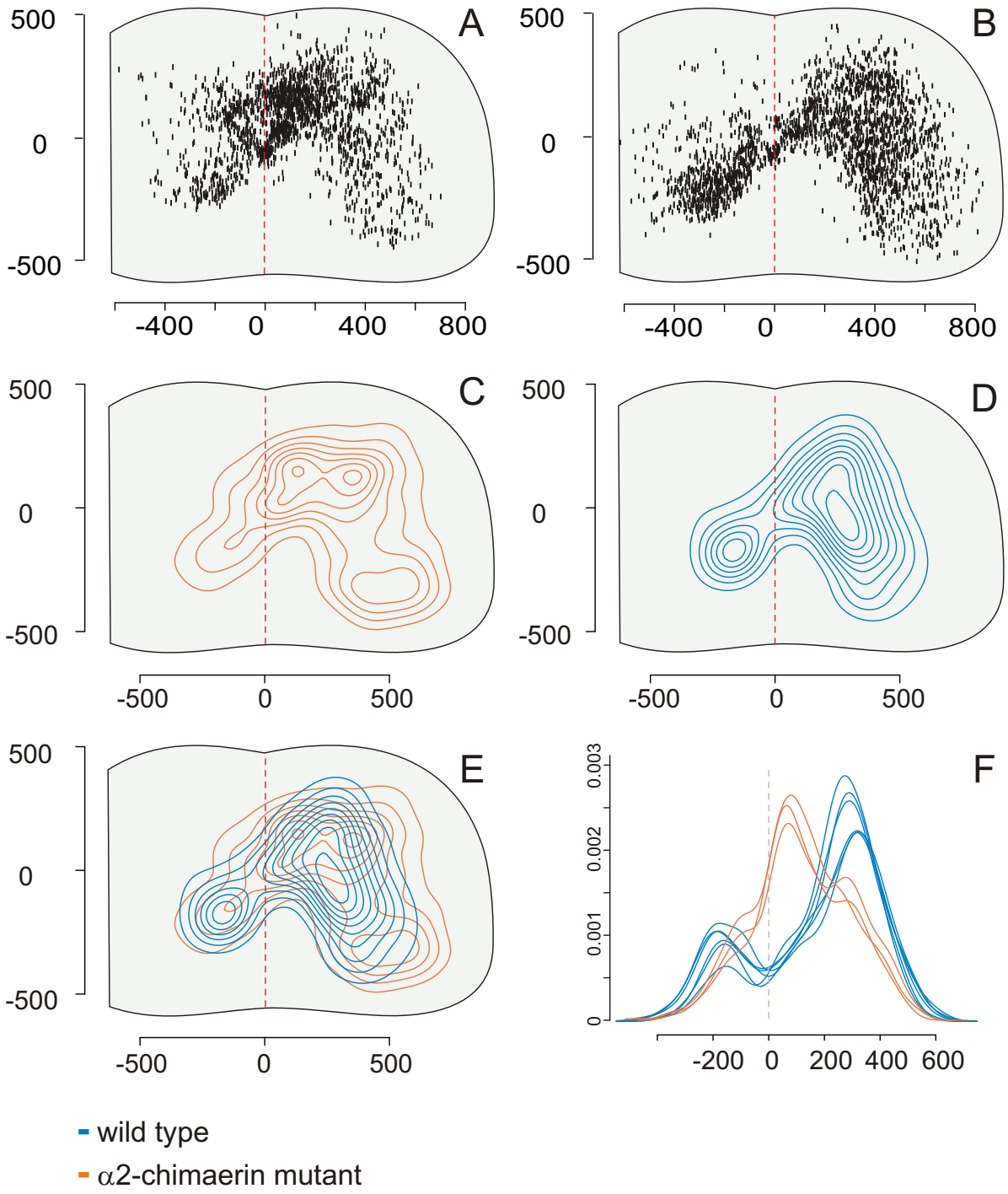

Figure 6.3: **Distribution pattern of premotor interneurons in α-2-chimaerin mutant mice.** Distribution of premotor interneurons on the crossection plane of α-2-chimaerin mutant mice: A and C, and wild type mice: B and D for the Q MNs; E - overlap of the contours, orange - α-2-chimaerin mutant, blue - wild type; F - density plots projection on medio-lateral axis, colors like in E

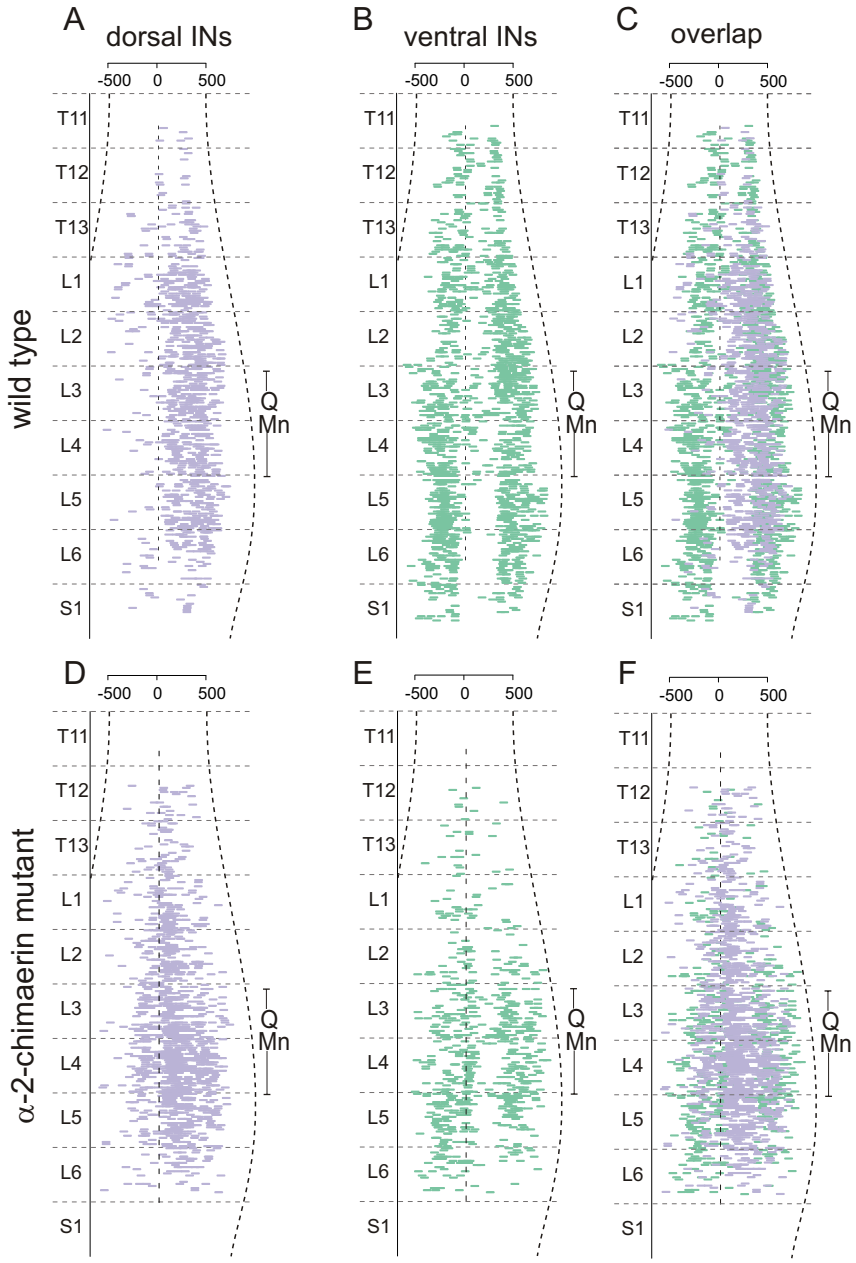

Figure 6.4: **Distribution pattern of Q premotor interneurons along the rostro-caudal axis in α-2-chimaerin mutant and wild type mice.** Distribution of dorsal subpopulation of premotor interneurons on the longitudinal plane in α-2-chimaerin mutants (D), and wild type (A) vs the ventral subpopulations, corresponding - (E and B). F and C - overlap, QMn indicates longitudinal spread of Q motor neurons

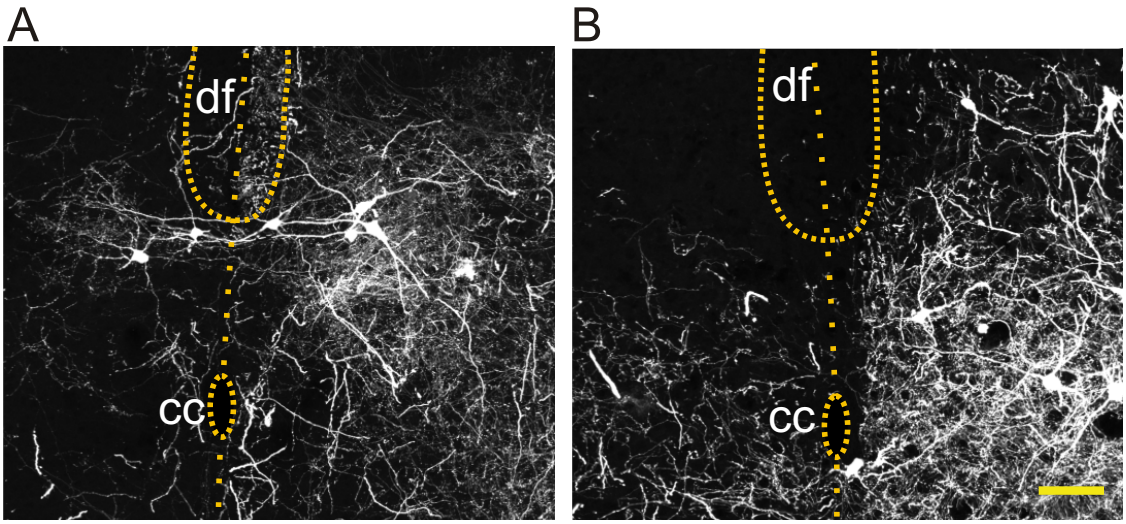

Figure 6.5: **Distribution of dorsal interneurons in α-2-chimaerin mutant mice and in wild type.** Premotor interneurons of the dorsal spinal cord in α-2-chimaerin mutants are found on ipsi- and contralateral side (A), in the wild type case they can be found only on the ipsilateral side (B). These interneurons do not extend their dendrites through the midline in wild type (B) but do that in the mutant case (A). Dorsal funiculus (df), midline and the central canal (cc) are indicated by the yellow dashed line. Scale bar, 100μm

both ipsi- and contralateral sides (Figure 6.5 A).

To test whether the input to the dorsal interneurons belongs to the inhibitory or excitatory category, antibody staining to vGlut2 and GAD67 were performed. Presence of both was noticed in the terminals around the cell bodies of the dorsal population of interneurons (Figure 6.6) indicating that the dorsal premotor population of interneurons receives input from both - glutamatergic and GABAergic projections In bilateral injections, in which monosynaptically spreading GFP-rabies was injected into the left Q and RFP-rabies into the right Q in α-2-chimaerin mutants, some double positive dorsal interneuron were found (Figure 6.7 A).

This observation is a sign that at least some of these interneurons connect bilaterally to motor neurons in α-2-chimaerin mutants. In contrast, in wild type, bilaterally projecting

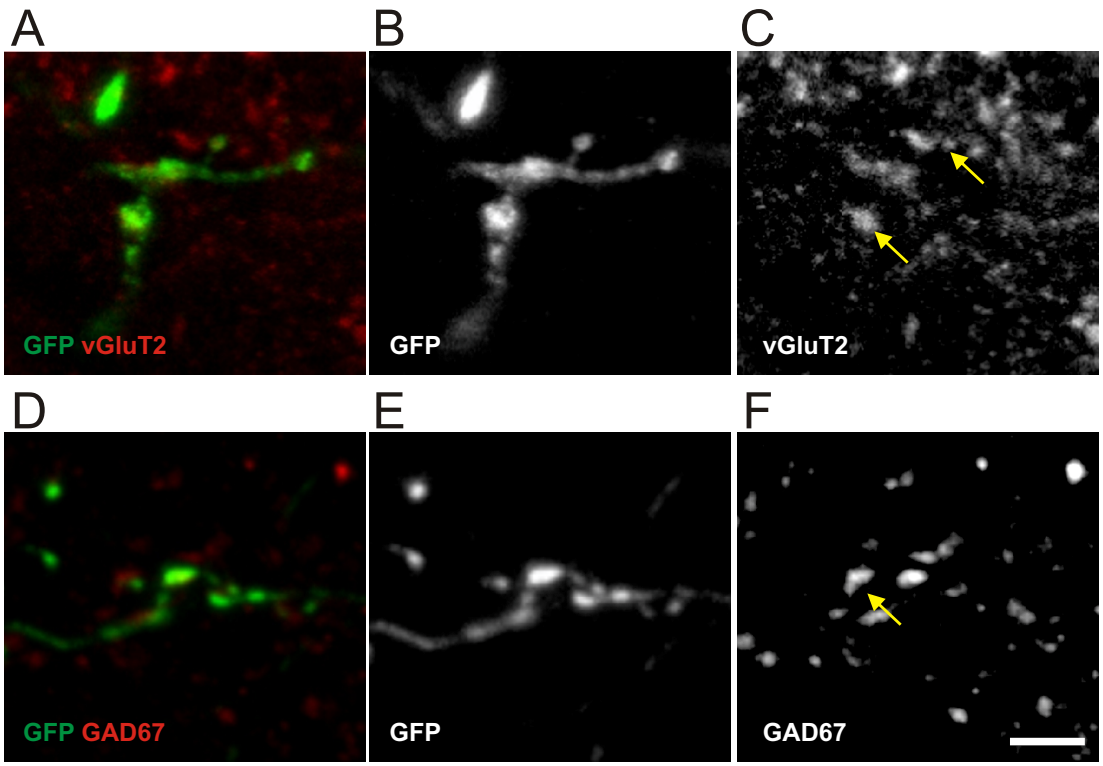

Figure 6.6: **Neurotransmitter phenotype of terminals in the dorsal spinal cord in α-2-chimaerin mutants.** Terminals in the dorsal spinal cord area, occupied by the ectopic contralateral premotor interneurons in the α-2-chimaerin mutants contain vGlut2 (A-C) and GAD 67(D-F). This shows their inhibitory and excitatory nature. Scale bar, 20μm

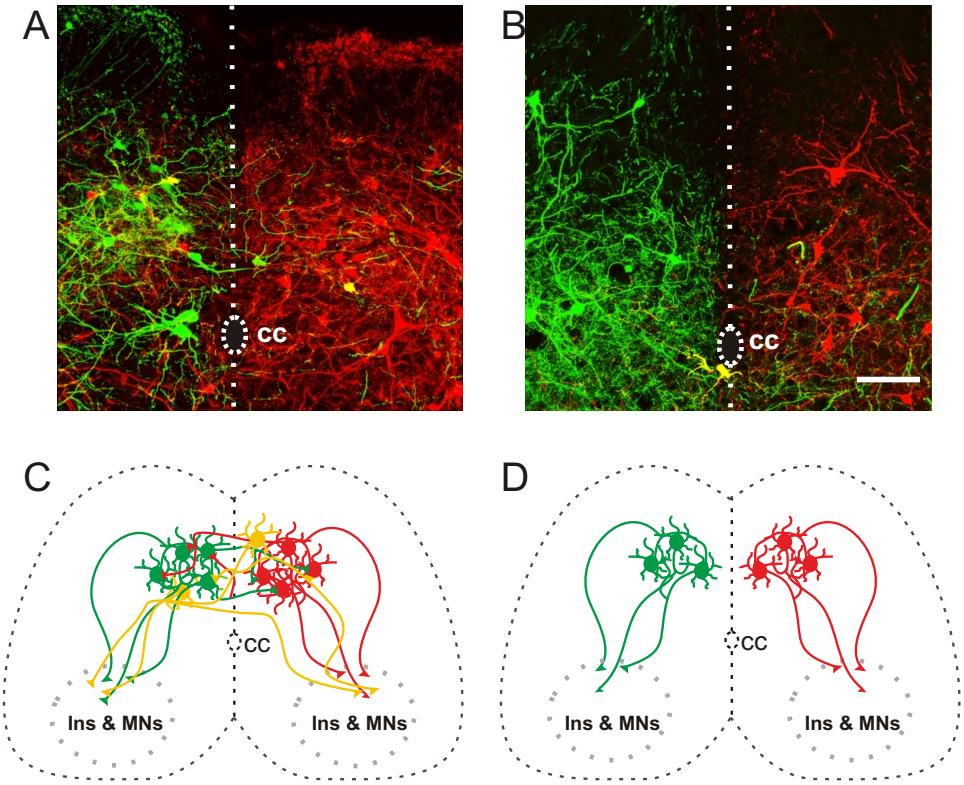

Figure 6.7: **Bilateral tracing from Q in α-2-chimaerin mutants and wild type mice.** In wild type mice bilaterally projecting cells (yellow) are found at the midline (B) but not in the dorsal spinal cord. In α-2-chimaerin mutants bilaterally projecting cells occupy the the dorsal spinal cord(A), C and D show proposed connectivity of the dorsal subpopulation of the Q premotor interneurons in α-2-chimaerin mutants and wild type, respectively. A,B - midline and central canal indicated by white dashed line

interneurons in this area are generally not detected in such assays (Figure 6.7 B). Above results show that a dorsal population of Q premotor interneurons in α-2-chimaerin mutant mice is characterized by different connectivity patterns than in wild type (Figure 6.7 C and D), but further experiments are needed to define the details of these observed phenotypes.

6.3.3 Bilaterally projecting interneurons in α-2-chimaerin mutants distribute differently than such interneurons in the wild type spinal cord.

To test whether lack of α-2-chimaerin influences interneurons connecting to motor neurons bilaterally, I performed massive bilateral injections of GFP-rabies and RFP-rabies into hindlimb muscles of wild type mice and α-2-chimaerin mutants. Bilaterally projecting

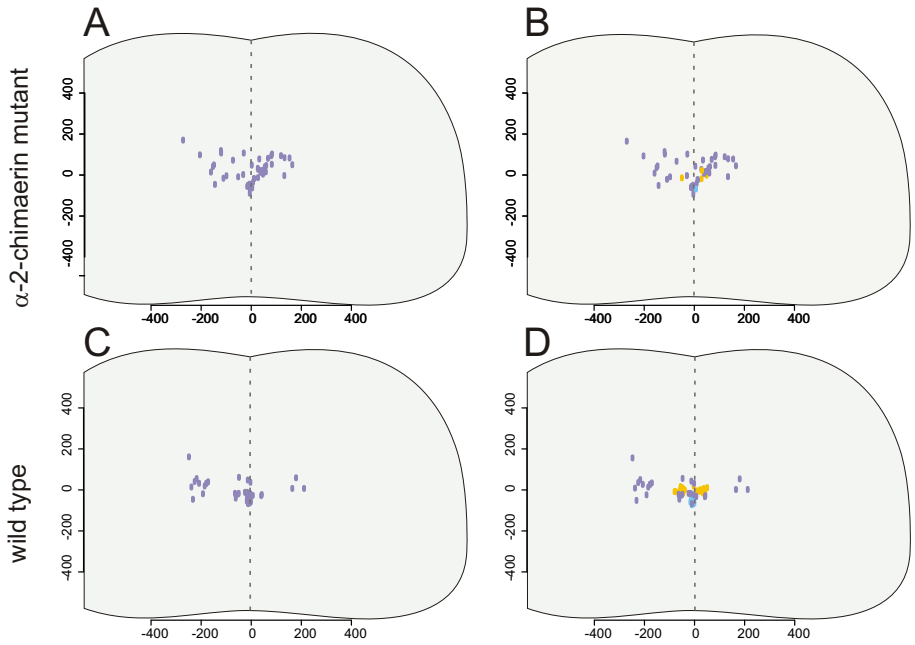

Figure 6.8: **Bilateral interneurons revealed by massive tracing from hindlimb muscles in α-2-chimaerin mutants and wild type mice.** In wild type mice bilaterally projecting cells are found at the midline (C), close to central canal(0,0) and laterally to it. In α-2-chimaerin mutants bilaterally projecting cells are found in the dorsal spinal cord (A) and at the midline. B and D show bilateral interneurons split in to classes: yellow - cholinergic partition cells, light blue - midline cells with elongated body shape, violet - not classified INs.

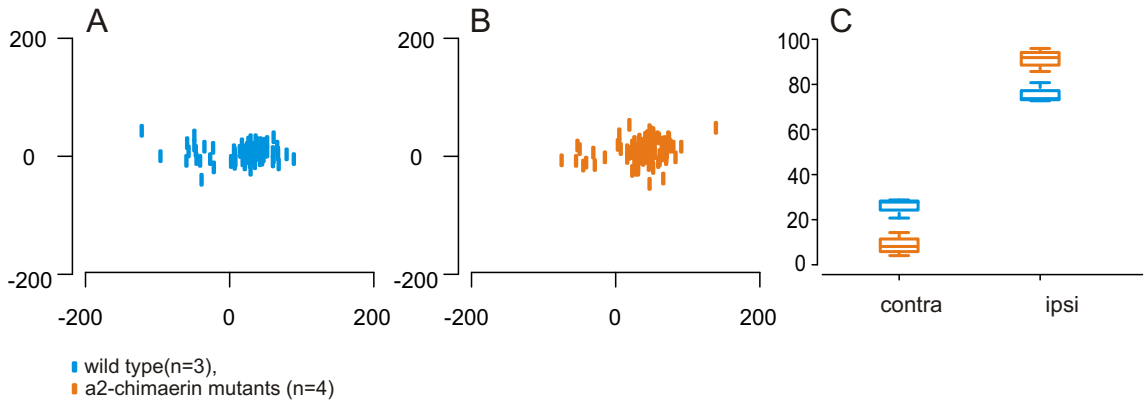

Figure 6.9: **Distribution of cholinergic partition cells after massive injection into hindlimb muscles in α-2-chimaerin mutants and wild type mice** In wild type mice 25% of cholinergic partition cells belongs to the contralateral population (A and C), in α-2-chimaerin mutants only about 10% (B and C).

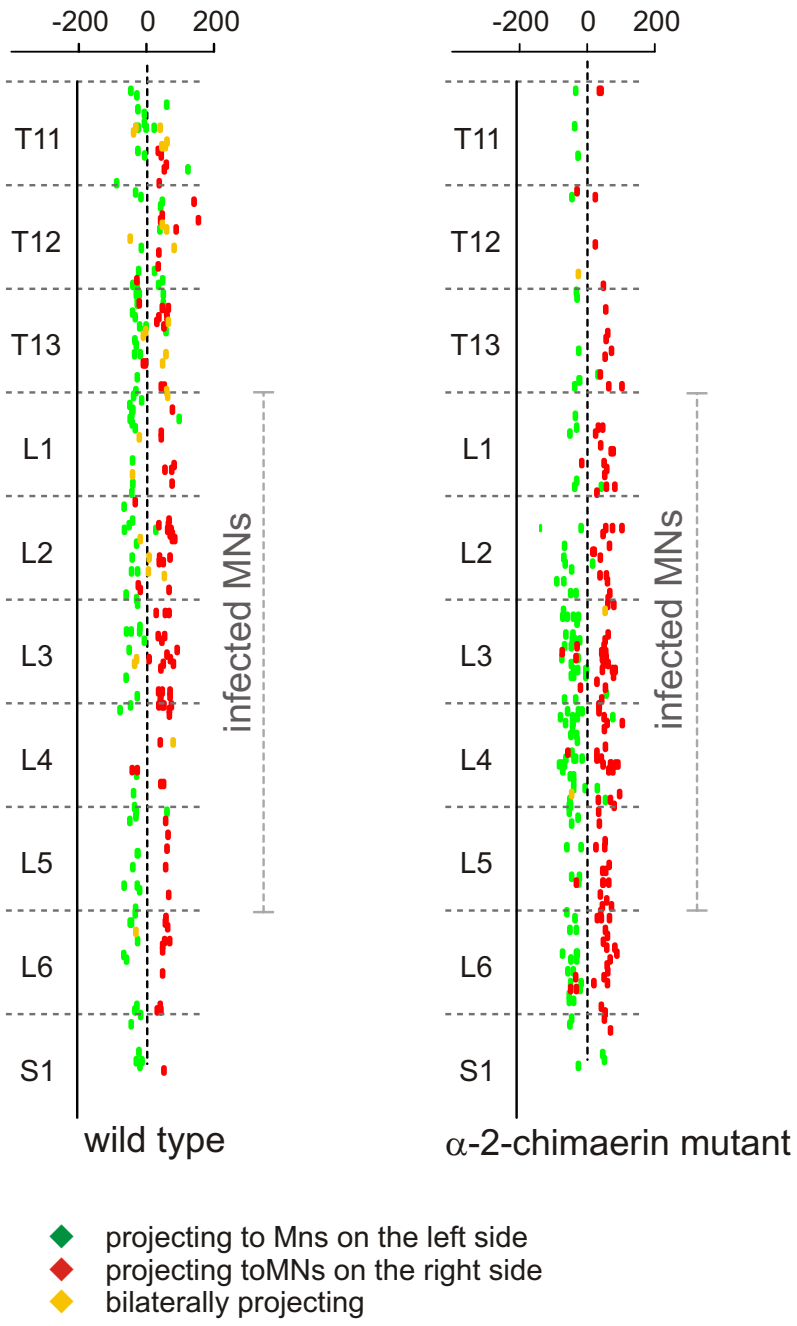

Figure 6.10: **Distribution of cholinergic partition cells after massive injection into hindlimb muscles in α-2-chimaerin mutants and wild type mice on the longitudinal plane.** In wild type mice (left) several bilaterally projecting cholinergic partition cells can be found (yellow) while in α-2-chimaerin mutants (right) they are sparse. Lack of contralateral partition cells is also pronounced in α-2-chimaerin mutants (right).

neurons found in wild type mice belonged mainly to 3 classes: midline cells positioned just under or above the central canal, some lateral neurons in the middle of the dorso-ventral extent of the spinal cord and cholinergic partition cells (described in previous chapter)(Figure 6.8 bottom). In α-2-chimaerin mutant mice, distribution patterns of bilaterally projecting premotor interneurons were different than in wild type (Figure 6.8 top). To our surprise, not many of the midline cells were found to be bilaterally connecting to motor neurons in mutant mice, the lateral population of bilaterally projecting interneurons was not observed and many bilateral interneurons were found in the dorsal half of the spinal cord. Also, only very few of the cholinergic partition cells in α-2-chimaerin mutants connected bilaterally to motor neurons. This result led me to check the general distribution of cholinergic partition cells in α-2-chimaerin mutants. Previously, I have shown that about 25% of cholinergic partition cells traced monosynaptically from one Q muscle have cell bodies with contralateral position in wild type mice. These results were also similar for the broad hindlimb injection experiments in wild type (Figure 6.9 A and C). In contrast, for α-2-chimaerin mutant mice, the ratio between ipsi- and contralateral interneurons was 9 to 1 (Figure 6.9 B and C). The lack of contralateral cholinergic partition cells may already suggest that not many bilaterally projecting cholinergic cells innervate Q motor neurons in α-2-chimaerin mutant mice. On the top-down view (Figure 6.10) on the the contra- versus ipsilateral and bilaterally projecting cells are illustrated.

6.4 Discussion

Lack of α-2-chimaerin in the nervous system has been shown to induce aberrant midline crossing of cortico-spinal tract and spinal interneurons (Beg et al. (2007); Wegmeyer et al. (2007)). Therefore, my expectation from the premotor neuronal tracing experiments in α-2-chimaerin mutants was to find additional midline crossing interneurons and bilaterally projecting cells with cell body position close to the midline. My arguments for this expectation were that premotor interneurons that normally are responsive to EphA4, in the mutant may cross the midline by mistake and connect to contralateral motor neurons, responding to the cues that normally influence their behavior on the ipsilateral side. Cells occupying the area close to the midline could do these mistakes even more often since their distance to the opposite side of the spinal cord is very close. Therefore, extra crossing of the midline by dorsal INs seemed to be a natural consequence of the lack of α-2-chimaerin. But further analysis of the cell body position of those interneurons brought some doubts

whether they belong to the same population as the dorsal premotor interneurons in wild type or maybe they are an ectopic population normally not connected to Q motor neurons. Such neurons may not have altered cell body position but normally would not connect to motor neurons and therefore not be revealed by our tracing approaches. A third possibility may be that not only the axon guidance process is disturbed in α-2-chimaerin mutant mice but also neuronal migration. A fourth possibility may be that α-2-chimaerin is also involved in axonal guidance in the periphery as is EphA4 (Luria et al. (2008), Kania and Jessel (2003)). In this case, part of the phenotype could be a result of mistakes in peripheral muscle innervation by motor neurons, normally innervating different muscles in wild type mice. Lack of midline cells and cholinergic partition cells among premotor interneurons projecting bilaterally was unexpected due to the reasons explained above. The findings also raised the possibility that α-2-chimaerin may be involved downstream of other axon guidance receptor systems and/or could play attractive roles. Another explanation could be aberrant migration of cell bodies, making themselves non-responsive to the attractive cues locally present at the midline.

In summary, at least three types of cellular interactions may have influence on the premotor distribution pattern in the α-2-chimaerin mutant mice: failures in axon guidance in the periphery, migration of the cells to the wiring area and/or wrong innervation of motor neuron pools in the spinal cord as a consequence of an aberrant recognition process between interneurons and motor neurons.

To avoid interaction between these factors, the best way to resolve the issue how lack of α-2-chimaerin influences connectivity of spinal circuits will be to make conditional knockouts specific for subpopulations of the INs in future experiments. It is now possible to cross specific mouse lines expressing Cre recombinase in subpopulations of interneurons with α-2-chimaerin mutants and a mouse line carrying a floxed allele of α-2-chimaerin . To study the influence of the lack of α-2-chimaerin on dorsal interneuronal populations, one could for example use Cre lines selectively removing α-2-chimaerin in defined interneuron populations. Specifically, it would be very interesting to study lack of α-2-chimaerin in the dorsal population of interneurons. It is interesting that dorsal premotor interneurons of the Q motor neuron pool in the wild type animals occupy an area in the spinal cord that is densely targeted by proprioceptive afferents (not shown). Therefore, it is possible that these interneurons receive strong proprioceptive input. My results suggest that in the α-2-chimaerin mutants, these dorsal premotor interneurons may project to both sides of the spinal cord. A set of publications from the lab of H. Hultborn have shown that stimulation of the proprioceptive afferents of flexor or extensor muscles can reset

the locomotor step cycle (Conway et al. (1987); Gossard et al. (1994); Schomburg et al. (1998)). It is therefore possible that the dorsal premotor interneurons revealed here are involved in resetting of the locomotor cycle. In the α-2-chimaerin mice, due to the bilateral projection of the dorsal premotor interneurons, this resetting mechanism may happen on both sides of the spinal cord at the same time, and may therefore contribute to the observed hopping gait locomotion. However, the synchrony in hindlimb activity does not have to be solely caused by premotor interneurons. This phenotype may be a result of mis-wired premotor interneurons, higher order interneurons and/or mis-wired sensory and motor projections. But it would be very interesting in the future, to study the premotor circuits of mice in which α-2-chimaerin or EphA4 are conditionally removed from particular subpopulations of premotor interneurons to determine the contribution of phenotypes in defined populations to the motor behavioral phenotype.

Chapter 7

Final discussion

Regulation of the motor behavior is possible due to neuronal networks in the spinal cord. During development, several molecules and intrinsic genetic programs influence specification, migration and connectivity of neurons encompassing these motor circuits. Previous studies brought along insight into spinal circuits by describing properties and molecular identities of distinct interneuron classes and others also focused on interneuronal connectivity. However, it is still hard to answer the question of how the spinal cord controls the activity of single muscles in such specific ways. In the course of my PhD thesis, I have developed a technique allowing for visualization of premotor interneurons connected to particular motor neuron pools and to shown that their three-dimensional distribution is specific and reproducible across animals. This technique allows to compare the distribution of premotor neurons connected to different motor neuron pools, and together with genetic and molecular approaches, one can identify distinct classes of interneurons connected to particular motor neuron pools.

7.1 What does premotor distribution pattern reveal?

Is it possible to look at the distribution pattern of premotor interneurons and deduce the function of the motor neuron pool? Comparison of the premotor interneurons distribution of the Cm and the Tri revealed that the premotor interneuron pattern differ significantly. Detailed analysis of the distribution of the premotor interneurons of other muscles may indeed help to recognize the muscle function based on the premotor pattern what may lead to identification of particular interneurons involved in particular motor tasks based on their position in the spinal cord. The organization of the spinal cord in terms of synaptic

input zones can be mapped by staining of neurotransmitters or transporters, specifically expressed in some neurons for example using mice expressing GFP in particular tracts, by dye injection to different brain and brainstem structures etc. Using such maps and comparing the patterns of premotor interneuron distribution one can already gain some information about the function of specific subsets of interneurons based on their synaptic input. For example, interneurons embedded in the area of high density of proprioceptive terminals in the intermediate spinal cord are expected to receive proprioceptive input while neurons embedded in the area of high density of sensory terminals from the skin are expected to receive sensory information from the skin. Studying mutant mice with different motor phenotypes and their premotor interneuronal distribution may bring important information about functions of interneuronal populations.

Tracing studies in α-2-chimaerin mice performed in this thesis revealed different premotor interneuron distribution patterns than in wild type mice. The motor phenotype is very clear in these mice and if it is due to premotor interneuron connectivity differences (which remains to be seen), the differences in dorsal interneuronal distributions may indicate that the alternation or coordination of the locomotor pattern can be directly dependent on these interneurons. It is possible that the bilaterally projecting dorsal premotor interneurons in α-2-chimaerin mutants act on motor neuron pools bilaterally at the same moment causing the synchrony in motor behavior between the two sides. Conditional mutagenesis experiments will have to be done in the future to determine whether this is indeed the case.

7.2 Variety of different locomotor patterns

It is known from comparative studies and paleontological data that all vertebrates come from common aquatic ancestor. The most primitive vertebrates are limbless and move due to alternating contractions of muscles on contralateral side. The bony fish that were ancestral to land tetrapods are Osteichthyes and they possess a true bony skeleton. Limbs evolved from bony fishs fins and a fact speaking for that, some species of fish (lung fish and mudskippers) use fins to move on land or climb on rocks. The fully terrestrial tetrapod body form was reached by the amphibians. Beyond amphibians, limbs improve for locomotor efficiency by more upright stance rather than the amphibian sprawling stance. Tetrapod forelimbs evolved from simple structures and became useful for grasping, climb-

ing, flying, burrowing and swimming as well as terrestrial locomotion. Information coming from fossils also suggests that the present limb construction was reached by their elongation and straightening. Together with the reduction of the peripheral weight during evolution, it allowed for more energy efficient movements. But together with the development of the construction of the limbs, the nervous system controlling these structures had to develop. It is interesting to think about the movement in terms of synchronization or alternation of muscle contractions. For symmetric movements, synchronization of the same muscles on contralateral sides is needed while for alternation of limb movements, the activity of the contralateral muscles cannot happen at the same moment. Thinking about terrestrial animals, one can find mainly two types of locomotion. The alternating pattern of limbs is very frequent in all groups of vertebrates, the locomotor pattern, where limb activity is synchronized can be found also in many vertebrate groups but nevertheless seems to be less frequent.

How is it possible to generate such different motor behaviors? The theory of central pattern generators assumes the existence of intraspinal circuits that are responsible for the coordination of the activity of particular motor neuron pools (Grillner (2006)). This can explain smooth changes in activation of segments of the fish body, so that they are active in a form of wave, starting at the first segments and successively reaching the tail. Activation of motor neurons on one side implies the inactivation of the other side, at the same time interacting with segments in front or behind. Spinal circuits that regulate limb activity must have evolved from those regulating the segmental activity of the body wall muscles in fish. So did the circuits regulating the alternating patterns of activity between flexors and extensors and the ones regulating movements of both sides of the body.
How evolution of spinal circuits happened is not known since not much is known about these circuits. However, studies on the properties of V1 derived interneurons in fish and in mammals provide evidence that from a primitive inhibitory interneuron regulating swimming speed (Higashijima et al. (2004); Li et al. (2004)), these molecularly defined interneurons evolved in mammals into several premotor inhibitory circuits for limb control and terrestrial locomotion (Alvarez et al. (2005); Goulding (2009)).
The different locomotor patterns like alternation or synchronization of the limbs evolved probably independently in many different groups of vertebrates. One can assume that the alternating pattern is the primary one, because the primary movement control system depends on the alternation of left and right body segments. However observing mudskippers, one can see clearly that although they alternate the body wall muscles, their fin

movements are synchronized. They coextend their left and right fins in front and then move ahead by a typical for fish body movement. So it may actually be that synchronous limb activity is the primary one. Amphibians show already alternating or synchronic patterns of limb activity. Another interesting group of animals are birds. All birds exhibit synchronized activity of their forelimbs (wings), while some of them also synchronize hindlimbs (e.g sparrow) and others do not (e.g.chicken). Therefore, it is very likely that synchronized hindlimb activity can be reached by several ways but each time depends on a very simple change. The monosynaptic tracing experiments on EphA4 mutants or α-2-chimaerin mutants may deliver information about interneuronal groups possibly involved in the control of the symmetric movements by simple switches. It would indeed be very interesting to compare premotor neuron distributions between wild type mice and rodents that naturally exhibit a hopping gait.

7.3 When does the locomotor pattern fully develop?

Most of the electrophysiological data obtained from spinal cord recordings in mice are collected from p0-p4 animals. Studies on RCs and IaINs were primarily done on adult cats and only lately, these cells and their molecular properties were studied in mice. Our monosynaptic tracing experiments were performed on p7 animals and terminated at p15, when adult locomotor pattern is observed. But we do not know whether and how the motor circuits change in early postnatal life. Observing young animals like kittens or lambs, one can see that they very often move by hopping- like but not walking like adult animals and this behavior disappears after a certain age. One possibility why this transition happens may be the refinement in spinal circuit organization. Some evidence that motor circuits still develop in postnatal life comes from studies by the Alvarez lab on proprioceptive input to RCs. Initially, it was thought that RCs do not receive proprioceptive input, but (Siembab et al. (2010)) have shown that such input exists and disappears with time, a feature not present anymore in the adult (Alvarez et al. (1999)). Monosynaptic rabies tracing currently does not work in mice older than p8 and the reason is probably the maturation of the immune system and/or myelination of the neuro-muscular junctions. However if one could overcome this problem, and perform monosynaptic rabies virus tracing experiments in adult animals, it would be very valuable to study whether the premotor spinal circuits are similar to the postnatal ones.

7.4 Are different circuit modules active for different speeds?

Locomotor patterns can also differ according to the speed of the locomotion. Locomotor patterns of horses have been of great interest to humans around the world. There are several natural gaits in horse locomotion depending on the speed. This phenomenon can be also observed in other animals and was not particularly well studied in mice. But data from EMG recordings in cats and in rats for different locomotor speed show that the activity duration of extensors scales with cycle duration and is low for short cycle duration (and high speed of locomotion) and high for long cycle duration while animals move slowly. At the same time the activation of flexors remains constant throughout different cycle duration and animal speed (Courtine et al. (2009) for rats; Halbertsma (1983) for cats). These findings would suggest that there may be differences in spinal circuits controlling movements at different speeds. Recruitment of some interneurons seems to be dependent on the locomotor cycle speed (V2a) (Crone et al. (2009)). In zebrafish, distinct subpopulations of interneurons are activated during fast and slow swimming. The circumferential descending interneurons (CiDs) that are homologues of the V2a INs in mammals are divided into ventral and dorsal subpopulations and are active preferentially during slow swim (ventral) or fast swim (dorsal) (Kimura et al. (2006); McLean et al. (2008); McLean et al. (2007)). In the turtle, there is a group of locomotor-related INs that are active either during scratching or swimming, while others are recruited during both behaviors (Berkowitz (2008)). Task dependent or gait-dependent differential recruitment of interneurons may be a general feature of spinal motor circuitry in vertebrates. Dissection of the circuits underlying behavioral modules in the spinal cord could be possible by ablation of different interneuronal classes in the fully developed spinal cord and subsequent behavioral testing. It is therefore impossible to distinguish such circuits by only premotor tracing experiments, but together with specific ablation of distinct interneuronal populations, it can be a valuable tool for spinal circuit studies.

7.5 Methodological considerations of the rabies virus method

In our studies, we have used rabies viruses as a tool to study neuronal circuits. These viruses have been shown to have high affinity for motor neurons and to spread in a

retrograde manner in the nervous system (Ugolini (1995)). However, exact molecular mechanisms underlying viral infection and spreading are currently not known. Therefore, we cannot be completely sure whether all interneurons presynaptic to particular motor neurons can be infected. We also do not know whether the number of synaptic connections between the presynaptic and postsynaptic cell influences somehow the probability of presynaptic infection but so far, for all known to be directly connected with motor neurons and possible to identify interneuronal classes, we have found examples of infected interneurons.

The advantage of the motor system is its anatomy that allows infecting motor neurons from the periphery with high specificity. Studies of premotor interneurons for distinct motor neuron pools can bring a lot of insight into which classes of interneurons are involved in locomotor control, and which ones are shared by different systems like flexor and extensor premotor control or how they are different. So far, it is not known, which interneurons underlie the control of the body wall muscle contraction and what is their relation to interneurons controlling limb muscles. In addition, monosynaptic rabies tracing from distinct motor neurons can bring more insight to our understanding of the role of higher motor control areas in the nervous system in brain and brainstem. In my study, the brainstem and cortical neurons monosynaptically connected to the motor neuron pools were not analyzed in detail but I have observed labeled neurons in cortex and other brain and brainstem areas.

As mentioned previously, studies on premotor circuits using monosynaptically restricted rabies viruses have the advantage that the injection of the rabies into a muscle can be very precise and no other cells than those of interest become infected. However, restrictions of this system specificity are possible. One possibility described previously (Wickersham et al. (2007b)), is to create a virus pseudotyped with sarcoma leucosis virus glycoprotein EnvA and express their receptors (TVA) from a cell of interest. Another possibility was published lately also from the lab of E.Callaway. Wall et al. (2010) generated helper viruses that target gene expression to Cre-expressing cells, allowing the control of initial rabies virus infection and subsequent monosynaptic retrograde spread. In this new approach, Cre-dependent helper virus carries a glycoprotein G gene and TVA. Injected into a nervous tissue, where specific type of neurons express Cre, this system allows the spread only from the Cre expressing neurons. Following Cre recombination, helper virus expresses both proteins (TVA and glycoprotein G). Rabies virus is also pseudotyped with EnvA in this approach. Here the limitation is the access to different Cre-lines and their specificity to defined cell types.

One could also imagine that expression of light sensitive dyes by rabies virus (instead of RFP or GFP) could allow functional analysis of neuronal circuits labeled monosynaptically for example from motor neurons, however the distributed anatomy of motor control circuits in the spinal cord also revealed by my PhD thesis does not make it easy to access these circuits all at once. Future developments and more sophisticated uses of currently existing and additional viruses, in combination with mouse transgenesis will certainly produce a wide variety of applications to use this system also in the future for many questions in the studies of motor circuitry and beyond.

List of Figures

Bibliography

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

Alvarez, F. J., Dewey, D. E., McMillin, P. and Fyffe, R. E. (1999). Distribution of cholinergic contacts on Renshaw cells in the rat spinal cord: a light microscopic study. J. Physiol. (Lond.) *515*, 787–797.

Alvarez, F. J. and Fyffe, R. E. W. (2007). The continuing case for the Renshaw cell. The Journal of Physiology *584*, 31–45.

Alvarez, F. J., Jonas, P. C., Sapir, T., Hartley, R., Berrocal, M. C., Geiman, E. J., Todd, A. J. and Goulding, M. (2005). Postnatal phenotype and localization of spinal cord v1 derived interneurons. The Journal of Comparative Neurology *493*, 177.

Andén, N. E., Jukes, M. G., Lundberg, A. and Vyklick, L. (1966). The effect of DOPA on the spinal cord. 1. Influence on transmission from primary afferents. Acta Physiol Scand *67*, 373–386.

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T. M. and Sockanathan, S. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron *23*, 659–674.

Arber, S., Ladle, D. R., Lin, J. H., Frank, E. and Jessell, T. M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell *101*, 485–498.

Bannatyne, B. A., Edgley, S. A., Hammar, I., Jankowska, E. and Maxwell, D. J. (2003). Networks of inhibitory and excitatory commissural interneurons mediating crossed reticulospinal actions. Eur. J. Neurosci. *18*, 2273–2284.

Barbeau, H., McCrea, D. A., O'Donovan, M. J., Rossignol, S., Grill, W. M. and Lemay, M. A. (1999). Tapping into spinal circuits to restore motor function. Brain Res. Brain Res. Rev. *30*, 27–51.

Barnard, R. J., Elleder, D. and Young, J. A. (2006). Avian sarcoma and leukosis virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. Virology *344*, 25–29.

Bates, P., Young, J. A. and Varmus, H. E. (1993). A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell *74*, 1043–1051.

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

Bel-Vialar, S., Itasaki, N. and Krumlauf, R. (2002). Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development *129*, 5103–5115.

Bergemann, A. D., Zhang, L., Chiang, M. K., Brambilla, R., Klein, R. and Flanagan, J. G. (1998). Ephrin-B3, a ligand for the receptor EphB3, expressed at the midline of the developing neural tube. Oncogene *16*, 471–480.

Berkowitz, A. (2008). Physiology and morphology of shared and specialized spinal interneurons for locomotion and scratching. J. Neurophysiol. *99*, 2887–2901.

Borgius, L., Restrepo, C. E., Leao, R. N., Saleh, N. and Kiehn, O. (2010). Atransgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. Mol. Cell. Neurosci. *45*, 245–257.

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

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell *96*, 795–806.

Brown, M. C., Enberg, I. and Matthews, P. B. (1967). The use of vibration as a selective repetitive stimulus for Ia afferent fibres. J. Physiol. (Lond.)Phy *191*, 31–32.

Burke, R. E. and Glenn, L. L. (1996). Horseradish peroxidase study of the spatial and electrotonic distribution of group Ia synapses on type-identified ankle extensor motoneurons in the cat. J. Comp. Neurol. *372*, 465–485.

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

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

Card, J. P. and Enquist, L. W. (2001). Transneuronal circuit analysis with pseudorabies viruses. Curr Protoc Neurosci *Chapter 1*, Unit1.5.

Carlin, K. P., Bui, T. V., Dai, Y. and Brownstone, R. M. (2009). Staircase currents in motoneurons: insight into the spatial arrangement of calcium channels in the dendritic tree. J. Neurosci. *29*, 5343–5353.

Carr, P. A., Alvarez, F. J., Leman, E. A. and Fyffe, R. E. (1998). Calbindin D28k expression in immunohistochemically identified Renshaw cells. Neuroreport *9*, 2657–2661.

Charron, F., Stein, E., Jeong, J., McMahon, A. P. and Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. Cell *113*, 11–23.

Chen, H., Zhang, M., Tang, S., London, N. R., Li, D. Y. and Zhang, K. (2010). Slit-robo signaling in ocular angiogenesis. Adv. Exp. Med. Biol. *664*, 457–463.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The role of the floor plate in axon guidance. Annu. Rev. Neurosci. *18*, 497–529.

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

Conway, B. A., Hultborn, H. and Kiehn, O. (1987). Proprioceptive input resets central locomotor rhythm in the spinal cat. Exp Brain Res *68*, 643–656.

Coonan, J. R., Greferath, U., Messenger, J., Hartley, L., Murphy, M., Boyd, A. W., Dottori, M., Galea, M. P. and Bartlett, P. F. (2001). Development and reorganization of corticospinal projections in EphA4 deficient mice. J. Comp. Neurol. *436*, 248–262.

Courtine, G., Gerasimenko, Y., van den Brand, R., Yew, A., Musienko, P., Zhong, H., Song, B., Ao, Y., Ichiyama, R. M., Lavrov, I., Roy, R. R., Sofroniew, M. V. and Edgerton, V. R. (2009). Transformation of nonfunctional spinal circuits into functional states after the loss of brain input. Nat. Neurosci. *12*, 1333–1342.

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

Crone, S. A., Zhong, G., Harris-Warrick, R. and Sharma, K. (2009). In mice lacking V2a interneurons, gait depends on speed of locomotion. J. Neurosci. *29*, 7098–7109.

Cullheim, S. and Kellerth, J. O. (1981). Two kinds of recurrent inhibition of cat spinal alpha-motoneurones as differentiated pharmacologically. J. Physiol. (Lond.) *312*, 209–224.

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

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

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

Dasen, J. S., Tice, B. C., Brenner-Morton, S. and Jessell, T. M. (2005). ScienceDirect - Cell : A Hox Regulatory Network Establishes Motor Neuron Pool Identity and Target-Muscle Connectivity. Rev Prat *55*, 574–577.

Deschamps, J., van den Akker, E., Forlani, S., De Graaff, W., Oosterveen, T., Roelen, B. and Roelfsema, J. (1999). Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. Int. J. Dev. Biol. *43*, 635–650.

Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick, T., Bartlett, P. F., Murphy, M., Kontgen, F. and Boyd, A. W. (1998). EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. Proc. Natl. Acad. Sci. U.S.A. *95*, 13248–13253.

Dougherty, K. J. and Kiehn, O. (2010). Firing and cellular properties of V2a interneurons in the rodent spinal cord. J. Neurosci. *30*, 24–37.

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

Eccles, J. C. (1964). Presynaptic inhibition in the spinal cord. Prog. Brain Res. *12*, 65–91.

Eccles, J. C., Eccles, R. M., Iggo, A. and Ito, M. (1961). Distribution of recurrent inhibition among motoneurones. J. Physiol. (Lond.) *159*, 479–499.

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

Eccles, J. C., Fatt, P. and Landgren, S. (1956). Central pathway for direct inhibitory action of impulses in largest afferent nerve fibres to muscle. J. Neurophysiol. *19*, 75–98.

Eccles, J. C. and Pritchard, J. J. (1937). Journal of Neurophysiology *89*, 43.

Eccles, R. M. and Lundberg, A. (1959). Supraspinal control of interneurones mediating spinal reflexes. J. Physiol. (Lond.) *147*, 565–584.

Egea, J. and Klein, R. (2007). Bidirectional Eph-ephrin signaling during axon guidance. Trends Cell Biol. *17*, 230–238.

Eide, E., Lundberg, A. and Voorhoeve, P. (1961). Monosynaptically evoked inhibitory post-synaptic potentials in motoneurones. Acta Physiol. Scand. *53*, 185–195.

Ekstrand, M. I., Enquist, L. W. and Pomeranz, L. E. (2008). The alpha-herpesviruses: molecular pathfinders in nervous system circuits. Trends Mol Med *14*, 134–140.

Eldred, E., Granit, R. and Merton, P. A. (1953). Observations on intact', de-afferented and de-efferented muscle spindles. Acta Physiol. Scand. *29*, 83–85.

Engberg, I., Lundberg, A. and Ryall, R. W. (1968). Reticulospinal inhibition of interneurones. J Physiol *194*, 225–236.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V. and Jessell, T. M. (1997). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. Cold Spring Harb. Symp. Quant. Biol. *62*, 451–466.

Etessami, R., Conzelmann, K. K., Fadai-Ghotbi, B., Natelson, B., Tsiang, H. and Ceccaldi, P. E. (2000). Spread and pathogenic characteristics of a G-deficient rabies virus recombinant: an in vitro and in vivo study. J. Gen. Virol. *81*, 2147–2153.

Federspiel, M. J., Bates, P., Young, J. A., Varmus, H. E. and Hughes, S. H. (1994). A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukosis virus-based retroviral vectors. Proc. Natl. Acad. Sci. U.S.A. *91*, 11241–11245.

Fyffe, R. E. (1991). Glycine-like immunoreactivity in synaptic boutons of identified inhibitory interneurons in the mammalian spinal cord. rain Res. *547*, 175–179.

Gale, N. W., Flenniken, A., Compton, D. C., Jenkins, N., Copeland, N. G., Gilbert, D. J., Davis, S., Wilkinson, D. G. and Yancopoulos, G. D. (1996a). Elk-L3, a novel transmembrane ligand for the Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments. Oncogene *13*, 1343–1352.

Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G., Pawson, T., Davis, S. and Yancopoulos, G. D. (1996b). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. Neuron *17*, 9–19.

Geiman, E. J., Knox, M. C. and Alvarez, F. J. (2000). Postnatal maturation of gephyrin/glycine receptor clusters on developing Renshaw cells. J. Comp. Neurol. *426*, 130–142.

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

Gossard, J. P., Brownstone, R. M., Barajon, I. and Hultborn, H. (1994). Transmission in a locomotor-related group Ib pathway from hindlimb extensor muscles in the cat. Exp Brain Res *98*, 213–228.

Goulding, M. (2009). Circuits controlling vertebrate locomotion: moving in a new direction. Nature Reviews Neuroscience *10*, 507–518.

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

Granit, R. (1950). Autogenetic inhibition. Electroencephalogr Clin Neurophysiol *2*, 417–424.

Granit, R. and Henantsch, H. D. (1956). Gamma control of dynamic properties of muscle spindles. J. Neurophysiol. *19*, 356–366.

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

Grillner, S. (2006). Biological pattern generation: the cellular and computational logic of networks in motion. Neuron *52*, 751–766.

Haase, G., Dessaud, E., Garces, A., de Bovis, B., Birling, M., Filippi, P., Schmalbruch, H., Arber, S. and deLapeyriere, O. (2002). GDNF acts through PEA3 to regulate cell body positioning and muscle innervation of specific motor neuron pools. Neuron *35*, 893–905.

Halbertsma, J. (1983). The stride cycle of the cat: the modelling of locomotion by computerized analysis of automatic recordings. Acta Physiol. Scand. *521*.

Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., Biag, J., Dong, H.-W., Deisseroth, K., Callaway, E. M., Fanselow, M. S., Lüthi, A. and Anderson, D. J. (2010). Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature *468*, 270–276.

Heekyung Jung, J. L. E. O. M. K. F. L. J. J. G. S. M. D. M. D. K. G. R. A. Y. K. V. A. H. W. and Dasen, J. S. (2010). Global Control of Motor Neuron Topography Mediated by the Repressive Actions of a Single Hox Gene. Neuron *67*, 781.

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

Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F. M., Chambon, P. and Charnay, P. (1998). Hoxa1 and Krox-20 synergize to control the development of rhombomere 3. Development *125*, 4739–4748.

Higashijima, S., Masino, M. A., Mandel, G. and Fetcho, J. R. (2004). Engrailed-1 expression marks a primitive class of inhibitory spinal interneuron. J. Neurosci. *24*, 5827–5839.

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

Holstege, G., van Neerven, J. and Evertse, F. (1987). Spinal cord location of the motoneurons innervating the abdominal, cutaneous maximus, latissimus dorsi and longissimus dorsi muscles in the cat. Exp Brain Res *67*, 179–194.

Holstege, J. C. and Kuypers, H. G. (1987). Brainstem projections to spinal motoneurons: an update. Neuroscience *23*, 809–821.

Hongo, T., Jankowska, E. and Lundberg, A. (1969). The rubrospinal tract. II. Facilitation of interneuronal transmission in reflex paths to motoneurones. Exp Brain Res *7*, 365–391.

Hultborn, H., Illert, M. and Santini, M. (1976). Convergence on interneurones mediating the reciprocal Ia inhibition of motoneurones. I. Disynaptic Ia inhibition of Ia inhibitory interneurones. Acta Physiol Scand *96*, 193–201.

Hultborn, H., Jankowska, E. and Lindstrom, S. (1968). Inhibition in IA inhibitory pathway by impulses in recurrent motor axon collaterals. Life Sci. *7*, 337–339.

Hultborn, H., Jankowska, E. and Lindstrom, S. (1971). Recurrent inhibition from motor axon collaterals of transmission in the Ia inhibitory pathway to motoneurones. J. Physiol. (Lond.) *215*, 591–612.

Hultborn, H., Pierrot-Deseilligny, E. and Wigstrom, H. (1979). Recurrent inhibition and afterhyperpolarization following motoneuronal discharge in the cat. J. Physiol. (Lond.) *297*, 253–266.

Hultborn, H. and Udo, M. (1972). Convergence of large muscle spindle Ia afferents at interneuronal level in the reciprocal Ia inhibitory pathway to motoneurones. Acta Physiol. Scand. *84*, 493–499.

Hunt, C. C. (1952). The effect of stretch receptors from muscle on the discharge of motorneurons. J. Physiol. (Lond.) *117*, 359–379.

Illert, M. and Tanaka, R. (1976). Transmission of corticospinal IPSPs to cat forelimb motoneurones via high cervical propriospinal neurones and La inhibitory interneurones. Brain Res *103*, 143–146.

Itoh, A., Miyabayashi, T., Ohno, M. and Sakano, S. (1998). Cloning and expressions of three mammalian homologues of Drosophila slit suggest possible roles for Slit in the formation and maintenance of the nervous system. Brain Res. Mol. Brain Res. *62*, 175–186.

Jankowska, E. (1992). Interneuronal relay in spinal pathways from proprioceptors. Prog. Neurobiol. *38*, 335–378.

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

Jankowska, E., Hammar, I., Slawinska, U., Maleszak, K. and Edgley, S. A. (2003). Neuronal basis of crossed actions from the reticular formation on feline hindlimb motoneurons. J. Neurosci. *23*, 1867–1878.

Jankowska, E. and Lindstrom, S. (1972). Morphology of interneurones mediating Ia reciprocal inhibition of motoneurones in the spinal cord of the cat. J. Physiol. (Lond.) *226*, 805–823.

Jankowska, E. and Roberts, W. J. (1972). Synaptic actions of single interneurones mediating reciprocal Ia inhibition of motoneurones. J. Physiol. (Lond.) *222*, 623–642.

Jankowska, E. and Skoog, B. (1986). Labelling of midlumbar neurones projecting to cat hindlimb motoneurones by transneuronal transport of a horseradish peroxidase conjugate. Neurosci. Lett. *71*, 163–168.

Jaworski, A. H. L. and Tessier-Lavigne, M. (2010). Collaborative and Specialized Functions of Robo1 and Robo2 in Spinal Commissural Axon Guidance. J. Neurosci. *30*, 9445–9453.

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

Kania, A. and Jessel, T. M. (2003). Topographic Motor Projections in the Limb Imposed by LIM Homeodomain Protein Regulation of Ephrin-A:EphA Interactions. Neuron *38*, 581–596.

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

Kaprielian, Z., Runko, E. and Imondi, R. (2001). Axon guidance at the midline choice point. Dev. Dyn. *221*, 154–181.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425–435.

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

Kiehn, O., Dougherty, K. J., Hagglund, M., Borgius, L., Talpalar, A. and Restrepo, C. E. (2010). Probing spinal circuits controlling walking in mammals. Biochem. Biophys. Res. Commun. *396*, 11–18.

Kimura, Y., Okamura, Y. and Higashijima, S. (2006). alx, a zebrafish homolog of Chx10, marks ipsilateral descending excitatory interneurons that participate in the regulation of spinal locomotor circuits. J. Neurosci. *26*, 5684–5697.

Kjaerulff, O. and Kiehn, O. (1997). Crossed rhythmic synaptic input to motoneurons during selective activation of the contralateral spinal locomotor network. J. Neurosci. *17*, 9433–9447.

Klausberger, T. and Somogyi, P. (2008). Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. Science *321*, 53–57.

Kudo, K., Yamada, M., Imai, H., Katagiri, M., Ozawa, S., Ida, Y. and Okada, R. (1987). [Familial cardiomyopathy with different clinical features in individual members]. J Cardiol *17*, 907–914.

Kuffler, S. W., Hunt, C. C. and Quilliam, J. P. (1951). Function of medullated small-nerve fibers in mammalian ventral roots; efferent muscle spindle innervation. J. Neurophysiol. *14*, 29–54.

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

Kullander, K., Butt, S. J. B., Lebret, J. M., Lundfald, L., Restrepo, C. E., m, A. R., Klein, R. d. and Kiehn, O. (2003b). Role of EphA4 and EphrinB3 in Local Neuronal Circuits That Control Walking. Science *299*, 1889–1892.

Kullander, K., Mather, N. K., Diella, F., Dottori, M., Boyd, A. W. and Klein, R. (2001). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron *29*, 73–84.

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

Lance-Jones, C. and Landmesser, L. (1981a). Pathway selection by chick lumbosacral motoneurons during normal development. Proc. R. Soc. Lond., B, Biol. Sci. *214*, 1–18.

Lance-Jones, C. and Landmesser, L. (1981b). Pathway selection by embryonic chick motorneurons in an experimentally altered environment. Proc. R. Soc. Lond., B, Biol. Sci. *214*, 19–52.

Landmesser, L. (1978). The distribution of motoneurones supplying chick hind limb muscles. J. Physiol. (Lond.) *284*, 371–389.

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

Laporte, Y. and Lloyd, D. P. (1952). Nature and significance of the reflex connections established by large afferent fibers of muscular origin. Am. J. Physiol. *169*, 609–621.

Lee, K. J. and Jessell, T. M. (1999). The specification of dorsal cell fates in the vertebrate central nervous system. Annu Rev Neurosci *22*, 261–294.

Li, H. S., Chen, J. H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z. H., Nash, W., Gick, C., Ornitz, D. M., Wu, J. Y. and Rao, Y. (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell *96*, 807–818.

Li, W. C., Higashijima, S., Parry, D. M., Roberts, A. and Soffe, S. R. (2004). Pimitive roles for inhibitory interneurons in developing frog spinal cord. J. Neurosci. *24*, 5840–5848.

Liem, K. F., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFbeta-related proteins in neuronal patterning in the dorsal spinal cord. Cell *91*, 127–138.

Lin, J. H., Saito, T., Anderson, D. J., Lance-Jones, C., Jessel, T. M. and Arber, S. (1998). Functionally Related Motor Neuron Pool and Muscle Sensory Afferent Subtypes Defined by Coordinate ETS Gene Expression. Cell *95*, 393–407.

Liu, J. P., Laufer, E. and Jessell, T. M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron *32*, 997–1012.

Livet, J., Sigrist, M., Stroebel, S., De Paola, V., Price, S. R., Henderson, C. E., Jessell, T. M. and Arber, S. (2002). ETS Gene Pea3 Controls the Central Position and Terminal Arborization of Specific Motor Neuron Pools. Neuron *35*, 877–892.

Lloyd, D. P. (1946). Facilitation and inhibition of spinal motoneurons. Journal of Neurophysiology *9*, 421.

Lloyd, D. P. (1951). After-currents, after-potentials, excitability, and ventral root electrotonus in spinal motorneurons. J.gen. Physiol *35*, 289.

Long, H., Sabatier, C., Ma, L., Plump, A., Yuan, W., Ornitz, D. M., Tamada, A., Murakami, F., Goodman, C. S. and Tessier-Lavigne, M. (2004). Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron *42*, 213–223.

Lucas-Meunier, E., Fossier, P., Baux, G. and Amar, M. (2003). Cholinergic modulation of the cortical neuronal network. Pflugers Arch. *446*, 17–29.

Lundberg, A. and Winsbury, G. (1960). Selective adequate activation of large afferents from muscle spindles and Golgi tendon organs. Acta Physiol. Scand. *49*, 155–164.

Lundfald, L., Restrepo, C. E., Butt, S. J. B., Peng, C.-Y., Droho, S., Endo, T., Zeilhofer, H. U., Sharma, K. and Kiehn, O. (2007). Phenotype of V2-derived interneurons and their relationship to the axon guidance molecule EphA4 in the developing mouse spinal cord. European Journal of Neuroscience *26*, 2989–3002.

Luo, W., Enomoto, H., Rice, F. L., Milbrandt, J. and Ginty, D. D. (2009). Molecular identification of rapidly adapting mechanoreceptors and their developmental dependence on ret signaling. Neuron *64*, 841–856.

Luria, V., Krawchuk, D., Jessell, T. M., Laufer, E. and Kania, A. (2008). Specification of motor axon trajectory by ephrin-B:EphB signaling: symmetrical control of axonal patterning in the developing limb. Neuron *60*, 1039–1053.

M Douglas Benson, M. I. R. M. E. L. Q. R. L. M. H. and Parada, L. F. (2005). Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. Proceedings of the National Academy of Sciences of the United States of America *102*, 10694.

Maltenfort, M. G., Heckman, C. J. and Rymer, W. Z. (1998). Decorrelating actions of Renshaw interneurons on the firing of spinal motoneurons within a motor nucleus: a simulation study. J. Neurophysiol. *80*, 309–323.

Marder, E. and Bucher, D. (2007). Understanding circuit dynamics using the stomatogastric nervous system of lobsters and crabs. Annu. Rev. Physiol. *69*, 291–316.

Marshel, J. H., Mori, T., Nielsen, K. J. and Callaway, E. M. (2010). Targeting Single Neuronal Networks for Gene Expression and Cell Labeling In Vivo. Neuron *67*, 562–574.

Mattei, B., Schmied, A., Mazzocchio, R., Decchi, B., Rossi, A. and Vedel, J. P. (2003). Pharmacologically induced enhancement of recurrent inhibition in humans: effects on motoneurone discharge patterns. J. Physiol. (Lond.) *548*, 615–629.

Matthews, B. H. (1933). Nerve endings in mammalian muscle. J. Physiol. (Lond.) *78*, 1–53.

Mazzocchio, R. and Rossi, A. (2010). Role of Renshaw cells in amyotrophic lateral sclerosis. Muscle nerve *41*, 441–443.

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

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. Cell *68*, 283–302.

McHanwell, S. and Biscoe, T. J. (1981). The localization of motoneurons supplying the hindlimb muscles of the mouse. Philos. Trans. R. Soc. Lond., B, Biol. Sci. *293*, 477–508.

McLean, D. L., Fan, J., Higashijima, S., Hale, M. E. and Fetcho, J. R. (2007). A topographic map of recruitment in spinal cord. Nature *446*, 71–75.

McLean, D. L., Masino, M. A., Koh, I. Y., Lindquist, W. B. and Fetcho, J. R. (2008). Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. Nat. Neurosci. *11*, 1419–1429.

Mears, S. C. and Frank, E. (1997). Formation of specific monosynaptic connections between muscle spindle afferents and motoneurons in the mouse. J. Neurosci. *17*, 3128–3135.

Mebatsion, T., Konig, M. and Conzelmann, K. K. (1996). Budding of rabies virus particles in the absence of the spike glycoprotein. Cell *84*, 941–951.

Mentis, G. Z., Siembab, V. C., Zerda, R., O'Donovan, M. J. and Alvarez, F. J. (2006). Primary afferent synapses on developing and adult Renshaw cells. J. Neurosci. *26*, 13297–13310.

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

Miyamichi, K., Amat, F., Moussavi, F., Wang, C., Wickersham, I., Wall, N. R., Taniguchi, H., Tasic, B., Huang, Z. J., He, Z., Callaway, E. M., Horowitz, M. A. and Luo, L. (2010). Cortical representations of olfactory input by trans-synaptic tracing. Nature *472*, 191–196.

Nishimaru, H., Restrepo, C. E. and Kiehn, O. (2006). Activity of Renshaw cells during locomotor-like rhythmic activity in the isolated spinal cord of neonatal mice. J. Neurosci. *26*, 5320–5328.

Nordstrom, U., Maier, E., Jessell, T. M. and Edlund, T. (2006). An early role for WNT signaling in specifying neural patterns of Cdx and Hox gene expression and motor neuron subtype identity. PLoS Biol. *4*, e252.

Orioli, D. and Klein, R. (1997). The Eph receptor family: axonal guidance by contact repulsion. Trends Genet. *13*, 354–359.

Panayi, H., Panayiotou, E., Orford, M., Genethliou, N., Mean, R., Lapathitis, G., Li, S., Xiang, M., Kessaris, N., Richardson, W. D. and Malas, S. (2010). Sox1 is required for the specification of a novel p2-derived interneuron subtype in the mouse ventral spinal cord. J. Neurosci. *30*, 12274–12280.

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

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

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell *97*, 903–915.

Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron *29*, 367–384.

Price, S. R., De Marco Garcia, N. V., Ranscht, B. and Jessell, T. M. (2002). Regulation of Motor Neuron Pool Sorting by Differential Expression of Type II Cadherins. Cell *109*, 205–216.

Quinlan, K. A. and Kiehn, O. (2007). Segmental, synaptic actions of commissural interneurons in the mouse spinal cord. J. Neurosci. *27*, 6521–6530.

Rabe, N., Gezelius, H., Vallstedt, A., Memic, F. and Kullander, K. (2009). Netrin-1-dependent spinal interneuron subtypes are required for the formation of left-right alternating locomotor circuitry. J. Neurosci. *29*, 15642–15649.

Rastad, J., Gad, P., Jankowska, E., McCrea, D. and Westman, J. (1990). Light microscopical study of dendrites and perikarya of interneurones mediating la reciprocal inhibition of cat lumbar alpha-motoneurones. Anat. Embryol. *181*, 381–388.

Renshaw, B. (1940). Influence of discharge of motorneurons upon excitation of neighboring motorneurons. Journal of Neurophysiology *n.a.*

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

Romanes, G. (1964). The motor pools of the spinal cord. Prog. Brain Res. *11*.

Rousso, D. L., Gaber, Z. B., Wellik, D., Morrisey, E. E. and Novitch, B. G. (2008). Coordinated actions of the forkhead protein Foxp1 and Hox proteins in the columnar organization of spinal motor neurons. Neuron *59*, 226–240.

Sabatier, C., Plump, C., Le Ma, A., Brose, K., Tamada, A., Murakami, F., Lee, E. and Tessier-Lavigne, M. (2004). The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. Cell *117*, 157–69.

Sakmann, B. (2006). Patch pipettes are more useful than initially thought: simultaneous pre- and postsynaptic recording from mammalian CNS synapses in vitro and in vivo. Pflugers Arch. *453*, 249–259.

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

Saueressig, H., Burrill, J. and Goulding, M. (1999). Engrailed-1 and netrin-1 regulate axon pathfinding by association interneurons that project to motor neurons. Development *124*, 4201–4212.

Schneider, S. P. and Fyffe, R. E. (1992). Involvement of GABA and glycine in recurrent inhibition of spinal motoneurons. J. Neurophysiol. *68*, 397–406.

Schomburg, E. D., Petersen, N., Barajon, I. and Hultborn, H. (1998). Flexor reflex afferents reset the step cycle during fictive locomotion in the cat. Exp Brain Res *122*, 339–350.

Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M. and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell *78*, 409–424.

Shah, V., Drill, E. and Lance-Jones, C. (2004). Ectopic expression of Hoxd10 in thoracic spinal segments induces motoneurons with a lumbosacral molecular profile and axon projections to the limb. Developmental Dynamics *231*, 43–56.

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

Sharrocks, A. (2001). The ETS-domain transcription factor family. Nat. Rev. Mol. Cell Biol. *2*, 827–837.

Shirasaki, H., Asakura, K., Narita, S. I. and Kataura, A. (1998). Expression of substance P (NK1) receptor mRNA in human nose. Acta Otolaryngol. *118*, 717–722.

Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci *25*, 251–281.

Siembab, V. C., Smith, C. A., Zagoraiou, L., Berrocal, M. C., Mentis, G. Z. and Alvarez, F. J. (2010). Target selection of proprioceptive and motor axon synapses on neonatal V1-derived Ia inhibitory interneurons and Renshaw cells. J. Comp. Neurol. *518*, 4675–4701.

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

Stein, E. and Tessier-Lavigne, M. (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science *291*, 1928–1938.

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

Tamamaki, N., Yanagawa, Y., Tomioka, R., Miyazaki, J., Obata, K. and Kaneko, T. (2003). Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. J. Comp. Neurol. *467*, 60–79.

Tarchini, B., Huynh, T. H., Cox, G. A. and Duboule, D. (2005). HoxD cluster scanning deletions identify multiple defects leading to paralysis in the mouse mutant Ironside. Genes Dev. *19*, 2862–2876.

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

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J. and Pfaff, S. L. (1999). Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron *23*, 675–687.

Theriault, E. and Diamond, J. (1988a). Nociceptive cutaneous stimuli evoke localized contractions in a skeletal muscle. J. Neurophysiol. *60*, 446–462.

Theriault, E. and Diamond, J. (1988b). Intrinsic organization of the rat cutaneus trunci motor nucleus. J. Neurophysiol. *60*, 463–477.

Towne, C., Schneider, B. L., Kieran, D., Redmond, D. E. and Aebischer, P. (2010). Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6. Gene Ther. *17*, 141–146.

Trainor, P. and Krumlauf, R. (2000). Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm. Nat. Cell Biol. *2*, 96–102.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell *79*, 957–970.

Ugolini, G. (1995). Specificity of rabies virus as a transneuronal tracer of motor networks: transfer from hypoglossal motoneurons to connected second-order and higher order central nervous system cell groups. J. Comp. Neurol. *356*, 457–480.

Ugolini, G. (2010). Advances in viral transneuronal tracing. J. Neurosci. Methods *194*, 2–20.

Venables, W. and Ripley, B. (2002). Modern Applied Statistics with S. Fourth edition, New York: Spiringer.

von Engelhardt, J., Eliava, M., Meyer, A. H., Rozov, A. and Monyer, H. (2007). Functional characterization of intrinsic cholinergic interneurons in the cortex. J. Neurosci. *27*, 5633–5642.

Vrieseling, E. and Arber, S. (2006). Target-Induced Transcriptional Control of Dendritic Patterning and Connectivity in Motor Neurons by the ETS Gene Pea3. Cell *127*, 1439–1452.

Wall, N. R., Wickersham, I. R., Cetin, A., De La Parra, M. and Callaway, E. M. (2010). Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. Proc. Natl. Acad. Sci. U.S.A. *107*, 21848–21853.

Wang, Z., Li, L., Goulding, M. and Frank, E. (2008). Early postnatal development of reciprocal Ia inhibition in the murine spinal cord. J. Neurophysiol. *100*, 185–196.

Wang, Z., Li, L. Y., Taylor, M. D., Wright, D. E. and Frank, E. (2007). Prenatal exposure to elevated NT3 disrupts synaptic selectivity in the spinal cord. J. Neurosci. *27*, 3686–3694.

Wegmeyer, H., Egea, J., Rabe, N., Gezelius, H., Filosa, A., Enjin, A., Varoqueaux, F., Deininger, K., Schnütgen, F. and Brose, N. (2007). EphA4-Dependent Axon Guidance Is Mediated by the RacGAP 2-Chimaerin. Neuron *55*, 756–767.

Whelan, P., Bonnot, A. and O'Donovan, M. J. (2000). Properties of rhythmic activity generated by the isolated spinal cord of the neonatal mouse. J. Neurophysiol. *84*, 2821–2833.

Wickersham, I. R., Finke, S., Conzelmann, K.-K. and Callaway, E. M. (2007a). Retrograde neuronal tracing with a deletion-mutant rabies virus. Nature methods *4*, 47.

Wickersham, I. R., Lyon, D. C., Barnard, R. J. O., Mori, T., Finke, S., Conzelmann, K.-K., Young, J. A. T. and Callaway, E. M. (2007b). Monosynaptic Restriction of Transsynaptic Tracing from Single, Genetically Targeted Neurons. Neuron *53*, 639–647.

Wickersham, I. R., Sullivan, H. A. and Seung, H. S. (2010). Production of glycoprotein-deleted rabies viruses for monosynaptic tracing and high-level gene expression in neurons. Nat Protoc *5*, 595–606.

Windhorst, U. (1990). Activation of Renshaw cells. Prog. Neurobiol. *35*, 135–179.

Windhorst, U. (2007). Muscle proprioceptive feedback and spinal networks. Brain Res. Bull. *73*, 155–202.

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

Yonehara, K., Balint, K., Noda, M., Nagel, G., Bamberg, E. and Roska, B. (2010). Spatially asymmetric reorganization of inhibition establishes a motion-sensitive circuit. Nature *469*, 407–410.

Young, J. A., Bates, P. and Varmus, H. E. (1993). Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. *67*, 1811–1816.

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

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

Zhong, G., Sharma, K. and Harris-Warrick, R. M. (2011). Frequency-dependent recruitment of V2a interneurons during fictive locomotion in the mouse spinal cord. Nat Commun *2*, 274.

Zou, Y., Stoeckli, E., Chen, H. and Tessier-Lavigne, M. (2000). Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell *102*, 363–375.