Specific connectivity and molecular diversity of mouse rubrospinal neurons

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ABSTRACT

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While much progress has been made in understanding the development, differentiation, and organization of the spinal motor system, the complex circuitry that is integrated to determine a motor behavior has yet to be fully understood. The activity of motor neurons is influenced by sensory feedback, excitatory and inhibitory interneurons, and supraspinal control from higher brain regions in the CNS. Descending pathways from the cortex and midbrain are involved in the control of voluntary motor output. This is made possible by their projections onto spinal interneurons and, to a degree that varies between species, directly onto motor neurons. However, the somatotopic organization and molecular diversity of supraspinal projection neurons, and the circuitry that underlies their contribution to motor output, remain incompletely understood.

The evolutionary emergence of direct descending projections onto motor neurons has been considered to reflect a specialized level of organization for precise control of individual forelimb muscles. Unlike their polysynaptic counterparts, monosynaptic connections represent direct, unfiltered access to the motor neuron circuit. The direct circuit is thought to represent a neural specialization for the increase in fractionated digit movements exhibited by primates and humans. The

progressive realization that rodents have a greater degree of manual dexterity than was previously thought has evoked renewed interest in the role of direct supraspinal projections in other mammalian species. Lesion studies in the rodent indicated that, of the two major supraspinal pathways involved in the control of voluntary movement, the rubrospinal tract had a greater role in control of distal forelimb musculature. However, the degree to which this reflected direct projections onto motor neurons was not clear

Earlier anatomical tracing studies in the rat indicated that there are close appositions between labeled rubrospinal axons and motor neurons projecting to intermediate and distal forelimb muscles. To confirm that these contacts correspond to synapses, I developed a viral tracing strategy to visualize projections from the midbrain. Using an established technique of high-magnification confocal imaging combined with co-localization of the rubrospinal synaptic terminal marker, vglut2, I established the existence of monosynaptic connections from the ventral midbrain at the level of the red nucleus onto a restricted population of forelimb motor neurons at a single spinal level (C7-C8) in the rodent.

To determine whether the motor neurons that receive synaptic input correspond to specific motor pool(s), I first established a positional map of forelimb muscle motor pools in the cervical enlargement of the mouse spinal cord. A single motor pool, that which innervates the extensor digitorum muscle, appeared to be situated in the dense dorsolateral termination zone of rubrospinal ventral fibers. The

extensor digitorum muscle plays a key role in digit extension and arpeggio movements during skilled reaching. Anterograde labeling of rubrospinal descending fibers combined with retrograde labeling of extensor digitorum motor neurons revealed a direct circuit from the red nucleus onto this population of motor neurons. Surprisingly, neighboring motor pools innervating digit flexor muscles did not receive rubrospinal inputs. Moreover, other modulatory inputs onto motor neurons, including corticospinal, proprioceptive, and cholinergic interneuron afferents did not distinguish between extensor and flexor digitorum motor neurons. My data therefore reveal a previously unrecognized level of motor pool specificity in the direct rubrospinal circuit.

The identification of a small number of rubrospinal fibers that project onto extensor digitorum motor neurons suggested a considerable degree of heterogeneity between rubrospinal neurons. I therefore investigated the anatomical and molecular organization of subpopulations of rubrospinal neurons using retrograde labeling to identify subpopulations of rubrospinal neurons projecting, respectively, to cervical and lumbar levels of the spinal cord. Two rubrospinal populations could be identified within the red nucleus: a rostral population of intermingled cervical and lumbar projection neurons which express the Pou transcription factor Brn3a, and a caudal population containing segregated cervical and lumbar domains, which co-express Brn3a and a novel member of the C1q/TNF protein family, C1qL2. Following laser capture microdissection and genetic profiling of these three populations, I identified and validated molecular correlates of the topographic domains within the rodent red

nucleus. The transcription factors *tshz3* and *mafB* are expressed in the caudal cervical domain, whereas the chemokine *fam19a4* is restricted to the caudal lumbar domain. KitL is an axon guidance molecule that is expressed in both the rostral population and the caudal cervical population. Finally, I identified two genes, *cxcl13* and *gpr88*, that characterize subpopulations within these topographic divisions. Although the functional role of these genes in the establishment of the rubrospinal circuit remains to be determined, the data reveal a high level of molecular heterogeneity within the red nucleus. I hypothesize that this diversity allows rubrospinal neurons to form circuits in a precise and specific manner during development.

Overall, my data provide evidence for a novel organization within the rodent motor system in which direct projections from the rubrospinal tract onto motor neurons appear to control a very specific aspect of skilled movement: the stereotypic extension and separation of the digits in preparation for a task requiring digit manipulation. Identifying molecular correlates of the direct rubrospinal population is the logical next step in further understanding the specific circuitry that encodes descending motor commands. My results will provide a basis for the dissection of the rubro-motoneuronal circuit, enabling the establishment of a direct link between neural connectivity and individual muscle control during a skilled movement

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Dedication

I dedicate this thesis to our dear friends, Ted and Karen Bee, whose enthusiastic support and encouragement over the years has meant the world to me.

Chapter 1: Introduction

In this thesis, I will investigate the specific circuitry of one of the major descending motor pathways, the rubrospinal tract, in relation to individual muscle activation during skilled movement. This project will involve an exploration of two important aspects of the rubrospinal circuit: the specificity of post-synaptic targets contacted by descending rubrospinal fibers, and the molecular identity and organization of rubrospinal neurons underlying the establishment of this circuit.

The functional consequences of descending motor signals from higher brain regions on motor output are encoded by their projections onto spinal neurons. The overall effects of supraspinal commands are thus constrained by the intraspinal circuits through which this information is relayed. This introductory chapter will begin by describing the fundamental organization within the motor system. I will then focus my attention on the specific connections between supraspinal motor regions and their post-synaptic spinal counterparts responsible for implementation of the descending commands. Finally, I will detail the strategies employed by the developing nervous system to establish the precise wiring within this circuit required for the appropriate execution of a motor behavior.

Multiple levels of control of motor output

While much progress has been made in understanding the development, differentiation, and organization of the motor system, the complex circuitry that is integrated to ultimately generate a motor behavior has yet to be fully understood. The main components of the motor system are the following: the spinal motor system comprised of local interneurons and motor neurons, and the supraspinal motor system, consisting of the brain regions dedicated to sensorimotor control and the descending axonal tracts that relay information from the brain to the spinal cord (Kuypers, 1964). An emergence of increasingly sophisticated motor output is seen as one advances along the neocortical evolutionary spectrum, and supraspinal motor circuits result in devastating motor consequences (Lawrence and Kuypers, 1968a). Thus, it has been postulated that a top-down command system forms the basis of neural control of movement.

In both clinical and scientific settings, supraspinal pathways have been classically referred to as 'upper motor neurons.' However, many scientists now believe that this hierarchical view of motor control needs to be adjusted, given the amount of independent integration that takes place within the spinal cord. For example, the monosynaptic stretch reflex responsible for the stabilization of muscle length is mediated at a spinal level (Brown, 1981). Furthermore, studies have demonstrated that an isolated spinal cord preparation is capable of maintaining a normal fictive locomotion pattern in the absence of supraspinal input (Bonnot and Morin, 1998; Grillner and Zangger, 1979). The fundamental importance of supraspinal control is not being questioned. Rather, as Roger Lemon notes in his

seminal review, “we need to understand that the descending pathways function as part of a large network rather than as separate controllers of the spinal cord” (Lemon, 2008). We therefore need to first direct our attention to understanding how the descending pathways integrate with pre-established spinal circuits.

Spinal motor pools as the final link in the chain of motor control

The fundamental basis of organization within the motor system is the control of individual muscles. Over 100 years ago, Sherrington made the striking observation that motor neurons in the lumbar enlargement of the spinal cord were grouped into distinct elongated columns that extended across multiple spinal segments (Sherrington, 1892). More than 50 years would pass before the seminal work of Romanes and others demonstrated that the columnar organization in fact consisted of clustered ‘pools’ of motor neurons, each of which innervated a single muscle (Romanes, 1951). The location of the motor pool corresponding to each muscle was reproduced from animal to animal along both a longitudinal and a transverse axis (Landmesser, 1978b; Romanes, 1951). The one-to-one relationship between motor pool activity and contraction of individual muscles provides the simplest anatomical foundation of motor output.

The assembly of the motor circuit begins with the assignment of motor pool identity and the establishment of accurate motor neuron-muscle connectivity (Dasen et al., 2008; Dasen et al., 2005; De Marco Garcia and Jessell, 2008). Cell types within the spinal cord are generated in discrete cellular columns along a dorso-ventral

axis in response to a gradient of secreted molecular signals, including sonic hedgehog (SHH) from the floorplate, and retinoic acid (RA) from the paraxial mesoderm (Novitch et al., 2003; Roelink et al., 1995). This molecular gradient induces the expression of transcription factors, which define spinal progenitor domains through a combinatorial code, including motor neuron progenitors in a ventral domain (Briscoe et al., 2000) (Figure 1.1a).

From this generic population of motor neurons, subtype identity is generated along a rostro-caudal axis. Motor neurons are first classified into columns based on the identity of their target tissue (Hollyday, 1980; Landmesser, 1978b). A medial motor column (MMC) of MNs projecting to axial muscles is present at all spinal levels whereas cervical and lumbar levels contain a lateral motor column (LMC) corresponding to limb-projecting motor neurons. Within the LMC, MNs are further segregated into a medial and lateral division depending on their innervation of dorsal or ventral musculature (Landmesser, 1978a). Finally, MNs projecting to individual muscles are segregated into motor pools (Hollyday, 1980; Hollyday and Jacobson, 1990).

Insight into the mechanisms by which MN subtype identity is induced has come from a number of experiments in which regions of the spinal cord were transposed at during a critical early time period prior to muscle innervation (Ensini et al., 1998; Matise and Lance-Jones, 1996). The transplanted spinal segments acquired

Figure 1.1

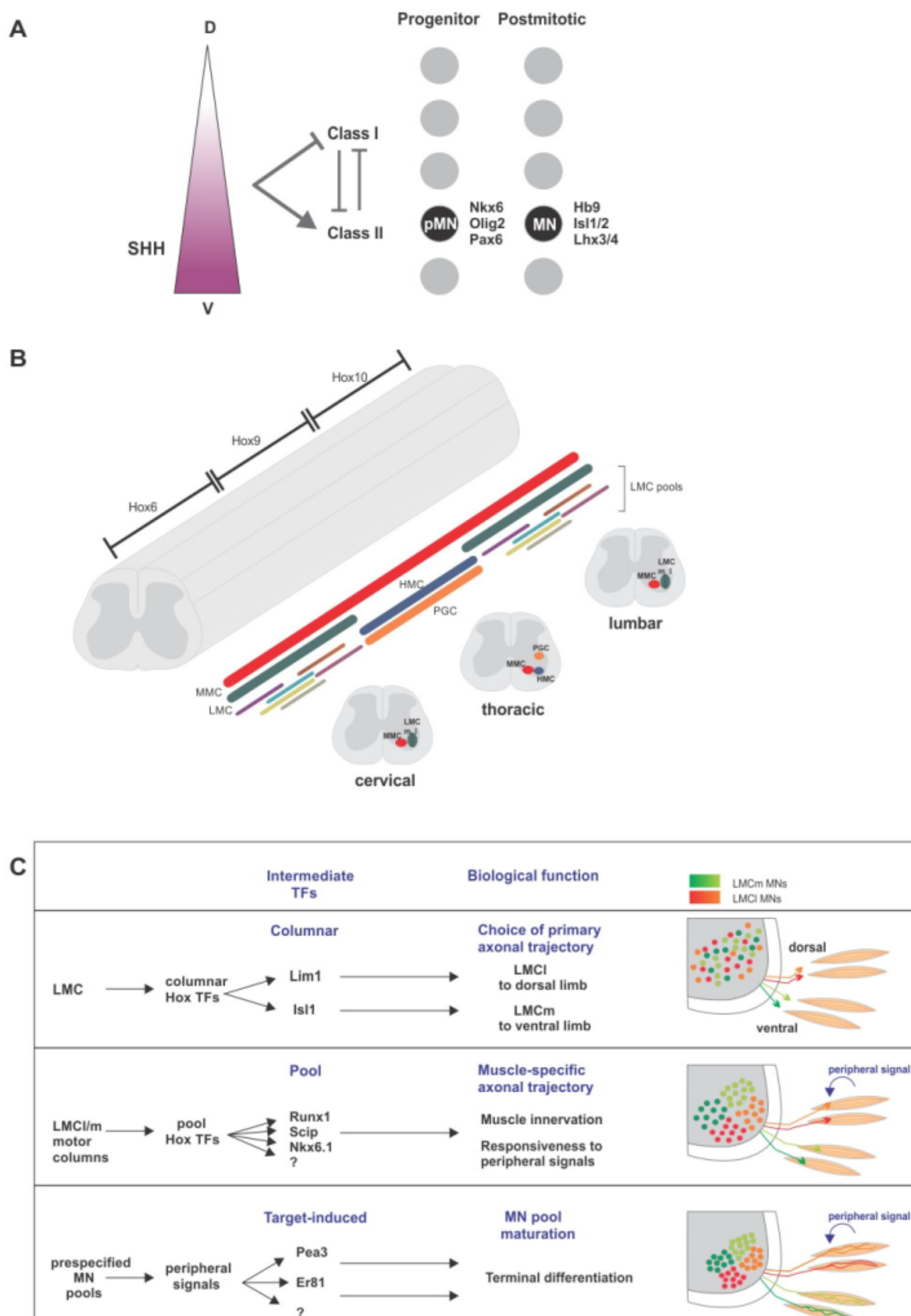


Figure 1.1 Development of motor neuron subtype identity

A. A gradient of SHH specifies progenitor domains in the ventral spinal cord Progenitor domains within the ventral spinal cord are specified along a dorso-ventral (DV) axis by a gradient of secreted molecular factors, including SHH from the ventral floorplate. Shh induces the expression of Class II transcription factors, and represses Class I factors. Further cross-repression between these two classes of TF's results in a unique transcriptional profile in each progenitor domain.

From: Dasen and Jessell, 2009

B. Motor neuron columnar identity is determined by Hox gene expression Establishment of motor neuron columnar identity is specified along a rostro-caudal (RC) axis by the graded expression of FGF8, retinoic acid (RA), and Gdf11 (not shown). These signals induce the expression of longitudinal domains of Hox proteins, whose RC distribution mirrors their chromosomal order within the Hox cluster. At segmental levels, the combinatorial expression of Hox genes specifies individual motor pool identity.

Adapted from: Dasen et al., 2003, 2005

C. Establishment of motor neuron-muscle connectivity Accurate motor neuron-muscle connectivity is determined by the activation of transcriptional programs at various stages of development. The initial choice of axon trajectory through the developing limb depends on the activity of columnar Hox TFs. Motor pool specific Hox TFs induce the expression of pool specific TFs such as runx1, scip, and nkx6.1, that result in the innervation of a specific muscle target. Finally, peripheral signals induce the expression of ETS TFs such as pea3 and er81, which control late aspects of motor neuron differentiation.

From: Dalla Torre di Sanguinetto et al., 2008

a MN identity appropriate for their new location within the spinal cord, suggesting that subtype identity is specified through rostro-caudal signals extrinsic to the spinal cord. It is now known that various molecular signals from the surrounding mesoderm are responsible for the induction of longitudinal domains of gene expression defined by members of the Hox family of homeobox proteins (Dasen et al., 2003; Ensini et al., 1998; Liu et al., 2001) (Figure 1.1b). Within each broader domain, cross-repressive activities of individual Hox genes direct the expression of transcription factor profiles that specify individual motor pool identity (Dasen et al., 2005; Jung et al., 2010).

These downstream regulatory networks are part of a cell-intrinsic repertoire that controls the early patterning of motor neuron-muscle connectivity (De Marco Garcia and Jessell, 2008). Target muscle-derived cues also induce transcriptional programs in developing MNs that further refine aspects of the motor circuit (Lin et al., 1998; Vrieseling and Arber, 2006). For example, the induction of ETS transcription factors, such as Pea3 and Er81, in individual motor pools is responsible for cell body clustering, dendritic arborization, axonal branching, and sensory innervation (Arber et al., 2000; Livet et al., 2002; Vrieseling and Arber, 2006) (Figure 1.1c).

Control of motor pool activity by local circuits

Although the final output of the motor system is ultimately determined by the activity of motor neurons, this activity represents an integration of inputs from many

sources: sensory feedback from the muscle, excitatory and inhibitory spinal interneurons, and supraspinal commands from higher brain regions in the CNS. Each of these modulatory circuits regulates the activity of motor neurons in two ways: indirectly, by feeding into intraspinal circuits that project onto motor neurons, and directly, through monosynaptic activation of motor neurons (Figure 1.2).

The organization of motor neurons into muscle-specific motor nuclei likely simplifies the development of this precise circuitry. Clustering of motor neuron cell bodies is required for cell-cell communication via gap junctions, thought to be responsible for the synchronous firing of motor neurons in a given pool during embryonic development (Personius and Balice-Gordon, 2001). In addition to facilitating coordinated output of motor neurons within a pool, the cell body and dendritic location of a given motor pool may also direct the patterning of afferent inputs (Arber et al., 2000; Vrieseling and Arber, 2006). For example, recent work in the Jessell lab has demonstrated that incoming sensory axons initially select a post-synaptic motor neuron target region based on laminar positioning in the ventral spinal cord (Gulsen Surmeli and Tom Jessell, personal communication). Precise matching of pre-synaptic inputs with the appropriate post-synaptic motor target maintains an organized flow of information through the motor circuit.

Proprioceptive information from a muscle is relayed back to its corresponding motor pool via discrete sensory-motor circuits (Brown, 1981). The proprioceptive neurons involved form a subset of the sensory neurons grouped together in dorsal root

Figure 1.2

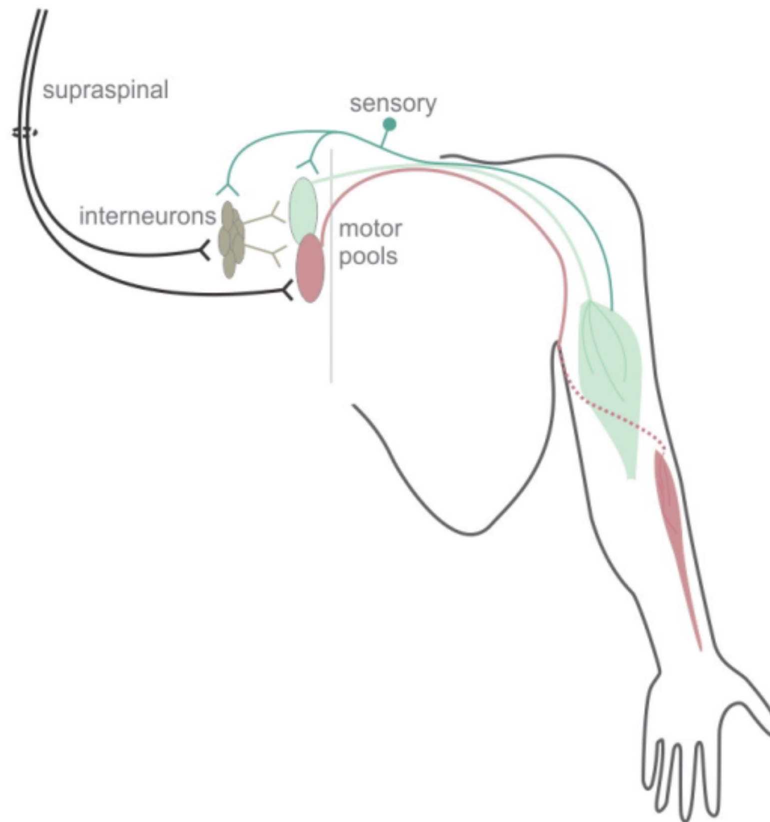


Figure 1.2 control of motor pool activity by local and supraspinal circuits Individual motor pool activity can be regulated through monosynaptic and polysynaptic circuits. Proprioceptive feedback from the muscle is relayed back to the motor pool through monosynaptic Ia circuits and polysynaptic Ia and Ib circuits through interneuron pathways. Likewise, supraspinal commands modulate motor pool activity indirectly, through interneuron intermediaries, and directly, through monosynaptic inputs onto motor neurons.

Adapted from: Kanning et al., 2010

ganglia (DRG) at each spinal segment. Group Ia and II proprioceptors innervate specialized sensory organs called muscle spindles, and respond to an increase in spindle length (Matthews, 1964). Through a monosynaptic reflex arc, group Ia sensory afferents then excite the alpha motor neurons innervating the muscle, signaling the muscle to contract (Burke and Nelson, 1966). The monosynaptic stretch reflex is thus responsible for maintaining individual muscle contractility. Group Ib proprioceptors innervate Golgi tendon organs, and respond to an increase in muscle tension. However, rather than directly regulating motor neuron activity, 1b proprioceptors utilize interneuron circuits to excite antagonistic muscles and inhibit synergistic muscles (Eccles et al., 1957).

Within the spinal cord too, “pre-motor” interneurons, the final neurons in a pathway prior to the motor neuron target, constitute only a subset of interneurons. Although the organization of interneuron networks in the spinal cord is just starting to be addressed, recent studies have demonstrated that “pre-motor” interneurons are preferentially linked to an individual motor pool target (Stepien et al., 2010). Interneurons that are further removed from motor neurons would likely have more widespread effects, given the cross talk between interneuron circuits.

The modulatory effects of different classes of pre-motor interneurons on motor neuron activity are wide-ranging, based on their neurotransmitter class, cell body or dendritic location of synaptic input, temporal activity, and input/output circuitry. For example, Renshaw cells are a specialized class of inhibitory pre-motor

interneurons that receive collateral inputs from motor neurons, and also send recurrent collaterals back to motor neurons, inhibiting their further activity in a feedback loop (Renshaw, 1941). Alternatively, cholinergic interneurons within the spinal cord give rise to large, so-called c-bouton inputs onto motor neurons. Recent work has demonstrated that this circuit provides an excitatory drive to motor neurons during CPG-stimulated locomotor behavior (Zagoraiou et al., 2009). Thus, the diverse nature of pre-motor inputs onto motor neurons provide a mechanism for the differential regulation of motor neuron activity based on behavioral requirements.

Control of motor pool activity by descending pathways

In addition to their regulation through local modulatory circuits, spinally-directed motor behaviors depend critically on the specificity with which supraspinal descending pathways activate motor neurons (Lawrence and Kuypers, 1968a). Descending long-range pathways from cortical and sub-cortical brain regions relay motor commands to the spinal cord, and these descending circuits are also capable of modulating motor neuron activity indirectly by feeding into established intraspinal pre-motor circuits, or directly through monosynaptic projections onto motor neurons. However, while lesion studies have implicated both the cerebral cortical and midbrain motor centers in the control of voluntary, skilled movement, the organization and intraspinal circuitry of these regulatory systems remains unclear.

It has been suggested that the emergence of the direct projections from higher brain regions onto motor neurons represents an evolutionary specialization facilitating

the voluntary activation of individual muscles (Lemon, 2008). However, the strongest evidence supporting this hypothesis is largely correlative. A significant expansion of the direct circuit is seen in species exhibiting the highest capacity for fractionated digit movements (Bortoff and Strick, 1993; Heffner and Masterton, 1975). Similarly, lesion studies have demonstrated a permanent loss of individual digit control following removal of descending inputs (Lawrence and Kuypers, 1968a, b). Nonetheless, a direct causative link between the monosynaptic circuit and individual muscle activation does not yet exist.

Previous studies of descending pathways have approached each tract as a single entity. Although these anatomical and functional studies have provided much needed insight into the functional role of supraspinal control, and will be described in detail, the specific connections of individual descending neurons have been largely ignored. In a review of the post-synaptic targets of one of the major descending pathways, the rubrospinal tract, the spinal physiologist Elizabeta Jankowska states: "…we would understand its mode of operation much better by knowing the role played by individual neurons of this circuit in activating various neuronal circuits." (Jankowska, 1988).

The proposed function of supraspinal monosynaptic activation of motor neurons in regulating individual muscle activation would rely heavily on the specificity of descending inputs onto motor pools. In this thesis, I will explore whether a motor pool-specific level of organization exists within one of the major

descending motor circuits, the rubrospinal tract. I hope to provide a direct link between the functional output of descending motor control, and the underlying neural circuitry required for its execution.

Supraspinal control of movement

Classification of descending pathways based on spinal termination zones

The supraspinal pathways regulating motor control originate in the cortex, midbrain, and hindbrain, and each has a distinct role to play in the development of a motor repertoire, encoded by their synaptic connections onto intraspinal circuits. In his review of descending motor systems, Roger Lemon describes the seminal approach of Hans Kuypers, one of the pioneers of the field: "Kuypers study of descending pathways convinced him that the key to understanding their function was to examine their termination pattern within the spinal gray matter: to define the address to which descending activity is sent" (Lemon, 2008). With this distinction in mind, the descending pathways have historically been categorized into four major classifications based on their spinal termination zones: the ventromedial brainstem group, the emotional motor system, the dorsolateral brainstem group, and the most recent emergence in evolutionary terms, the corticospinal and corticobulbar pathways.

As their name implies, the ventromedial brainstem pathways descend in the ventral and ventrolateral funiculi of the spinal cord and terminate bilaterally in intermediate laminae VII and VIII, which give rise to long-range bilateral

propriospinal neurons. The proposed function of the ventromedial pathways in motor control is to provide coordinated postural support for head, neck, trunk, and limb movements. The ventromedial pathways are comprised of spinal projection neurons from the interstitial nucleus of Cajal, the tectum, the vestibular nuclei, and the diffuse midbrain and hindbrain reticular formation. These pathways are found in primitive vertebrates, and their function is largely conserved through evolution.

The emotional motor system comprises a number of pathways from non-motor regions of the brainstem, such as the Raphe nucleus. These descending tracts generally express modulatory neurotransmitters (5-HT, noradrenaline), and terminate either in the dorsal horn or on autonomic and somatic motor neurons, where they can influence motor neuron excitability. These pathways play a role in the mobilization of motor behaviors in response to emotional and physiological cues.

The dorsolateral brainstem group is thought to play a greater role in limb movements, based on their target zone in the dorsolateral intermediate spinal cord. At the level of the cervical enlargement, this termination zone is also situated in close proximity to the dorsolateral populations of motor neurons. The major dorsolateral brainstem pathway is the rubrospinal tract, which arises from the red nucleus in the midbrain, and a second, smaller pathway, the pontospinal tract, originates from the pontine nucleus in the medulla. The rubrospinal tract is found in all vertebrate animals with limbs or pseudolimbs, indicating a long evolutionary history of this pathway (ten Donkelaar, 1988).

Lastly, the most recent descending pathway to emerge is the corticospinal tract, which originates from layer 5 in the cortex. This projection is first seen in mammals, and its contribution to motor output has significantly increased throughout evolution. In more primitive species, the corticospinal termination zone largely overlaps with that of dorsolateral brainstem pathways, in particular from the rubrospinal tract. In higher species such as primates and humans, the corticospinal termination zone has expanded to include a larger portion of the dorsal horn and a dense projection into the ventral horn (Bortoff and Strick, 1993). The large overlap between the termination zones of the cortical projections and the dorsolateral brainstem pathways, the two pathways involved in the control of voluntary movement, raises the question as to whether these two systems represent parallel or complementary motor control centers, and whether their functional roles are maintained in the face of shifting evolutionary motor demands.

Overall, the descending pathways are classified by their brain region of origin, and their termination zone within the spinal cord. Nonetheless, a deeper understanding of the functional contribution of higher brain regions to motor control requires a detailed description of the post-synaptic circuits through which supraspinal commands are relayed.

Descending modulation of motor output

Descending projections exert their influence over voluntary motor output in a number of ways: through monosynaptic synapses onto motor neurons (direct

pathway) (Kuypers, 1958), by feeding into polysynaptic intraspinal circuits (indirect pathway) (Jankowska, 1988), or by inputs onto neurons that give rise to ascending projections to higher brain regions (Hantman and Jessell, 2010). In the latter two circuits, descending input is coalesced with inputs from other sources, including sensory information and commands from other descending pathways. The polysynaptic, indirect pathway might represent a mechanism for dispersion of the descending command at a spinal level, as interneuron circuits might communicate the descending command to multiple motor pools, whereas the direct, monosynaptic pathway would converge directly onto the target motor pool. This distinction between the indirect and direct descending projections onto motor neurons can be used to highlight two divergent motor control strategies that have both been attributed to descending pathways: the activation of muscle synergies (or action patterns) and the control of individual muscles.

Descending control of motor behavior through the activation of muscle synergies

Descending pathways play a critical role in the execution of motor tasks. However, the mechanisms by which they accomplish this are largely unknown. A question that has long been debated is whether the organizational scheme of the descending pathways centers on the activation of individual muscle targets, or whether the output is encoded through local spinal networks such as those governing "muscle synergies," the activation of a group of muscles in concert to accomplish a certain movement. In the former case, each element of a movement would need to be centrally encoded, whereas muscle synergies organized at the spinal level could be

recruited by descending pathways in the combinations and sequences necessary to execute a behavior.

The strongest current evidence that descending pathways control motor output through the activation of muscle synergies comes from experiments correlating cortical activity with the coordinated activation of multiple muscle groups (Armstrong and Drew, 1985; Graziano et al., 2002), For example, the firing of individual pyramidal tract neurons was recorded during a skilled forelimb task (Holdefer and Miller, 2002). When compared with EMG recordings of individual muscles, the activity of single neurons could be tightly correlated with that of a restricted subset of muscles. In some cases, these muscle groupings could be associated with a functional synergy required for a component of the movement.

Drew and colleagues have proposed a model in which motor cortex activation of muscle synergies may be instrumental during visually-guided gait modification in the cat (Drew et al., 2008). Lesioning the descending corticospinal inputs results in an immediate defect in obstacle avoidance during overground locomotion, reflecting a loss of ability to modify movements in response to a changing environment (Drew et al., 2002; Muir and Whishaw, 1999). Gait modification requires a complex pattern of timing, amplitude, and duration in the activity of various limb muscles. By using a novel clustering method to identify muscle co-activation, Drew and colleagues grouped the forelimb muscles into 11 distinct “synergies” during the locomotor cycle, with each synergy representing a specific movement component of the lift, swing, and step phases. These synergistic groups included muscles acting across different joints

(multiarticular synergies), and individual muscles could be included in more than one synergy (Figure 1.3 a,b).

Motor cortex recordings have demonstrated that individual pyramidal tract neurons are activated in a phasic manner during the gain modification step. Rather than correlating individual pyramidal tract neuron activity with the activation of an individual muscle, Drew and colleagues hypothesize that a more efficient way to execute the movements required for gait modification would be for the motor cortex to instead activate a subset of the 11 identified muscle synergies. Individual descending tract neurons could produce necessary gait and behavioral modifications by altering the amplitude and phase of an individual synergy. This idea is favorably supported by both the phasic activity of corticospinal neurons and the extensive collateral branching of individual corticospinal and rubrospinal fibers within the spinal cord (Li and Martin, 2001; Shinoda et al., 1976; Shinoda et al., 1977), which could provide the anatomical substrate necessary for the ultimate activation of multiple motor pools. As the authors judiciously point out, the case for descending control of movement through muscle synergies must be strengthened by a direct linkage of pyramidal tract neuron activity with the actual phase and amplitude changes observed in synergistic muscle groups during gait modification.

Although the neural basis of muscle synergies is unknown, they are likely to be encoded by spinal interneurons that recruit multiple motor neuron targets. Unfortunately, we are only just beginning to develop the tools necessary to

Figure 1.3

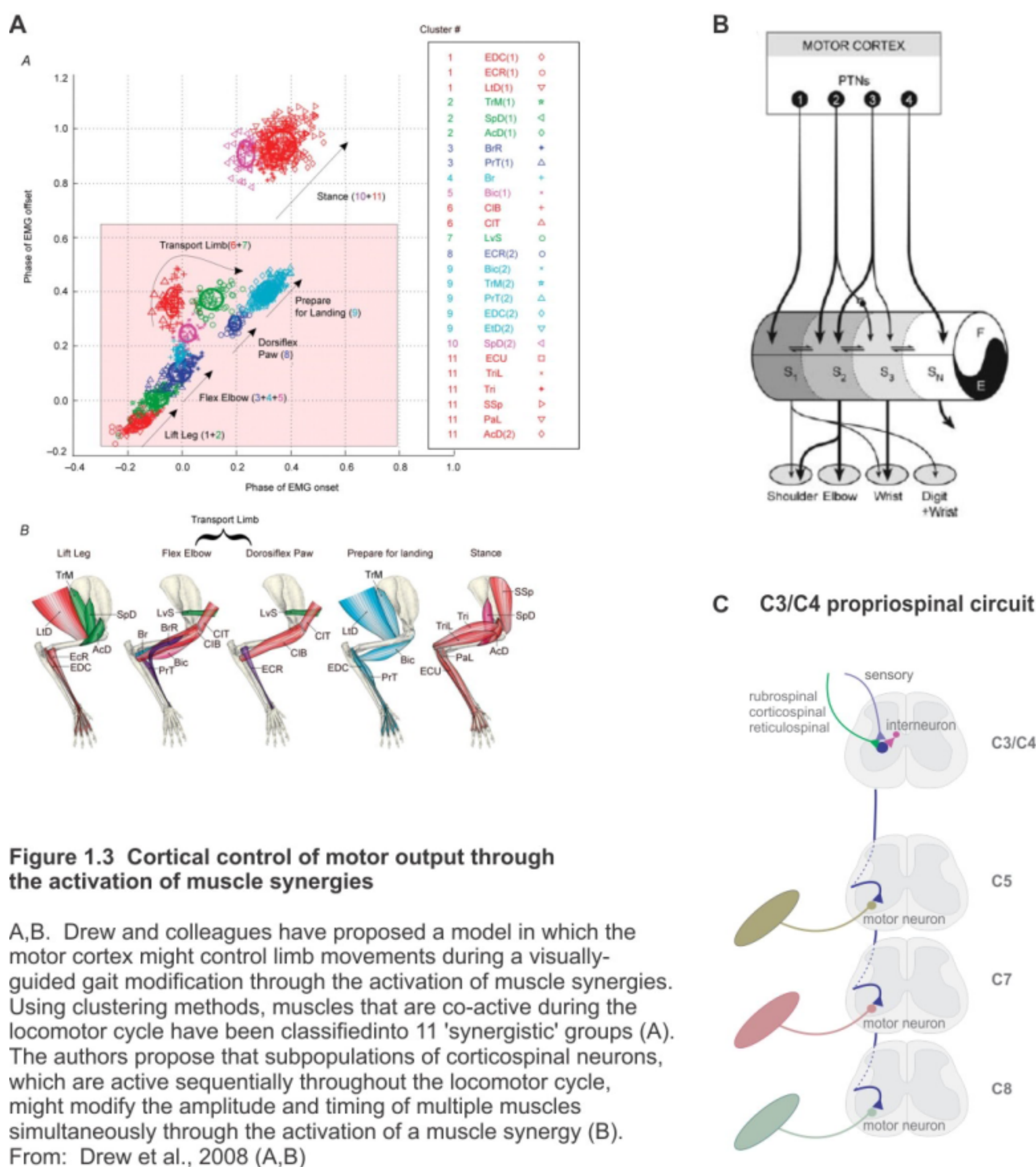


Figure 1.3 Cortical control of motor output through the activation of muscle synergies

A,B. Drew and colleagues have proposed a model in which the motor cortex might control limb movements during a visually-guided gait modification through the activation of muscle synergies. Using clustering methods, muscles that are co-active during the locomotor cycle have been classifiedinto 11 'synergistic' groups (A). The authors propose that subpopulations of corticospinal neurons, which are active sequentially throughout the locomotor cycle, might modify the amplitude and timing of multiple muscles simultaneously through the activation of a muscle synergy (B). From: Drew et al., 2008 (A,B)

C. Muscle synergies might be encoded at a spinal level through the C3/C4 propriospinal circuit. These neurons receive converging inputs from many sources,including supraspinal inputs from the corticospinal, rubrospinal, and reticulospinal tracts, and have been demonstrated to project onto multiple motor pool targets, thus providing a potential anatomical substrate for the dispersion of a descending signal to multiple motor pools.

understand the complexity of intraspinal circuits. We do know that although last-order interneurons usually display a preference for a single motor pool target, these interneurons also modulate to a lesser extent motor pools that innervate synergistic muscles (Stepien et al., 2010). Cortical and rubral projections are capable of accessing both first- and last-order interneurons (Jankowska, 1988).

One candidate population that might mediate the distribution of descending signals to multiple motor pools is the system of propriospinal neurons (PN) (Figure 1.3c). Propriospinal neurons are a specialized group of interneurons located at C3-C4 spinal levels and have been identified in the cat, monkey, and human (Alstermark et al., 2007). PN neurons receive inputs from the major descending pathways, including the rubrospinal and corticospinal tracts, and integrate these supraspinal commands with information from sensory afferents and intrinsic spinal interneurons (Alstermark et al., 1999; Illert et al., 1978). Propriospinal neurons are last-order interneurons, and directly contact motor neurons. The demonstrated branching patterns of propriospinal axons are consistent with the requirements for an anatomical substrate of muscle synergies. Single labeled propriospinal axons send collateral branches to multiple motor pools including combinations of muscles affecting up to 3 joints (Tantisira et al., 1996). It remains to be determined whether descending pathways utilize propriospinal circuits in the recruitment of multiple motor pools.

Even in species that possess a significant direct descending projection, the majority of descending input is mediated primarily through interneurons. The

evolution of the descending tracts to include direct projections onto motor neurons suggests that perhaps the strategy of individual muscle control vs. muscle synergies may function in parallel depending on the behavioral repertoire demands of each species. Although it will not be addressed in this thesis, our knowledge of descending control will remain incomplete without a fundamental understanding of how the indirect and direct descending circuits combine forces to regulate motor output.

Direct descending circuits

The most striking difference in descending inputs between species is the development of direct synaptic inputs onto forelimb motor neurons. Although historically this direct circuit was thought to first emerge in the primate lineage, recent work has indicated that direct projections from the cortex and red nucleus are present in other species to a lesser extent (Bareyre et al., 2005). These monosynaptic connections onto motor neurons bypass the pre-motor integration that occurs within the local spinal circuits, and provide an intriguing potential neuronal basis for the increase in individual motor control and dexterity exhibited by primates and humans.

Functional significance of monosynaptic projections onto motor neurons

Although it had long been known that the cortex influenced motor output through long-range spinal projections, it was not until 1958 that Hans Kuypers first demonstrated evidence of corticospinal fibers terminating amongst the ventral motor nuclei in the rhesus monkey (Kuypers, 1958). Fifty years later, we know now that the two main supraspinal motor centers involved in voluntary movement, the cortex and

the red nucleus, both send a direct projection to the distal forelimb motor nuclei in primates (Bortoff and Strick, 1993; Holstege et al., 1988; Ralston et al., 1988).

The direct circuit from the motor cortex has expanded over the course of evolution; although monosynaptic contacts in the primate lineage are restricted to distal forelimb motor nuclei, in humans they extend to include proximal forelimb and even hindlimb motor neurons (Colebatch et al., 1990; Nielsen et al., 1995). Even within different primate species, the extent of the direct CM circuit onto motor neurons varies. Anatomical tracing of the corticospinal tract in a primate with a low index of dexterity, the squirrel monkey, reveals the presence of only a few corticospinal terminals which extend into the ventral horn at lower cervical levels (Bortoff and Strick, 1993). In comparison, the macaque monkey has corticospinal fibers extending in the dorsal and dorsolateral–most ventral horn at C8-T1 (Ralston and Ralston, 1985). Motor pool maps at this spinal level suggest that these locations house motor neurons projecting to distal forelimb flexors and intrinsic hand muscles (Jenny and Inukai, 1983). Finally, the capuchin monkey, which is capable of executing a precision grip between its thumb and index finger, displays a high density of corticospinal labeling throughout the entirety of the lateral ventral horn from C6-T1, suggesting the presence of corticospinal inputs onto motor neurons innervating a number of distal forelimb muscles (Bortoff and Strick, 1993) (Figure 1.4).

Rubro-motoneuronal projections to distal forelimb motor nuclei have also been anatomically demonstrated in the macaque monkey (Holstege et al., 1988).

Figure 1.4

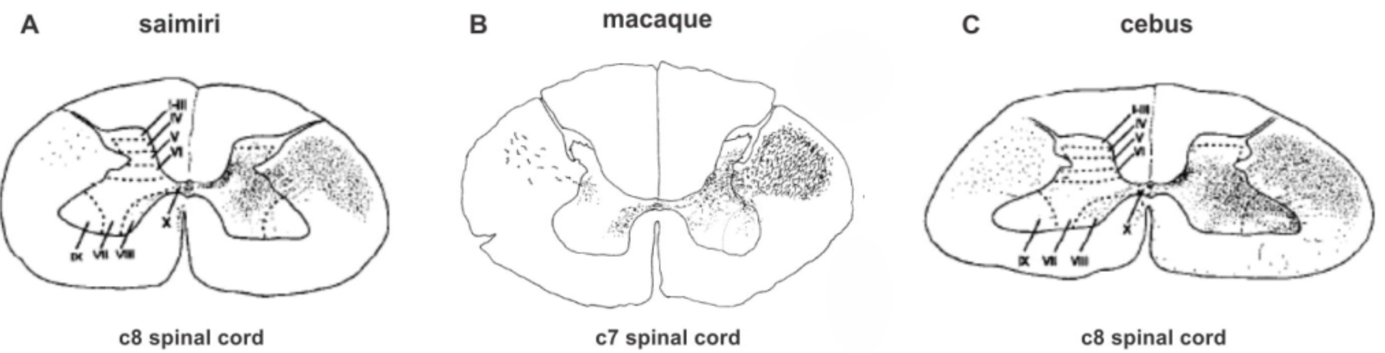


Figure 1.4 Cortico-motoneuronal projection varies across primate species The emergence of direct cortical projections onto motor neurons is an evolutionary specialization thought to increase the fine control of distal musculature. Across primate species, the density of the corticospinal projection into the ventral horn varies, and can be correlated with the level of dexterity exhibited by each species. The highly dextrous cebus monkey has the densest laminae IX CST projection. From Bortoff and Strick 1993 (A,C) and Ralston and Ralston 1985 (B).

Injection of an anterograde tracer into the red nucleus results in the labeling of a subset of rubrospinal fibers projecting into the ventral horn at C8-T1. Similar to the ventral horn corticospinal projection in the macaque, rubrospinal fibers appear restricted to the area containing the dorsolateral populations of motor neurons. This region contains forearm and digit extensor and flexor motor nuclei, and those projecting to the intrinsic hand muscles. Electron microscopic analysis has demonstrated ultrastructural evidence of labeled rubrospinal fibers making synaptic contacts with the proximal dendrites and cell bodies of large neurons in this region (Ralston et al., 1988).

The existence of rubro-motoneuronal connections in the human is largely unknown, as the location of the red nucleus deep within the cranium has prevented trans-cranial stimulation of the rubrospinal tract, and detailed tract tracing experiments in human material has not been performed. The decreased relative size of the red nucleus in humans has been taken to suggest that the corticospinal tract has evolved to provide the majority of descending control of voluntary movement (Nathan and Smith, 1982).

The emergence of direct cortical and rubral projections onto motor neurons is thought to reflect a specialized level of organization centered on the supraspinal activation of individual forelimb muscles. Unlike their polysynaptic counterparts, monosynaptic connections represent direct, unfiltered access to the motor neuron circuit. The actual contribution of the direct circuit to motor neuron activity is limited

by the quality and quantity of their inputs onto motor neurons. The latter measurement encompasses both the number of synaptic inputs converging onto a single motor neuron and the extensiveness of the direct projection to a given motor pool (Lemon, 2008). In experimental contexts, several studies have found that the post-synaptic effect of cortico-motoneuronal stimulation motor neurons is significant, and shown that discharge from a single CM neuron is capable of evoking a change in single motor unit or EMG recordings from distal forelimb muscles (Cheney et al., 1991a; Cheney et al., 1991b; de Noordhout et al., 1999).

Despite the documented effects of experimental stimulation, the functional significance of direct projections onto motor neurons in the execution of skilled forelimb movement is still a matter of debate. Support for a specialized role for direct descending projections in individual distal muscle control comes from the following lines of correlative evidence: the species-specific relationship between dexterity and the size of the cortico-motoneuronal projection, and the deficits in distal muscle functioning observed after lesioning the corticospinal or rubrospinal tracts in primates. The evidence linking the direct circuit with the activation of individual distal forelimb muscles will be explored in the following sections. Much of the most precise work has been performed using primate models, which show a greater incidence of direct CM projections. I will review these first, and at the end of the section contrast the findings with our relatively limited knowledge of these projections in rodent models.

Relationship between manual dexterity and direct supraspinal circuits

Manual dexterity can be thought of as a movement that has two components: the mechanical component comprised of bone and joint structure, and musculature, and the neural component encompassing anatomical circuitry and cerebral volition (Lemon, 1999). If we compare rodents and humans, for example, there is a clear difference in the mechanical elements of dexterity. Control of individual digit movements in humans is enabled by the expansion of the intrinsic hand musculature, including lumbricals and interosseus muscles on the digits themselves, facilitating both a flexion/extension movement and abduction/adduction. Furthermore, differences in radial and ulnar bone structure in humans allow for additional movement around the intermediate and distal forelimb joints. However, when primates and humans are compared, the mechanical aspects of hand movement seem insufficient to alone explain the increase in dexterity across these species.

One of the early anatomists, Wood Jones, suggested that the main difference between man and monkey was rooted neither in the mechanical nor neural aspects of limb movement, but in the cerebral control dictating voluntary repetition, purpose, and motivation. While this is certainly a significant consideration, more recent studies have emphasized the anatomical constraints of the motor system through which higher centers must exert their influence. Foremost is the emergence of monosynaptic projections from higher brain regions onto forelimb motor neurons. In a historical study by Heffner and Masterson, the extent and depth of the corticospinal projection into the ventral horn was found to be highly correlated with

the capacity for relatively independent finger movements (RIFM) exhibited across species (Heffner and Masterton, 1975; Heffner and Masterton, 1983).

While certainly a groundbreaking study of comparative biology, a number of key issues have been raised regarding the Masterton and Heffner study. The 'index of dexterity' scoring measurement used in the study was developed by Napier (Napier, 1961) and ranks species' dexterity on a scale of 1-7 based on various measurements of digit movement. It is now apparent that the dexterity of certain animals, in particular the rat, was underestimated by the study (see following sections). Furthermore, the tracing of the corticospinal tract was performed using older neurodegenerative techniques that underestimate the extent of the pathway. More advanced genetic and viral tract-tracing techniques can now be used to more accurately trace the descending pathways, increasing both specificity and completeness. Despite these drawbacks, the correlation between the direct CM circuit and digit dexterity first introduced a quarter of a century ago appears to have withstood the test of time. Recent anatomical tracing studies focusing on the primate lineage have demonstrated that the dextrous capuchin and macaque monkeys, which are capable of precision grip and tool use, have a much more extensive CM projection to the distal forelimb motor pools than does the less dextrous squirrel monkey (Bortoff and Strick, 1993; Maier et al., 1997).

Motor pools innervating hand muscles receive strong monosynaptic CM input

The correlative evidence linking the direct CM system and its proposed role in distal muscle control is further strengthened by physiological recordings indicating that the strength of monosynaptic inputs to forelimb motor pools increases along the proximo-distal limb axis (Porter and Lemon, 1993). In the macaque monkey, although monosynaptic EPSPs on motor neurons are evoked in most upper limb motor neurons following corticospinal stimulation, effects seen in MNs innervating intrinsic hand muscles were on the order of 5-7 mV, providing a considerable fraction of the excitatory drive required to bring a motor neuron to threshold. Likewise, single stimuli applied to the pyramidal tract were capable of evoking clear twitches in hand muscles (Porter and Lemon, 1993). Although the relative contributions of the direct and indirect corticospinal were not determined in these studies, the proximal vs. distal bias is clear.

Lesion studies implicate the CM system in distal muscle control

The functional consequences seen after lesioning a particular brain structure have been an instrumental tool in our ability to link behavioral processes and their underlying neural structures. Lesion studies of the descending pyramidal tract in primates have demonstrated a strong association between the corticospinal system and the control of distal hand movements. Although initial lesioning of the pathway results in the complete inability to move the forelimbs, the majority of proximal limb movement is eventually recovered. Although the mechanism is unknown, it likely involves plasticity and compensation from other circuits of the motor system, or sprouting of fibers above the lesion site. Despite the striking ability of the motor

system to regain the majority of limb movement following a corticospinal or rubrospinal lesion, independent digit and thumb movements are permanently lost, reflecting a striking deficit primarily in the control of intrinsic hand muscles (Lawrence and Kuypers, 1968a, b).

Evolutionary emergence of the direct circuit: evidence for monosynaptic inputs in non-primate species?

Corticospinal projections in the rat and cat

The ability to pinpoint the evolutionary emergence of direct supraspinal control of motor neuron activity would provide insight into the functional role of this neural specialization. If we can identify the earliest emergence of monosynaptic projections onto motor neurons, it might be possible to correlate this neural specialization with the corresponding emergence of a novel motor adaptation. This approach is similar to the comparative studies of Heffner and Masterson, but takes advantage of the tracing and imaging techniques developed in the three decades since the publication of their work to probe the species-specific emergence of the direct circuit. All members of the primate lineage that have been studied to date possess a direct descending projection onto motor nuclei corresponding to distal forelimb muscles (Bortoff and Strick, 1993; Holstege et al., 1988; Ralston and Ralston, 1985). Therefore, attention has been focused on species with a significantly decreased index of manual dexterity, the rat and cat.

Although the corticospinal tract sends dense projections to the dorsal and intermediate spinal cord in rats, projections extending further ventrally into the motor neuron nuclei are rarely observed (Brown, 1971; Yang and Lemon, 2003). A number of studies summarized below have indicated that the rodent lacks direct cortico-motoneuronal projections, but the subject has proved controversial (Alstermark et al., 2004; Bareyre et al., 2005; Liang et al., 1991).

In the rat, electron microscopic images of ventral-projecting corticospinal boutons reveal that although these terminals are located in close proximity to motor neuron cell bodies and proximal dendrites (Liang et al 1991), they do not make contact with a post-synaptic specialization (Yang and Lemon, 2003). In apparent agreement with this, electrophysiological evidence from intracellular motor neuron recordings at cervical levels demonstrates a lack of monosynaptic excitation following pyramidal tract stimulation (Alstermark et al., 2004). However, it is important to note that although the Alstermark experiments focused on CM connections in the lower cervical enlargement, which contains the distal forelimb motor neurons that are the most likely targets of direct inputs, there are also axial and proximal limb muscle motor neurons located at these spinal levels (Holstege et al., 1987). As only a fraction of motor neurons are analyzed for CM inputs, and the identity of motor neurons in these experiments is unclear, it remains a possibility that CM projections onto distal muscle pools were missed.

In fact, recent genetic labeling of the mouse corticospinal tract provided the first evidence of direct CST projections onto lumbar level motor neurons (Bareyre et al., 2005). A major advantage of genetic tract tracing experiments, which utilize gene expression studies and transgenic mouse engineering to restrict expression of a fluorescent reporter molecule in a subset of cells, is that – provided appropriate controls are performed - they specifically and robustly label a reproducible population of neurons. Interestingly, Bareyre et al. found the direct cortical connections to originate from the minor tracts of the rodent CST, the dorsolateral and ventral components, which descend separately from the main dorsal CST. In previous studies of the CST, the minor components were largely neglected, as they are few in number, and thus, rarely labeled by anterograde tracer injections into the cortex. The extent of the direct circuit in the rodent is unclear, and furthermore, the existence of direct cortical projections onto motor neurons at forelimb levels remains under investigation.

Anatomical evidence of cortico-motoneuronal projections has not been readily demonstrated in the cat (Armand et al., 1985). Although descending cortical projections terminate heavily in the dorsal and intermediate regions of the spinal cord, anterograde tracing of corticospinal projections from various areas of motor and somatosensory cortex in the cat reveal a sparse number of labeled fibers entering the ventral horn of the spinal cord throughout the cervical enlargement (Cheema et al., 1984). In support of the anatomical findings, stimulation of descending cortical fibers

in the cerebral peduncle rarely evokes short-latency forelimb motor neuron responses in the cat (Fujito and Aoki, 1995; Fujito et al., 1991).

Direct projections from the red nucleus onto motor neurons in the rat and cat

Given the emphasis placed on the cortical control of voluntary movement through the specialized CM projection, the importance of monosynaptic projections from subcortical brain regions has not been investigated in as much detail. Although the rubrospinal tract represents a more primitive motor control system, the overlapping termination zones of the rubrospinal and corticospinal projections in the spinal cord suggests that these two supraspinal motor centers likely play similar or complementary roles in the control of motor output.

The first anatomical tracing of the rubrospinal tract in the rat utilized silver staining techniques to identify degenerating rubrospinal fibers following lesions of the red nucleus (Brown, 1974). Although these early studies did not detail the existence of a rubrospinal projection onto motor neurons, more recent studies of the rubrospinal tract in both the rat and the cat, using injections of an anterograde tracer to visualize axons and terminals, have demonstrated the existence of a ventral projection in the vicinity of motor nuclei (Fujito and Aoki, 1995; Kuchler et al., 2002). These ventral projections are limited to cervical levels C7-T1. The striking similarity of the ventral rubrospinal projection seen in the rat and cat, with that of the monkey (Figure 1.5 e-g) indicates that direct projections onto forelimb motor neurons

originating from the red nucleus might be more widespread across species than previously thought.

The strongest evidence for monosynaptic projections onto motor neurons in the rat comes from anatomical studies in which the rubrospinal tract was anterogradely labeled by stereotaxic injection of biotinylated dextran amine (BDA) into the red nucleus while motor neurons were retrogradely identified by intra-muscular injections of cholera toxin B-subunit (CTB). Direct rubro-motoneuronal connections were defined by the following criteria: 1. Labeled bouton-like swelling at the presynaptic apposition site. 2. Rubrospinal bouton and motor neuron dendrite both visible in the same plane of section at high magnification and 3. No gap observed between the two structures (Kuchler et al., 2002). Evidence of such anatomically defined 'synapses' were found in the rodent cervical spinal cord, and were restricted to motor neurons innervating intermediate and distal forelimb muscles (Kuchler et al., 2002) (Figure 1.5 a-d). However, the exact muscle targets of these motor neurons were not identified. The existence of direct rubrospinal projections onto motor neurons in the lumbar spinal cord has not been investigated to date. Cat studies demonstrate a similar rubrospinal projection to the region of the spinal cord containing dorsolateral motor neurons, although direct rubro-motoneuronal synapses have not been anatomically demonstrated using the above criteria (Holstege, 1987; McCurdy et al., 1987) (Figure 1.5 f).

Anatomical studies of rubrospinal connectivity in the rodent were also

Figure 1.5

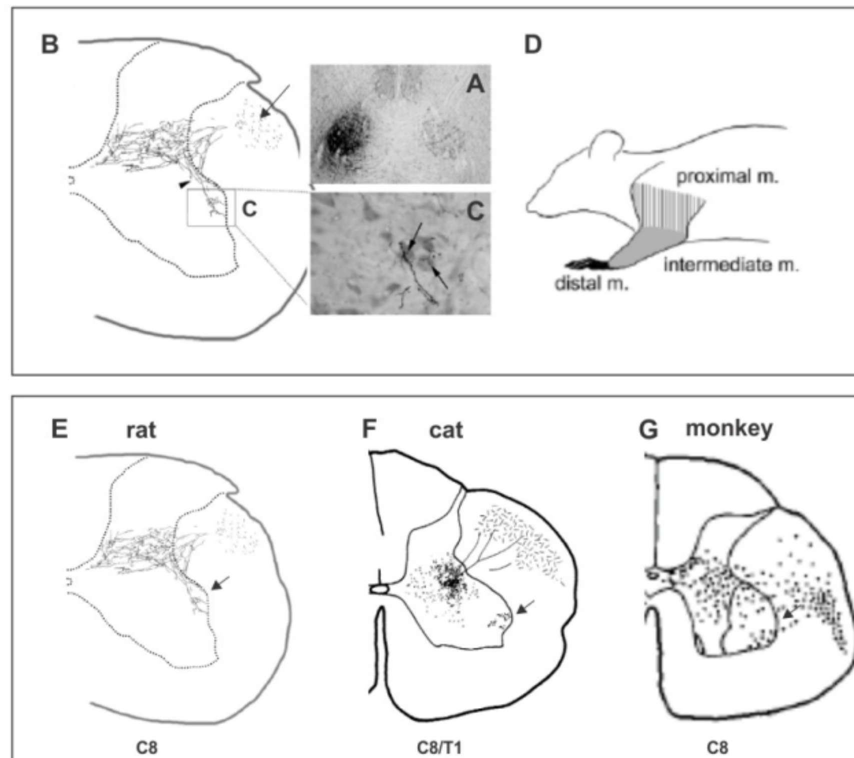


Figure 1.5 Rubrospinal projections to the ventral horn in rat, cat, monkey Anterograde injections of biotinylated dextran amine (BDA) into the red nucleus of the adult rat (A) label a descending projection in the dorsolateral funiculus (arrow in B). A small projection into the ventral horn is observed at C7-T1, and labeled rubrospinal neurons are demonstrated to form close appositions with labeled motor neurons in the ventral horn (C). Rubrospinal fibers are only observed to contact motor neurons projecting to intermediate or distal forelimb muscle targets (D). From Kuchler et al., 2002. An inter-species comparison of rubrospinal terminations demonstrates that a similar ventral projection is seen at C8 in the rat (Kuchler et al., 2002), cat (McCurdy et al., 1987), and monkey (Holstege et al., 1987) (arrows in E, F, G).

complimented by electrophysiological demonstration of direct projections onto motor neurons. Low-threshold stimulation of identified regions of the red nucleus resulted in a short latency EMG response in contralateral intermediate forelimb muscle groups, and evoked a visual wrist extension (Kuchler et al., 2002). Increasing the stimulation intensity led to an EMG response in the proximal forelimb muscles. However, the latency of this response was much longer than that of the intermediate forelimb muscle groups suggesting activation through a polysynaptic circuit. Likewise, physiological studies in the cat have demonstrated evidence of short latency inputs from the red nucleus onto a percentage of forelimb motor neurons that supply the ulnar and radial nerves (Fujito and Aoki, 1995; Fujito et al., 1991).

While these studies certainly suggest that the likelihood of cervical motor neurons receiving direct rubrospinal input is high, they also highlight areas in which additional studies are greatly needed. While rubrospinal terminals are present in the vicinity of motor neurons, such ‘close appositions’ do not necessarily represent an actual synaptic contact, a point which has been clearly demonstrated by electron microscopy studies of corticospinal tract tracing (Yang and Lemon, 2003). The steps required to amplify the signal of the tracer generally prohibit the combination of this technique with standard immunohistochemistry, which could definitively identify putative synapses by demonstrating co-localization with pre- and post-synaptic protein markers (Betley et al., 2009). Thus, until subjected to a more technically rigorous analysis, the existence of rubro-motoneuronal connections in the rodent remains likely rather than definitively established.

Nonetheless, the increasing evidence supporting the existence of a monosynaptic descending circuit in the rodent, particularly from the less-studied rubrospinal tract, brings into question the prevailing notion that direct projections onto motor neurons reflect an evolutionary specialization to enable fractionated distal forelimb movements. If this is so, why might such a direct circuit be present in rodents, a species thought to have a low index of dexterity? The answer to this question may lie in recent studies comparing forelimb movements between rodents and humans.

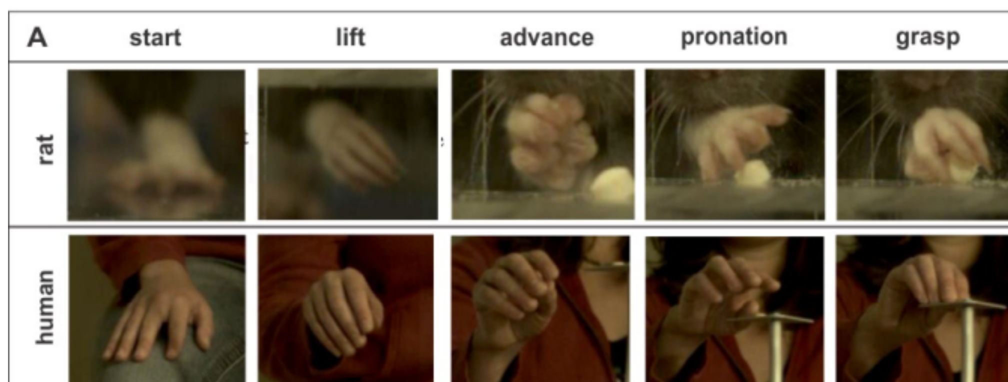
Homologous movements of rats and humans in the execution of a skilled forelimb reach-to-grasp task

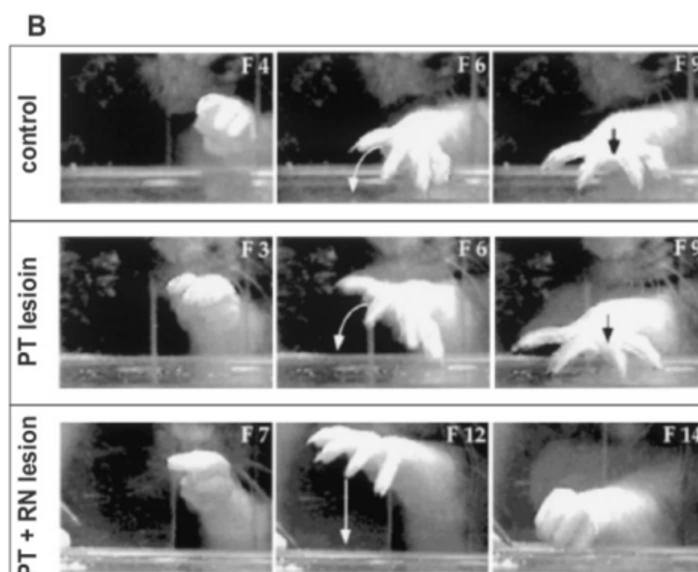
Recent work from the Whishaw lab has investigated the evolutionary origin of skilled forelimb reaching movements between different species, with the purpose of determining whether the movements used to accomplish a reach-to-grasp task are evolutionarily homologous, or developed in parallel given common behavioral requirements. Although behavioral homology can be difficult to classify, one of the established 'rules' is that "the greater the complexity and degree of correspondence, the more likely the homology" (Atz, 1970). This can be understood to mean that behavioral homology can be defined as a case in which each species uses homologous structures in a similar manner to accomplish a task. Following the observation that rodents and humans employ a number of hand (paw in the rodent) and digit adjustments over the course of a skilled reach (Whishaw et al., 1992), a temporal and spatial kinematic comparison of hand shaping was undertaken.

The skilled reach-to-grasp maneuver can be broken down into three phases: release (initial lifting of hand), collection (movement of limb towards target), and manipulation (hand pre-shaping and grasp). Across these distinct phases, ten hand shaping adjustments can be recognized and compared, based on timing and movement quality. Despite quantitative differences between rodent and human in the amount of time spent in each of the three phases, there is a remarkable similarity seen in hand shape as the forelimb progresses through the movement (Sacrey et al., 2009). In both species, the digits begin in an extended manner as they are lifted off the substrate, but are quickly flexed and closed during the subsequent limb advancement. Just prior to object grasp, the digits are fully extended and opened, and are then lowered onto the object in a pronated 'arpeggio' movement. The digits then flex around the object, before using a supination movement to retrieve the forelimb. Although a direct comparison to humans has not been made, non-human primates exhibit very similar movements during skilled reaching. Comparable quantification indices of the temporal components of hand 'flexion-extension', 'opening-closing', and 'supination-pronation' during skilled reach further support the claim of shared ancestral homology between rodent and human hand shaping (Figure 1.6a).

The authors of this study make the interesting observation that similarities and differences in hand shaping might reflect neural similarities and differences in the motor system of the rat and human: "Amongst the differences are the extensive direct connections of corticospinal neurons onto the motor neurons of the spinal cord in the more recent members of the primate lineage vs. more distance primate species and

Figure 1.6





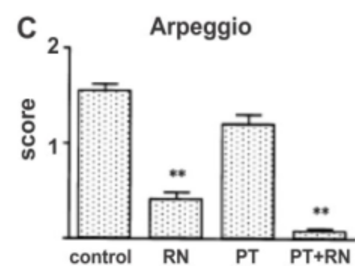


Figure 1.6 Skilled reaching in the rat

A. Homology in forelimb movements during a skilled reach-to-grasp task A comparison of stereotypic forelimb movements used to accomplish a skilled reaching task reveals striking similarities between rodent and human. In both cases, the forelimb is lifted and advanced with the digits in a flexed position. Just prior to object grasp, the hand pauses, and the digits are extended and separated. The paw is then lowered onto the object in a pronated 'arpeggio' movement. Finally, the object is grasped through a flexion movement, and the paw is then supinated and withdrawn.

From Sacrey et al., 2009

B. Deficits in distal limb movements following lesioning of the rubrospinal tract in rodents High resolution video analysis was used to access distal limb movement following a lesion of either the corticospinal tract, rubrospinal tract, or both pathways. Although corticospinal lesions resulted in limb guidance defects, attributed to proximal limb movements, only following a lesion of the red nucleus were deficits observed in distal limb movements. Instead of extending and separating the digits prior to the arpeggio movement, the animal simply lowered the paw onto the object, and grasped it during the retrieval phase.

C. Quantification of the arpeggio deficit following a red nucleus lesion The presence of arpeggio has been scored with ratings of 0 (not present), 1 (recognizable), and 2 (present). The arpeggio movement is essentially lost following a lesion of the red nucleus, or a combined pyramidal tract and red nucleus lesion. Arpeggio is not affected by a PT lesion.
PT pyramidal tract, RN red nucleus

From Whishaw et al., 1998 (B,C)

rodents. It is proposed that the direct connections mediate the fractionated movements of the limbs and digits; for example, independent digit movements used for grasping objects. Because all species shared similar hand shaping movements, it is likely that the hand shaping movements that occur during limb transport are related to more conserved spinal cord circuits and to multisynaptic pathways from the brain. Thus identification of static vs. changed characters in skilled reaching could prove useful in identifying the function of more conserved vs. changed neural pathways and connections" Pg. 159 (Sacrey et al., 2009).

Certainly, the differences between rodents and primates in regards to individual digit movements are uncontested. However, the 'hand shaping' manipulations, which reflect whole hand rather than fractionated digit movements, appear to be highly conserved. The underlying neural circuitry contributing to these movements in either the rodent or the human is at present, undefined. While it has long been suggested that direct cortico-motoneuronal connections are largely responsible for the increased digit dexterity demonstrated by higher species, there are two parallel descending systems, the corticospinal and the rubrospinal pathways, that play a role in the supraspinal control of voluntary movement, and their relative contributions remain undefined. Lesion studies in the rodent have provided insight into the division of labor by these two pathways.

Specific deficits in distal limb movements following a red nucleus lesion in rodents

The Whishaw lab performed the same video- and kinematic analysis following either a lesion of the pyramidal tract or a neurotoxic lesion of the red nucleus (Whishaw et al., 1998). Their initial prediction was that corticospinal tract lesions would have a greater impact on distal limb musculature, reflected by defects in the manipulation phase of the movement, whereas the rubrospinal tract might preferentially impair proximal limb movements. Surprisingly, the results were exactly the opposite. Removal of corticospinal inputs resulted in a decrease in rotatory movements of the limb, controlled by more proximal limb muscles, and an overall decrease in the success of the task, suggesting a role in limb guidance to the object. Although the red nucleus tract lesion also affected limb rotation, no effect was noted on overall success; unexpectedly, the most striking defect was seen in the arpeggio component of manipulation. The digits were no longer extended and opened prior to object grasp, and instead of lowering the paw onto the object in a pronated manner, the object was instead simply quickly grasped during limb retrieval (figure 1.6b). One caveat of the RN lesion experiments is that although the neurotoxin was stereotaxically delivered to the red nucleus, the canula must pass through overlying structures, leading to potential damage of other midbrain motor regions.

These lesion studies provide new insight into the overlapping and distinct roles of the corticospinal and rubrospinal tracts. Preferential corticospinal involvement in distal limb movements does not seem evident in these studies, although the authors do note that in primates, ‘distal’ is generally used to refer to digit movements. Given the relative paucity of direct forelimb cortico-motoneuronal

connections in the rodent, it is possible that the remainder of the indirect corticospinal circuit is dedicated to the control of proximal and intermediate limb musculature, perhaps through the activation of muscle synergies discussed previously.

The hypothesis that the rubrospinal projection is involved in the control of proximal limb musculature originates primarily from prior behavioral studies indicating that overall success in a skilled forelimb task is not affected following a loss of rubrospinal input (Whishaw et al., 1990). However, the development of an assay to provide a detailed kinematic analysis of the forelimb movements comprising a skilled reach has revealed a major deficit in wrist and digit movements following a rubrospinal lesion. Although perhaps not as essential as discrete finger movements for the execution of a skilled motor behavior, precise control of the extrinsic wrist and hand muscles controlling these 'whole hand' movements would certainly be expected to play an important role. In the rodent, these movements appear to be preferentially controlled by the rubrospinal tract, although whether that same distinction holds true in primates remains to be seen.

How exactly is this preferential control of distal musculature mediated by descending rubrospinal projections? The homology between 'whole hand' movements in rodents and higher species suggests that the neural mechanisms of control may be conserved. The recent revelation that rodents have a greater degree of manual dexterity than was previously thought has evoked renewed interest in the possibility that direct supraspinal projections onto motor neurons in the rodent control

certain distal forelimb movements. A complete knowledge of the motor pools that are targets of the rubrospinal tract would provide a necessary foundation for future experiments investigating the functional contribution of direct rubrospinal inputs onto motor neurons during a motor behavior.

In addition to providing insight into the mechanisms of descending control within the rodent motor system that may prove applicable to other species, this circuit would provide us with an excellent model for investigating the establishment of molecular specificity within a developing circuit. To achieve fine control of movements during a motor behavior, subsets of descending axons may target specific motor pools during development and refinement of the rubrospinal tract. How do these individual circuits emerge during development, and what are the molecular mechanisms that mediate the choice of post-synaptic partner? Before we can begin to address these questions, we must first understand the organization and molecular identity within the midbrain structure of origin, the red nucleus.

The red nucleus gives rise to the descending rubrospinal tract

Comparative biology of the red nucleus

We can note three major developments in the evolutionary history of vertebrate motor control. The first is the development of paired fins in water-based species, a deviation from the fundamental axial swimming pattern, and likely representing the primordial ancestor of the vertebrate limb. The second major development is the conversion of the fins to limbs in land- and air-based vertebrates,

and the final development is the specialization of the distal portions of the forelimb extremities to allow for increased environmental interactions. A comparative analysis relating the emergence or expansion of a particular pathway to a particular aspect of movement can provide valuable insight into its functional specialization.

A species is considered to possess a red nucleus if it contains a midbrain structure that fulfills the following three criteria: location in the midbrain tegmentum at the level of the oculomotor nucleus, mesencephalic recipient of crossed cerebellar output, and the site of origin of a crossed projection to the spinal cord (ten Donkelaar, 1988). Although the red nucleus is easily distinguishable in a histological preparation in the majority of species, given the predominance of large cells contained within it, it can be difficult to recognize in lower vertebrates in which cell size is more variable. By the above criteria, the earliest known example of a red nucleus in the vertebrate lineage is found in rays, which use alternating movements of their fins for locomotion. A red nucleus is absent from more primitive species that rely solely on segmental axial movements. Thus, the red nucleus and its efferent pathway, the rubrospinal tract, appear to emerge in evolutionary concordance with limb development.

A fascinating example of rubrospinal control over limb movement is seen in *Xenopus laevis*, which begins life as a larval tadpole before undergoing metamorphosis into an adult frog. Early in development, the tadpole uses alternating contractions of the axial muscles for forward propulsion. Later in development, the

hindlimbs are incorporated into the swimming movement, and eventually, only the extremities are used. Although the fibers of more primitive descending pathways, such as the reticulospinal, interstitiospinal, and vestibulospinal tracts, reach the spinal cord in the tadpole, the rubrospinal tract only projects to the spinal cord during the time period in which locomotion is shifting from axial to limb control (ten Donkelaar and de Boer-van Huizen, 1982).

The red nucleus contains two functionally distinct regions: parvocellular (RNp) and magnocellular (RNm)

The red nucleus is a heterogeneous structure populated by two functionally distinct neural subtypes: magnocellular neurons that give rise to the contralateral projecting rubrospinal tract, and parvocellular neurons that give rise to the ipsilateral projecting rubro-olivary tract (Massion, 1988). These neurons occupy anatomically distinct regions of the red nucleus in cats and primates, but their distribution has been poorly characterized in the mouse. In species that do contain this anatomical segregation, the rostral 1/3 of the red nucleus contains the smaller parvocellular neurons, which receive input from the dentate nucleus of the cerebellum and send projections to the inferior olive. The magnocellular red nucleus, whose name reflects the large cell body size of the neurons it contains, occupies the caudal 2/3 of the red nucleus, projects to the brainstem and spinal cord, and receives input from the interpositus nucleus of the cerebellum (Figure 1.7).

Within the red nucleus, the space allotted to RNm and RNp differs over the

course of evolution. The earliest primordial example of a red nucleus contains magnocellular neurons that give rise to a crossed spinal projection, but does not contain parvocellular neurons projecting to the inferior olive. These olivary projections emerge later, initially occupy only the rostral 1/3 of the red nucleus, but eventually expand their territory to include the majority of the red nucleus at the expense of the RNm. In fact, a limited number of human studies have indicated that only a small number (150-200) of magnocellular neurons remain, and that rubrospinal fibers are limited to upper cervical levels (Nathan and Smith, 1982). In primates, the ratio of large to small cells in the red nucleus is directly proportional to the relative sizes of the rubrospinal tract and central tegmental tract (projecting to the inferior olive) (ten Donkelaar, 1988).

Although a strict distinction between rostral rubro-olivary and caudal rubro-spinal projecting neurons is maintained in primates, in lower species this segregation appears to be less distinct. In a study of the avian red nucleus, spinal projection neurons of varying sizes were observed throughout the red nucleus following injections of a retrograde tracer into the spinal cord. Likewise, in the opossum, rubrospinal neurons are also found to be scattered throughout the red nucleus with no apparent somatotopic arrangement (Martin et al., 1981).

A recent study in the cat has provided further evidence that the functional distinction demonstrated in primates and humans does not hold true across species. Anterograde tracer injections into specific rostro-caudal regions of the red nucleus

Figure 1.7

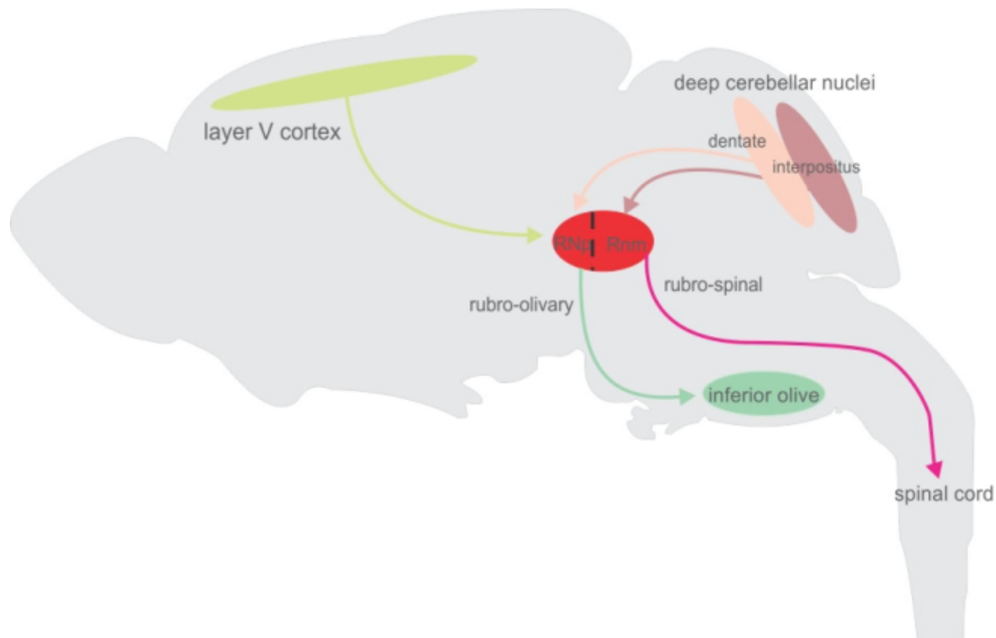


Figure 1.7 Input/output circuits from the red nucleus The red nucleus contains two functionally distinct populations of neurons: rubro-olivary neurons in the rostral parvocellular red nucleus (RNp), and rubro-spinal neurons in the caudal, magnocellular red nucleus (RNm). However, the distinction between these two populations in the rodent and cat is less clear. The parvocellular red nucleus receives input from the cortex and dentate nucleus in the cerebellum, whereas the magnocellular red nucleus receives the majority of its input from the interpositus nucleus. The inputs to the magnocellular red nucleus are topographically organized, with forelimb-specific afferent input to the dorsomedial RNm and hindlimb-specific afferent input to the ventrolateral RNm (see Figure 1.9 for rubrospinal topographic organization).

indicate that the parvocellular red nucleus contains a population of neurons that project to the contralateral spinal cord. This population is located in the caudal and lateral pole of the parvocellular red nucleus, and does not appear to be intermingled with the inferior olive-projecting classical parvocellular neurons (Pong et al., 2002). The RNp spinal-projecting neurons differ from the caudal RNm neurons in both location and cell body size. The authors also suggest that the two circuits are somatotopically organized, with the RNp projections mainly to the upper cervical levels of the spinal cord, where motor pools controlling proximal muscles are located, whereas the RNm projects mainly to lower cervical levels, with a greater effect on more distal forelimb muscles. However, the functional significance of two distinct spinal populations within the red nucleus is unclear. The existence of multiple populations of spinal-projecting neurons in the rodent red nucleus has not yet been investigated.

Molecular specification of the red nucleus

The observation of a migratory stream of neurons from the dorsal to the ventral midbrain led scientists to initially conclude that the red nucleus originated within the dorsal midbrain tectum. Although there is certainly precedence for the final address of a neuron to differ significantly from its original birthplace, more recent studies have demonstrated that the red nucleus derives from ventral midbrain explants in the complete absence of dorsal midbrain (Agarwala and Ragsdale, 2002). In addition to the ovoid-shaped red nucleus, the ventral midbrain consists of populations of neurons arrayed in discrete nuclear structures that deviate from the

longitudinal columnar organization displayed by cell types in the hindbrain and spinal cord. At hindbrain and spinal levels, an underlying source of sonic hedgehog (SHH), a secreted positional molecule, is responsible for the induction of neuronal populations in a graded manner along the dorso-ventral axis (Figure 1.1a). These longitudinal cell columns do not extend anterior to the mid-hindbrain junction, raising the question as to how the diverse cellular architecture within the midbrain is specified.

A striking observation was made when the developing chick brain was stained with acetylcholinesterase, a histochemical method capable of revealing brain structures in neuronal tissue . The ventral midbrain consisted of a series of rostro-caudal arcs, oriented parallel to the ventral midbrain (Bayly et al., 2007; Sanders et al., 2002) (Figure 1.8a). These arcs reflect the overall organization within the developing ventral midbrain, encompassing both the progenitor domains in the ventricular zone and the differentiated neurons in the mantle layer. As in the spinal cord, the midbrain ventral floorplate was a source of sonic hedgehog, indicating that despite a lack of similarity in the nuclear architecture between the midbrain and more posterior regions of the CNS, there appears to be a conserved mechanism for establishing positional identity (Agarwala et al., 2001) (Figure 1.8 e-g). Indeed, ectopic expression of SHH elsewhere in the midbrain results in the formation of a duplicate set of arcs, whose positions relative to each other and the SHH source are maintained (Agarwala et al., 2001) (Figure 1.8h).

Figure 1.8

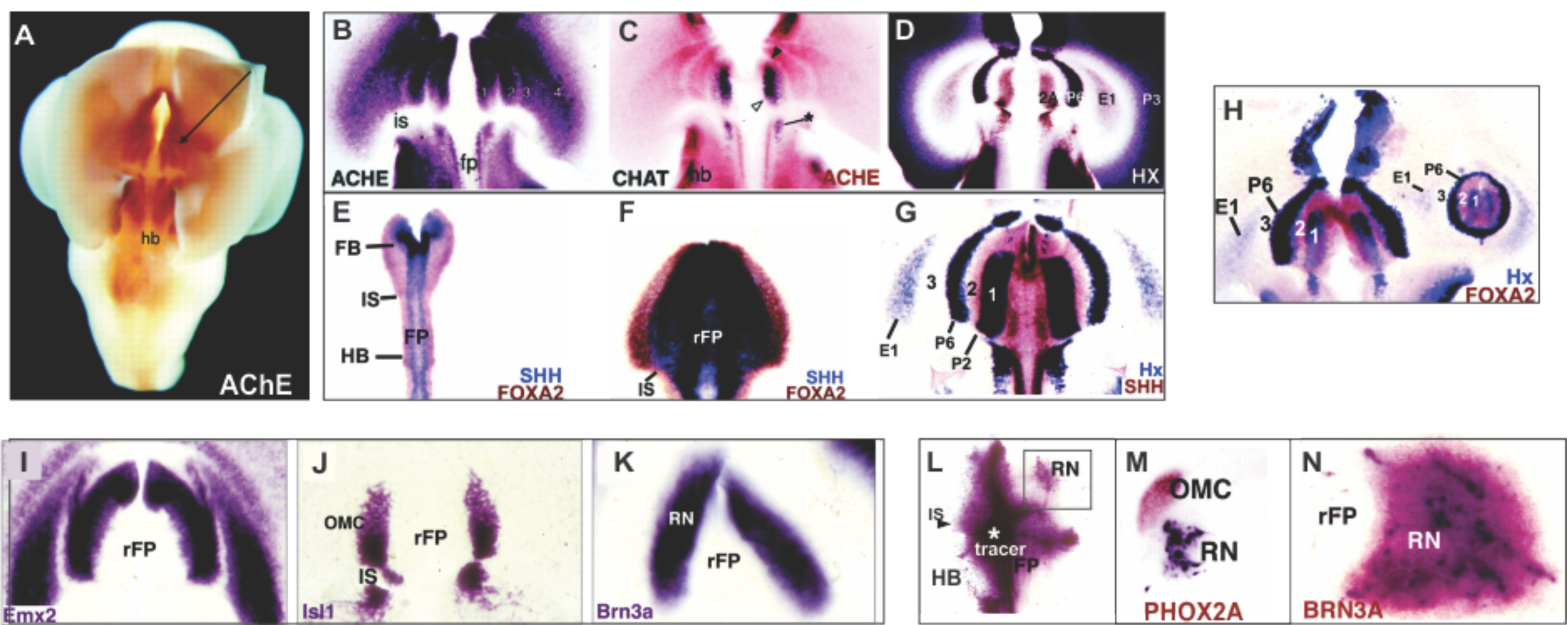


Figure 1.8 Early development of the red nucleus The ventral midbrain is organized into a series of acetylcholinesterase-rich 'arcs' early in development (A), aligned parallel to the midline. These arcs are apparent in whole mount preparations of the developing chick midbrain at E5 (B-D). CHAT expression reveals a population of cholinergic neurons in arc 1, likely the developing oculomotor neurons (C). The parallel arcs and the inter-arc spaces can be definedby the expression of homeobox transcription factors phox2a (arc1), pax6 (inter-arc 2/3), evx 1 (inter-arc 3/4 and arc 4) (D). Arc formation is controlled by a secreted SHH signal from the ventral floorplate (E,F). Arc position relative to the SHH signal is demonstrated in panel G. Ectopic expression of SHH is capable of inducing a complete set of arcs oriented relative to the SHH source. An ectopic point source of SHH results in a bulls-eye formation of ectopic arcs (H). Arc 1 contains populations of Isl1+ and Brn3a+ neurons, demostrated in chick E5 midbrain whole mounts (I-K). Midbrain dopaminergic neurons are also contained within Arc 1 (not shown). Injections of fluoresceinated dextran into the contralateral hindbrain (L) demonstrates the presence of retrogradely labeled neurons within the Brn3a+ red nucleus (M,N).

A-D: Sanders et al., 2002 E-H: Agarwala et al., 2001 I-N: Agarwala et al., 2002

A molecular and anatomical analysis of neurogenesis demonstrated that cells populating the oculomotor nucleus, red nucleus, and substantia nigra all derive from Arc1 (Agarwala and Ragsdale, 2002) (Figure 1.8 i-k). The antero-posterior distribution of the nuclei within the arc is established by an FGF8 signal localized to the midbrain-hindbrain isthmus (Agarwala and Ragsdale, 2002; Nakamura et al., 2005). These populations are further segregated along the ventricular-pial axis, and it is hypothesized that this third axis might reflect a temporal distinction in the birthdate of each population. Oculomotor neurons appear first, with red nucleus neurogenesis following a day later (E10.75 to E13), and midbrain dopaminergic neurons generated last (Agarwala and Ragsdale, 2002; Puelles et al., 2004).

Gene expression in the developing red nucleus

In order to generate discrete cellular subtypes, the positional signal must be translated into a transcriptional network capable of initiating and maintaining multiple, spatially distinct progenitor domains. In the developing spinal cord, the sonic hedgehog gradient is translated into the expression of homeodomain (HD) transcription factors in a dorso-ventral pattern. The homeodomain proteins are then capable of mutual cross-repression, preventing the activation of genetic programs reserved for neighboring cell types (Lee and Pfaff, 2001). Although the nuclear architecture within the midbrain differs from that of the hindbrain and spinal cord, a gradient positional signal is nonetheless used to generate both a general cytoarchitectonic organization (arcs), and distinct functional subtypes within each arc. Arc 1 gives rise to cholinergic motor neurons of the oculomotor nucleus,

glutamatergic spinal projection neurons of the red nucleus, and dopaminergic neurons of the substantia nigra. Within the constraints of the arc organization, what are the genetic signals that generate discrete nuclei?

Although less is known about the mechanisms by which nuclear identity is specified in the midbrain, it appears that a similar HD protein network is utilized. Nkx2.2, Nkx6.1 and Nkx6.2 are all expressed in the ventral midbrain ventricular zone (Prakash et al., 2009). Otx2 expression in the midbrain induces the expression of Nkx6.1, and the red nucleus does not develop in Otx2 or Nkx6.1 mutant animals (Prakash et al., 2009; Puelles et al., 2004). Furthermore, in Nkx6.1 mutants, there is an expansion of Dbx1 and Pax7, two HD proteins normally restricted to the dorsal midbrain, indicating that cross-repressive mechanisms may also play a role in D-V patterning in the midbrain.

Misexpression of Otx2 in the rostral hindbrain is capable of inducing ectopic Nkx6.1+ cells (although this likely requires a minimum level of SHH signaling), and eventually Brn3a+ RN neurons are also observed in this new location. Brn3a (Brn3.0, Pou4f1), a member of the Pou family of transcription factors, is a well-characterized marker of the differentiated red nucleus population. Retrograde tracing experiments in the chick CNS have confirmed that Brn3a is expressed in a population of midbrain neurons which give rise to a contralateral projection to the midbrain (Agarwala and Ragsdale, 2002) (Figure 1.8 l-n). Although Brn3a is expressed in the red nucleus from E11 onwards, analysis of Brn3a knock out animals has revealed that

this gene is not required for the migration and differentiation of this population. In the absence of Brn3a, however, red nucleus neurons die at E18, indicating that Brn3a is required for the late survival of the red nucleus (Xiang et al., 1996). It has not been determined whether Brn3a labels all rubrospinal neurons, or if it is specific to the magnocellular RN.

Development of the rubrospinal tract

Early-expressed transcription factors are crucial for setting in place the genetic profile that will establish later characteristics of neuronal identity, such as cell body migration, axon guidance, and choice of post-synaptic target. A knowledge of the developmental timecourse of rubrospinal maturation is necessary to enable the correlation of gene expression with molecular aspects of circuit formation. To date, the development and circuitry of the rubrospinal tract has been studied by injecting anterograde tracers into the red nucleus or retrograde tracers into the spinal cord, or through physiological studies. From this work, we know that the majority of rubrospinal axons cross the midline immediately upon exiting the confines of the red nucleus, at the level of the ventral tegmental decussation (Brown, 1974), and course through the hindbrain, lateral to the pyramidal tract. Rubrospinal fibers then descend in the dorsolateral funiculus of the spinal cord, ventral to the lateral component of the corticospinal tract. Retrograde tracing experiments in rat have demonstrated that projections from the red nucleus reach lumbar levels of the spinal cord by P0 (Shieh et al., 1983). However, the timing of invasion of rubrospinal axons into cellular

regions of the spinal cord is unknown, as is that of the onset of synaptogenesis of rubrospinal neurons with their post-synaptic spinal targets.

The initial projection of the rubrospinal tract at birth might differ from the final, mature circuit. In many species, newborn animals possess only the basic motor skills required for survival such as respiration and suckling. The development of a motor repertoire follows a species-specific trajectory; rats and cats establish these skills within the first few postnatal months, whereas human motor development is on the order of years. One hypothesis is that descending control from supraspinal motor centers contributes to fine motor skills, and that the refinement seen in these projections is partly responsible for the later establishment of motor control. For example, the corticospinal projection in neonatal rats has been described as exuberant: it extends diffusely throughout the dorso-ventral extent of the spinal cord. Corticospinal projections are also initially bilateral. Through activity-dependent refinement, corticospinal fibers eventually retract and the majority of ipsilateral projections are eliminated. Elimination of transient fibers is accompanied by branching and synaptic expansion of maintained corticospinal axons. Interestingly, in the monkey the opposite occurs: corticospinal fibers are initially restricted to the dorsal and intermediate spinal cord, and eventually invade the ventral horn to establish the direct corticospinal circuit (Armand et al., 1994). Although refinement during the pre-natal period has not been ruled out, the lack of corticospinal pruning seen in the postnatal period might reflect the precocious motor skills of the newborn monkey. Thus, the emergence of the mature descending circuits appears

tightly linked with the development of fine motor skills. We do not know whether the descending rubrospinal circuit undergoes a similar period of developmental maturation into its final, adult state, or whether refinement and/or expansion is limited to the indirect or direct rubrospinal circuits.

Although the rubrospinal tract is considered a crossed pathway, studies in rat and cat have revealed a few rubrospinal fibers that remain ipsilateral (Antal et al., 1992; Holstege, 1987), even in the adult animal. These projections are rarely in the focus of anatomical studies of the rubrospinal tract for a number of reasons: they might be sufficiently sparse such that they are rarely labeled via tracer injections, and critically, bilateral tracing experiments do not distinguish the ipsilateral and contralateral components. Direct projections onto motor neurons in the rodent originate from the ipsilateral corticospinal tract (Bareyre et al., 2005), and there may be similar functional distinctions between the ipsilateral and contralateral rubrospinal pathways. The combination of a genetic approach for labeling the entirety of the rubrospinal tract, with an additional mechanism for unilateral labeling, such as localized viral gene delivery (Ahmed et al., 2004), could enable identification of ipsilateral vs. contralateral components of the pathway.

Topographic organization of rubrospinal projections

The rubrospinal tract projects the entire length of the spinal cord, including dense projections to both forelimb and hindlimb spinal levels, and also to non-limb levels (ten Donkelaar, 1988). Rubrospinal projections at forelimb and hindlimb

levels might differ significantly, especially in species that predominantly use the distal forelimb in skilled movement. How then are the differing choices of post-synaptic spinal level represented within the red nucleus? Rubrospinal neurons also receive afferent input from the cerebellum, and the information being conveyed to an individual rubrospinal neuron must correspond to its efferent target. Is there an organization within the red nucleus that would facilitate the accurate flow of information throughout the cerebellar-rubrospinal circuit?

A common organizational scheme in the CNS is that neurons sharing similar characteristics within a population - such as the target they innervate - are positionally segregated into subpopulations. This is thought to enable communication between neurons and support the acquisition of similar synaptic inputs, thus facilitating the coordinated firing of a subpopulation of neurons with a conserved function. This organizational scheme can be further specialized by the existence of a topographic map, defined as the ordered set of projections within an afferent or efferent system to their particular anatomical target within the central nervous system. Topographic maps maintain spatial sensory information as it is transmitted from the periphery and processed by higher systems. This has been well demonstrated in the visual system, where adjacent retinal ganglion cells have unique but overlapping receptive fields, forming a representation of visual space. The projections from the retina are then guided to the appropriate target in the tectum through the utilization of guidance molecule gradients, ensuring that each step in the visual processing pathway will contain a representation of the visual field (the visuotopic map) (DeLong and

Coulombre, 1967).

The somatopic representations of the body within the somatosensory and motor cortices are referred to as the 'cortical homunculi.' The homunculus is an anatomically inclusive body map, although certain parts of the body such as hands and lips receive a disproportionately large representation. Descending spinal projections arising from the cortex have been shown to activate certain muscles based on their location within the topographic map (Liddell and Phillips, 1952). Whether such a detailed anatomic representation exists within the red nucleus is unclear. We do know that rubrospinal projections to forelimb and hindlimb levels in the developing and adult rat are segregated within the red nucleus, with the cervical-projecting neurons occupying a dorsomedial position, and those to caudal levels located in the ventrolateral region (Shieh et al., 1983) (Figure 1.9 a,b). The distribution of rubrospinal projections to non-limb levels is undefined. The somatotopic organization within the red nucleus appears to hold true across all species (Eccles et al., 1975; Larsen and Yumiya, 1980; Robinson et al., 1987).

The molecular strategy by which cervical and lumbar territories are established within the red nucleus is unknown. The segmental 'identity' of a rubrospinal neuron might be conferred through positional cues or birthdating. Alternatively, a target-derived signal might be required for the re-organization of rubrospinal cell bodies into a somatotopic map. Molecules that might mediate the segregation of rubrospinal subpopulations include cell surface adhesion molecules.

Figure 1.9

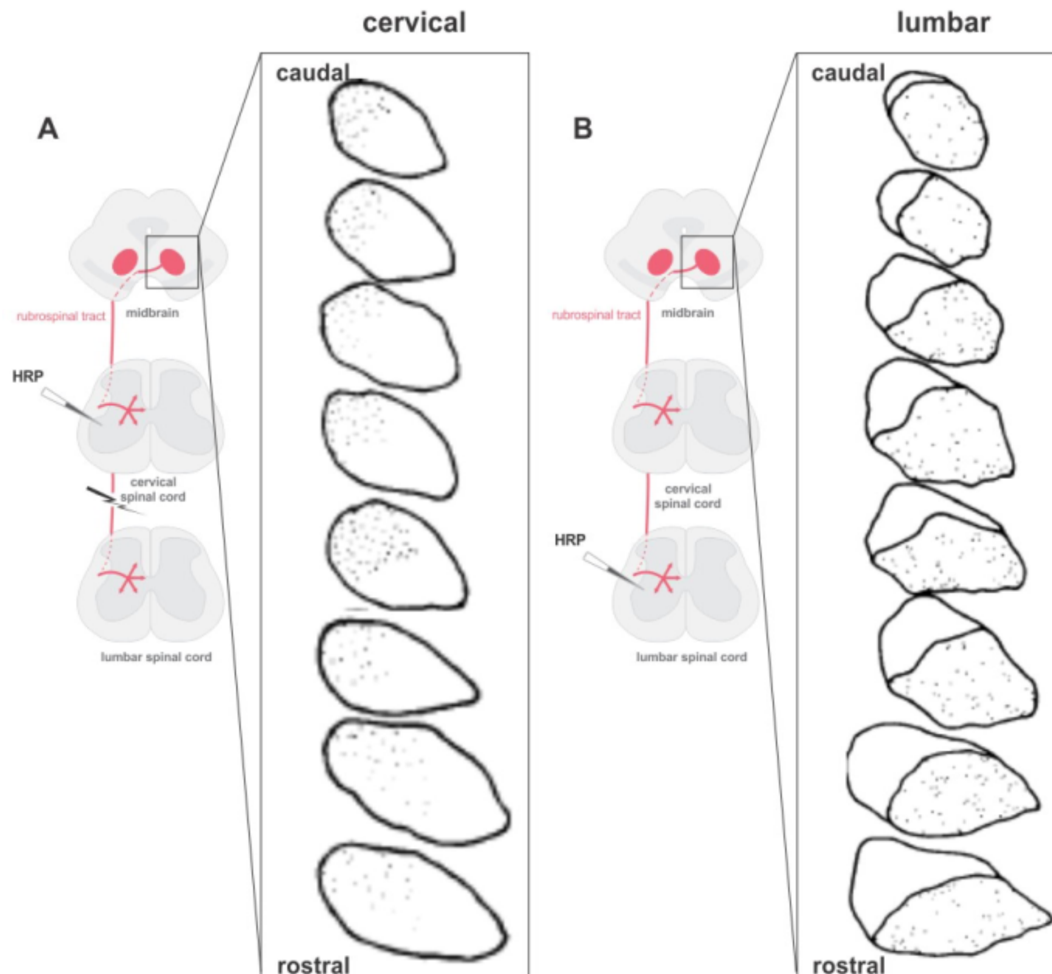


Figure 1.9 Somatotopic organization of rubrospinal neurons Injection of the retrograde tracer, horseradish peroxidase (HRP) into either the cervical or lumbosacral spinal cord of adult rats, and subsequent examination of the contralateral red nucleus 2-3 days later demonstrates that cervically-projecting rubrospinal neurons are located in the dorsomedial red nucleus (A) whereas lumbar-projecting rubrospinal neurons are positioned ventrolaterally (B). To prevent labeling of lumbar fibers in passing, the spinal cord was transected at T1 prior to tracer injection into the cervical spinal cord. This organization of forelimb and hindlimb regions within the red nucleus is seen in rats, cats, and monkeys.

From: Shieh et al., 1983

In particular, members of the cadherin family play an important role in motor pool clustering in the developing spinal cord (Price et al 2002, Elena Demireva and Tom Jessell). Within each domain, one can imagine that there might be a further level of organization based on the diversity of post-synaptic spinal targets.

Following this logic, an intriguing question that remains outstanding is whether rubrospinal neurons contacting individual motor pools are clustered together within the framework of the existing topography. This muscle-centric organization might hold true for both the direct and indirect rubrospinal circuits. Recent work examining the organization of last-order spinal interneurons revealed that the populations contacting individual motor pools are in fact quite separate, although there is some pre-motor overlap observed between motor pools innervating synergistic muscles (Stepien et al., 2010). Therefore, even the indirect rubrospinal circuit might be organized to reflect individual muscle targets. Understanding the organization of the rubrospinal tract in relation to motor pools and the muscles they innervate may provide insight into the mechanisms by which the RST regulates motor output.

Molecular patterning of the rubrospinal circuit

The establishment of appropriate rubrospinal connectivity is particularly complex, given the heterogeneity of the post-synaptic spinal targets. Decision points are seen at each stage of rubrospinal development: the initial decision to project ipsilaterally vs. contralateral, the segmental level of termination within the spinal

cord, choice of interneuron vs. motor neuron post-synaptic target, and further specification of interneuron or motor neuron subtype. Although there are a number of gene families that play a role in the establishment of specific connectivity within a neural circuit, there has been no indication of the molecules or mechanisms involved in the development of the rubrospinal circuit.

The distance that rubrospinal axons must travel to reach their final destination in the spinal cord encompasses almost the entire length of the CNS. Although this task appears daunting, molecular cues along the path likely play a key role in guiding the rubrospinal axons as they navigate their descent. Although we are not aware of the specific interactions that mediate rubrospinal pathfinding, axon guidance molecules in other systems have been well studied. For example, during various stages of descent, corticospinal projections respond to cell surface signaling molecules such as ephrins and semaphorins (Castellani et al., 2000; Chauvet and Rougon, 2009; Yokoyama et al., 2001). Of particular interest are the final molecular cues that determine whether an individual rubrospinal axon will terminate at cervical levels, or continue its descent to reach the lumbar spinal levels. Likewise, the mechanisms that promote axon collateral branching of a single rubrospinal neuron at multiple spinal levels may be crucial for the coordinated activation of multiple muscle groups.

Once rubrospinal fibers have entered the appropriate segmental level of the spinal cord, they must then select a synaptic partner from amongst the numerous

spinal subtypes. Specificity at this choice point is absolutely crucial to ensure that activation of the RST will have the correct effect on motor output. How then is this selection process mediated? Although the laminar termination zone may be determined by cell intrinsic mechanisms (see discussion in chapter 6) it is reasonable to conclude that synaptogenesis is mediated by specific interactions between the pre- and post-synaptic cells.

Given the numerous intraspinal networks accessed by the rubrospinal tract (Jankowska, 1988), there must be stringent mechanisms in place to establish precision within these microcircuits. Within the direct rubrospinal circuit, it appears that the more distal forelimb motor nuclei are preferentially targeted (Kuchler et al., 2002), but this grouping includes a number of individual motor pools. One candidate gene family that may be involved in rubrospinal tract connectivity is the semaphorins, a family of secreted and transmembrane proteins, which function as axonal repellants or attractants in a number of neuronal populations (Castellani and Rougon, 2002). The combinatorial expression of class 3 secreted semaphorins defines distinct motor pools in the developing (e13.5) mouse spinal cord (Cohen et al., 2005) (Figure 1.10 a,b). The specificity of the direct rubrospinal circuit could potentially be patterned by the unique expression of semaphorin receptors in subpopulations of rubrospinal neurons, which project to motor pools expressing a complimentary set of semaphorins (figure 1.10c). However, before we can even begin to explore these possibilities, we need to establish a much stronger foundation of the basic anatomy and organization of the rubrospinal circuit.

Finally, as was introduced in the previous section, the development of many neural circuits involves an initial period of exuberant growth, followed by a period of refinement and elimination leading to the eventual emergence of the mature circuit. Although we do not yet know the details of rubrospinal tract development, the molecular mechanisms responsible for this important aspect of circuit formation are beginning to emerge. Recent studies have implicated a wide range of molecules in the synaptic elimination process. Perhaps most surprisingly, the C1q protein, a member of the classical complement cascade, was found to play a key role in the developmental pruning of transient retinogeniculate projections (Stevens et al., 2007). A recent study has implicated an additional family member, cerebellin 1, as a key regulator of synaptic formation in the developing cerebellum through its interactions with the orphan glutamate receptor, GluD2 (Matsuda et al., 2010; Uemura et al., 2010). Although the exact mechanism of complement-mediated synaptic elimination is still being determined, this represents a novel role for immune system proteins in the establishment of CNS circuitry. The developing corticospinal tract also appears to rely on the postnatal expression of a neurotransmitter receptor subtype, the GluN2b component of the NMDA, for appropriate refinement of its extensive early projections (Ohno et al., 2010).

It is essential to determine whether the rubrospinal circuit undergoes any form of plastic rearrangement, as this is a necessary component in interpreting the circuitry that underlies the rubrospinal contribution to skilled movement. Given the difficulty of anterograde tracing from the red nucleus in embryos, this is likely best achieved by

Figure 1.10

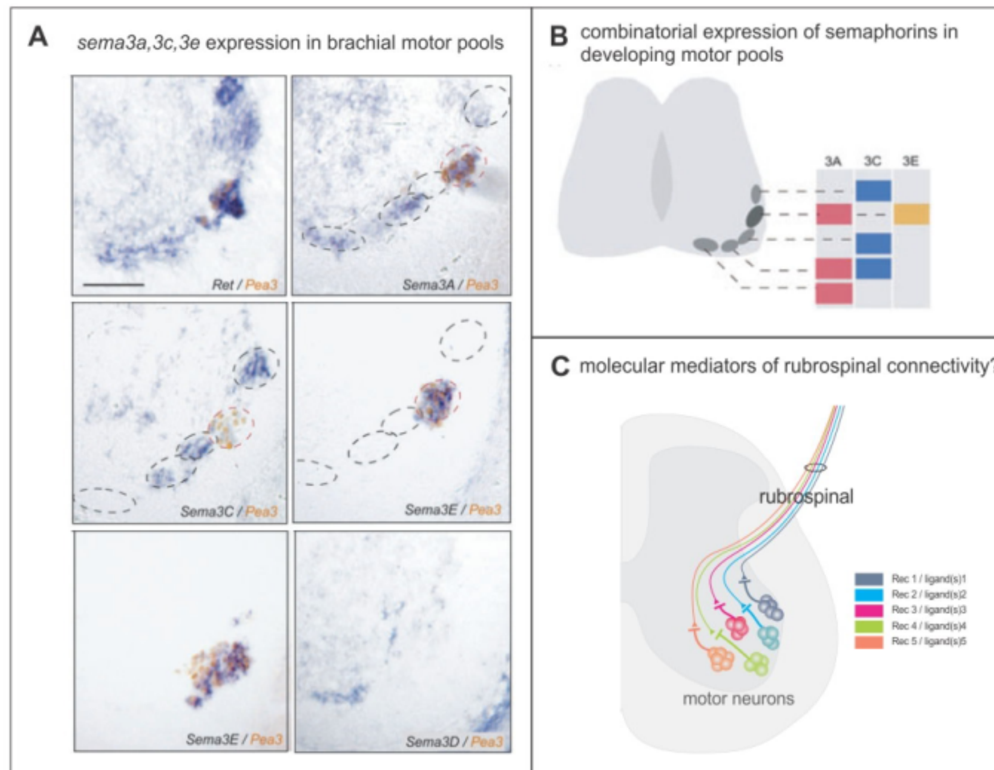


Figure 1.10

A,B. Combinatorial expression of semaphorins in developing spinal motor pools
In situ hybridization of class 3 semaphorins in the brachial spinal cord at e13.5 demonstrates that individual motor pools can be defined by their combinatorial expression of *sema 3a, 3c*, and *3e* (A). Motor neurons are defined by their expression of *ret*. Subpopulations of motor neurons at brachial levels also express the ETS transcription factor *pea3*. A combinatorial code of semaphorins defines individual motor pools (B)

From: Cohen et al., 2005

C. A role for semas in the establishment of rubrospinal connectivity? I hypothesize that individual circuits within the rubrospinal tract might be mediated through the interactions of receptors on rubrospinal neurons with their corresponding ligands on spinal target populations. Although semaphorins are used as an example here, other potential guidance molecules include Ephs/ephrins and chemokines/receptors, and cadherins.

the development of reporter strains of mice in which rubrospinal projections – or defined subsets – are specifically labeled from developmental stages. We can then determine whether any of the above mechanisms are conserved during rubrospinal circuit refinement.

Conclusions

Through advanced behavioral studies, it has recently emerged that the rodent possesses a much greater capacity for skilled forelimb movement than was previously known. As inputs from supraspinal motor control centers onto motor neurons are thought to be responsible for the execution of fine movements and the emergence of digit dexterity, these results suggest that the influence of direct descending control be re-examined in the rodent. Lesion studies predict an important role for the rubrospinal tract in the control of distal forelimb movements in the rodent, and anatomical tracing studies have suggested that the rodent rubrospinal tract might be capable of projecting directly onto forelimb motor neurons, a highly specialized circuit previously thought to be reserved for motor control only in higher species such as monkeys and humans.

Little is currently known about the circuitry of rubrospinal inputs onto spinal motor neurons, whose activity ultimately determines final motor output. A thorough evaluation of the extent and specificity of the rubrospinal projection onto motor neurons will provide an anatomical correlate for the suggested role of the rubrospinal tract in fine motor control in rodents, and will provide a necessary quantitative

foundation for future studies of motor circuit formation, the mechanisms by which motor output is regulated, and the loss of motor function in neurodegenerative disease.

Chapter 2: The rubrospinal tract projects directly onto motor neurons in the rodent

Introduction

Visualizing the rubrospinal tract

From the earliest studies of the CNS, scientists have been perfecting methods of visualizing the morphology and connectivity of brain cells. The first such method was developed by Golgi (Golgi, 1898), who, in parallel with Ramon y Cajal, conducted seminal studies revealing the vast morphological diversity amongst neuronal subtypes in the brain (cajal, 1899; Golgi, 1898). More recently, the focus has shifted towards visualizing connections between neurons that are wired together in a circuit, representing the fundamental and dynamic flow of information through the nervous system.

Early anatomical tracing of the descending pathways utilized the introduction of supraspinal lesions to identify degenerating axons in the spinal cord (Brown, 1974, 1971; Fink and Heimer, 1967; Nauta and Gygax, 1954). The discovery of dense supraspinal termination zones in the intermediate laminae of the spinal cord were instrumental in shaping the course of future anatomical and behavioral studies on the supraspinal control of movement. However, silver impregnation techniques are best suited to visualize nerve processes undergoing degeneration, such as axons and dendrites, and are less sensitive in labeling finer synaptic structures.

More recent circuit tracing methods have utilized the ability of small molecules such as horseradish peroxidase (HRP), dextran amines and cholera toxin B subunit (CTB) to be taken up by the cell and then transported to different compartments (e.g. axon to soma) via intracellular machinery or passive diffusion. Subsequent immunohistochemical or fluorescence detection of tracer distribution has provided unprecedented insight into the long-range connections between various brain regions. As one example, anterograde tracing of the rubrospinal tract has provided evidence of a projection to the ventral horn of the cervical spinal cord in the adult rat (Kuchler et al., 2002). Concurrent visualization of forelimb motor neurons by intramuscular injections of a retrograde tracer enabled the identification of pre- and post-synaptic members of a putative rubro-motoneuronal circuit.

Nonetheless, this study highlights the caveats of such anatomical tracing techniques that must be addressed. First, the midbrain contains a number of populations that give rise to major and minor descending spinal tracts. Do the observed projections onto motor neurons indeed arise from the red nucleus? Are the methods used to visualize the rubrospinal tract capable of revealing fine details of synaptic connectivity? Finally, and perhaps most importantly, do the demonstrated appositions between descending fibers and motor neurons represent definitive synaptic connections?

Specific labeling of descending projections from the red nucleus

Anterograde tracing of descending projections from the red nucleus have to date been accomplished via the injection of a tracer into the red nucleus using stereotaxic reference coordinates. The red nucleus is located in the ventral midbrain, and thus, the injection needle must pass through the various overlying structures before reaching its destination. Despite the stereotaxic targeting of the injection to the red nucleus, there is potential for tracer leakage along the needle tract, spread from the injection site, and variability in the injection location, all of which raise the possibility that additional populations might take up the tracer. Within the midbrain, there are various other structures that give rise to descending spinal projections, including the dorsal superior colliculus (tectospinal), and ventrally, the diffuse deep mesencephalic nucleus (DMN), the interstitial nucleus of Cajal (INC) (interstitiospinal), which borders the periaqueductal grey (PAG) dorsal to the red nucleus, and the midline Edinger-Westphal (EW) nucleus (figure 2.1 panel a).

The deep mesencephalic nucleus contains two populations of spinal projection neurons: a subset that project ipsilaterally in the ventral funiculus, and an additional population that project contralaterally in the ventral part of the lateral funiculus (Veazey and Severin, 1980a, b) (figure 2.1 d). The spinal termination zone of neither population has been investigated in detail. The interstitial nucleus of Cajal is primarily involved in the control of axial movements. Its projections to the medial ventral horn, via the interstitiospinal tract, terminate in the vicinity of motor neurons projecting to axial and trunk muscles (Fukushima et al., 1979b; Fukushima et al., 1979c) (figure 2.1 b). Neck muscle motor neurons at upper cervical levels receive

Figure 2.1

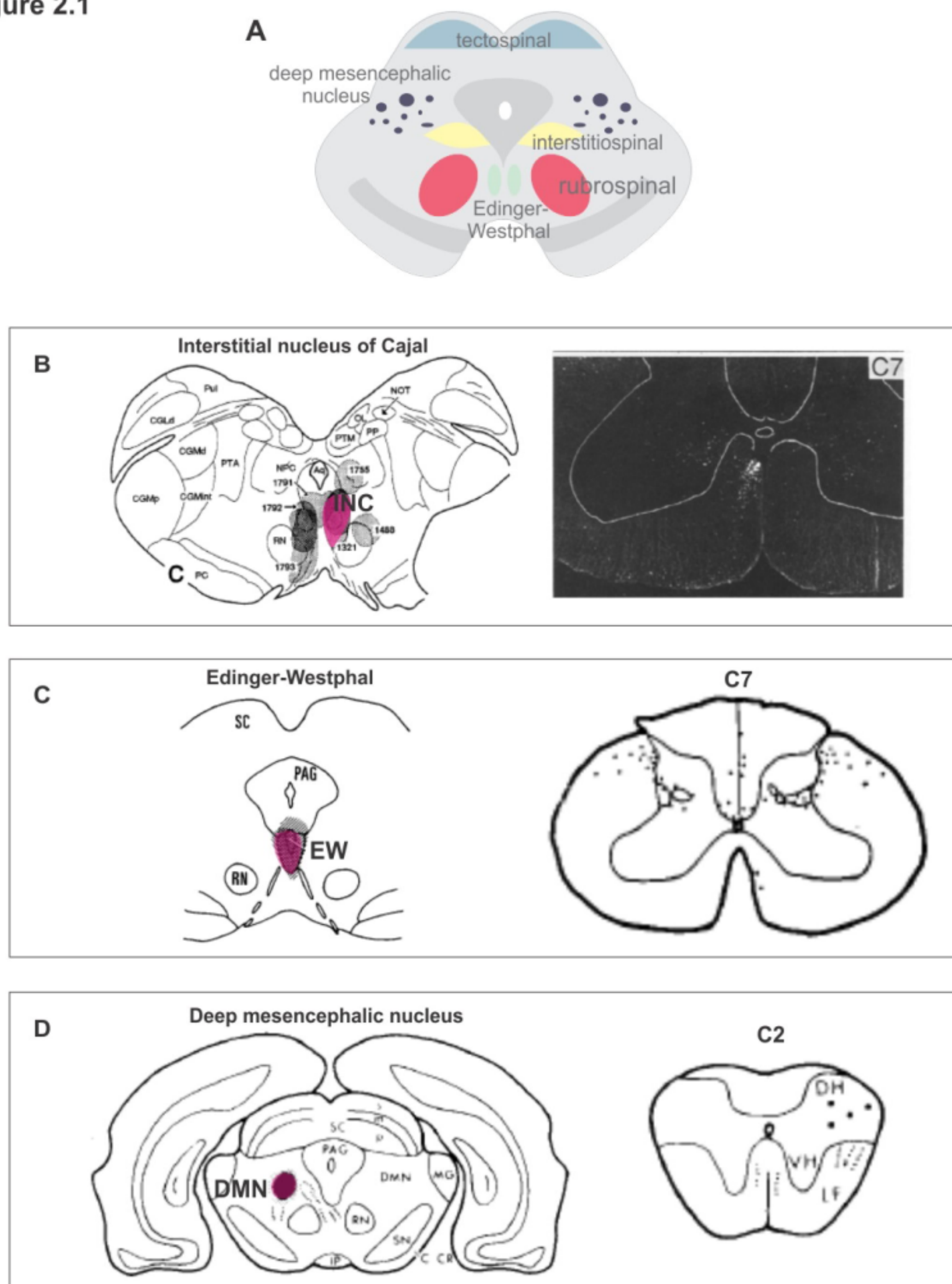


Figure 2.1 Descending projections from the midbrain to the spinal cord The midbrain gives rise to a number of descending spinal projections, illustrated in A. The dorsal superior colliculus is the originof the tectospinal tract. Ventrally, the interstitial nucleus of Cajal (INC) gives rise to the interstitiospinal tract, the deep mesencephalic nucleus contains two populations of descending projection neurons, andthe midline Edinger-Westphal nucleus also sends descending projections to the spinal cord. Anterograde tracing from specific midbrain motor centers in the cat labels descending tracts from each region at C7 in the spinal cord. The interstitial nucleus of cajal (INC) gives rise to an ipsilateral projection in the ventral funiculus that terminates bilaterally in the ventromedial region of the ventral horn (B). The midline Edinger-Westphal nucleus is the origin of a descending projection in the dorsal-most region of the dorsolateral funiculus that terminates in the dorsal horn (C). Neurons within the deep mesencephalic nucleus (DMN) give rise to two descending tracts, visualized here at C2 in the spinal cord: a bilateral projection descending in the ventral funiculus and a contralateral projection descending in the dorsolateral funiculus (D).

From: Holstege and Cowie 1988 (B), Loewy and Saper 1977 (C), Veazey and Severin 1980

direct monosynaptic inputs from the interstitiospinal tract in the cat (Fukushima et al., 1978). Given its close proximity to the red nucleus, it is not surprising that anterograde tracing of the rubrospinal tract frequently labels an additional descending tract that projects ipsilaterally in the ventral funiculus, and terminates bilaterally in the medial ventral horn, originating from the interstitial nucleus of Cajal (Holstege et al., 1988). Finally, the Edinger-Westphal nucleus, which is located in the ventral midline, gives rise to a projection descending in the dorsal-most aspect of the lateral funiculus, and terminating in the superficial layers of the dorsal horn (Loewy and Saper, 1978) (figure 2.1 c).

The development of transgenic mouse strategies that take advantage of differential gene expression to label specific populations of neurons provides an elegant solution to the question of specificity. The identification of a rubrospinal-specific gene promoter with which to drive expression of a fluorescent reporter protein would ensure the specific labeling of the rubrospinal tract. However, the only gene thus far identified as a marker of rubrospinal neurons is *brn3a* (Agarwala and Ragsdale, 2002; Xiang et al., 1996), which is broadly expressed throughout the midbrain and spinal cord (Helms and Johnson, 2003). Thus, this approach requires a more detailed analysis of gene expression in the red nucleus.

Visualization of rubrospinal axons and terminals

The success of genetic tracing techniques depends heavily on the labeled cells' ability to generate high enough levels of the reporter protein to fill cellular

processes. This is of increased importance in the labeling of long-range projection neurons such as the supraspinal pathways. Protein levels are determined by the activity of the genetic promoter, and can provide a significant advantage over non-genetic tracing techniques.

One successful example of genetic labeling is highly relevant to my own studies. Genetic labeling of the corticospinal tract was accomplished using a cortically restricted cre driver (emx1::cre*)* to express a fluorescent protein under the control of the robust Thy-1 promoter (Bareyre et al., 2005; Feng et al., 2000). Numerous prior studies have investigated the existence of a direct cortico-motoneuronal circuit in the rodent (Alstermark et al., 2004; Yang and Lemon, 2003), yet this study provided the first evidence of such direct connections in the mouse. Although there are numerous possible explanations as to why these direct projections were missed in previous tracing experiments, the enhanced visualization of corticospinal terminals likely increased the recognition and analysis of corticospinal projections onto motor neurons.

The emergence of viral vectors for anterograde and trans-synaptic neural circuit tracing has provided yet another avenue for robust labeling of neuronal populations. Although the small genomic capacity of commonly used neurotropic viruses such as adeno associated virus (AAV) limits promoter choice (Tal, 2000), well-characterized promoter sequences such as the cytomegalovirus (CMV) or synapsin promoters, have been used to drive high levels of gene expression . One

advantage of this approach is that viral infection results in stable gene expression over long time periods (McCown, 2005), unlike transgenic lines that frequently utilize genes whose expression is developmentally regulated. Moreover, AAV does not elicit an immune response within the brain (Bueler, 1999), an important consideration as a longer timecourse is required to produce and transport high enough levels of fluorescent protein to reveal synaptic morphology.

The introduction of genes through viral delivery can lack the specificity conferred by genetic labeling techniques, although strategies for restricted infectivity, such as envelope receptor pseudotyping (Wall et al., 2010), and cell-specific promoters (Oh et al., 2009) are being developed. Nonetheless, if combined with additional methods to identify neuronal subpopulations, such as distinguishing anatomical or molecular characteristics, viral tract tracing can be a powerful tool for visualizing neuronal circuits.

Anatomy of rubrospinal projections

The descending pathways have historically been grouped into medial and lateral systems based on proposed function. This distinction is anatomically represented by the white matter location of the descending pathway, and its subsequent termination zone in the spinal cord. The rubrospinal tract is classified as a lateral system and descends in the dorsolateral funiculus of the spinal cord. Thus, it can be readily distinguished from the medial midbrain systems, such as the interstitiospinal and tectospinal tracts by virtue of its physical distance.

Rubrospinal terminal markers

Neuronal populations can also be distinguished by the type of neurotransmitter released. Even within the larger categories of excitatory and inhibitory neurotransmitters are contained smaller populations of glutamatergic or cholinergic, GABAergic or glycinergic. It has also become apparent that even amongst neurons of the same neurotransmitter class, the cohort of synaptic proteins expressed is cell-type specific. For example, spinal interneurons responsible for presynaptic inhibition of incoming sensory neurons express the GABA synthesis enzyme GAD65, whereas neurons mediating post-synaptic inhibition co-express both GAD65 and an alternate isoform, GAD67 (Betley et al., 2009). Although the functional significance of varying isoform expression is yet to be determined, we can take advantage of these genetic differences to identify and manipulate populations within a circuit.

Rubrospinal neurons are excitatory, and utilize glutamate as their neurotransmitter (Beitz and Ecklund, 1988). Although all glutamatergic neurons require the vesicular glutamate transporter (vglut) to load glutamate into synaptic vesicles, there are 3 different isoforms (vglut1-vglut3) (Kaneko and Fujiyama, 2002; Liguz-Lecznar and Skangiel-Kramska, 2007). Vglut1 is expressed by group 1a sensory neurons and corticospinal neurons whereas vglut2 is the main isoform expressed by spinal interneurons (Oliveira et al., 2003). It remains to be seen which isoform is expressed at rubrospinal terminals, and whether this criterion can be used to distinguish rubrospinal neurons from other descending populations.

Defining synaptic contacts between rubrospinal and motor neurons

Lastly, the definitive establishment of a synaptic contact between descending rubrospinal neurons and spinal motor neurons has yet to be accomplished. The histochemical detection of small molecule tracers is quite robust, and has been used to demonstrate the localization of rubrospinal boutons in close proximity to identified motor neurons in the ventral horn. However, the optical resolution of light microscopic reconstruction of synaptic connections has been called into question (Yang and Lemon, 2003). Electron microscopic analysis of similarly labeled projections from the corticospinal tract in the vicinity of motor neurons revealed that purported sites of close apposition identified by light microscopy are not indicative of a synapse (Yang and Lemon, 2003).

Electron microscopy remains the gold standard for visualizing the ultrastructural components of a synapse (Charlton and Gray, 1966). However, although recent technological advances have demonstrated its potential to provide a high-throughput approach to circuit reconstruction (Denk and Horstmann, 2004) it currently remains an expensive and time-consuming method of analysis. The ability to efficiently image synaptic contacts between neuronal cells has been greatly enhanced by the development of high-resolution fluorescent microscopy. High-magnification imaging is capable of resolving physical contact between pre- and post-synaptic membranes, and furthermore, can be combined with immunohistochemical co-localization of cellular proteins that are known to be synaptically distributed to confirm the synaptic identity of axonal varicosities.

Although the technological advances in the field of neural circuit tracing over the past decade are impressive, for any given neuronal population it can still be a challenge to identify an appropriate approach that takes into consideration the molecular, anatomical, and technical constraints of the circuit of interest. In this chapter, I have established a method to visualize the rubro-motoneuronal circuit, and utilized it to confirm the existence of direct projections onto motor neurons in the mouse.

Results

Specific labeling of projections from the red nucleus

Previous tracing studies have suggested that descending rubrospinal axons form monosynaptic projections onto motor neurons in the rodent. To further investigate the post-synaptic targets of the rubrospinal tract, I first needed to establish an efficient and reproducible method for visualizing the rubrospinal tract. The generation of a transgenic mouse line in which a specific population of neurons is targeted to express a fluorescent or enzymatic reporter protein presents one method of doing so. This approach requires the identification of a gene fulfilling the following criteria: 1. expression in rubrospinal neurons, 2. exclusion from other neuronal populations that project to the spinal cord and 3. expression extending from development through adulthood.

Gene expression in the developing red nucleus

A previously characterized gene expressed in developing rubrospinal neurons is *brn3a,* a member of the POU family of transcription factors (Xiang et al., 1996). Although *brn3a* is expressed throughout the entirety of the red nucleus (Fig. 2.1 b), it is also expressed in a number of other spinal projection neurons, including intrinsic spinal interneurons (Helms and Johnson, 2003) and dorsal root ganglion sensory neurons (Xiang et al., 1996). In addition, *brn3a* expression in the red nucleus only extends through the third postnatal week (data not shown).

To identify additional genes expressed in both the developing and adult red nucleus, I performed an in-situ hybridization screen at e17.5 of 50 genes identified in the Allen Brain Atlas as being strongly expressed in the adult red nucleus. Using *brn3a* as a marker of the developing red nucleus, I identified a second putative red nucleus marker, *complement component 1 q subcomponent-like 2* (*c1ql2*), whose expression pattern matched that of *brn3a* (figure 2.2 c). C1qL2 is a secreted protein (Iijima et al., 2010) that shares homology with members of the complement cascade that have recently been implicated in synaptic elimination and refinement (Stevens et al., 2007). Whereas *brn3a* is expressed in multiple midbrain neuronal populations, *c1ql2* appears restricted to the red nucleus and an additional cell population in and around the periaqueductal gray (figure 2.2 b).

When I compared the developmental expression pattern of *brn3a* and *c1ql2* in more detail, I found that *brn3a* and *c1ql2* delineate two previously uncharacterized subpopulations of neurons within the red nucleus. *Brn3a* is expressed within the

rostral red nucleus, whereas both *brn3a* and *c1ql2* are expressed in the caudal red nucleus (Figure 2.2 d-i). To determine whether Brn3a and C1qL2 are co-expressed in the same population of neurons in the caudal domain, I examined protein co-localization. Antibody labeling demonstrated that all C1qL2^{+} neurons within the caudal red nucleus co-expressed Brn3a (Figure 2.2 j-l) at e17.5. Finally, I established that C1qL2 remains expressed throughout adulthood (figure 2.2 n)

The expression pattern of Brn3a and C1qL2 is particularly interesting given that the red nucleus is a heterogeneous structure populated by at least two functionally distinct neuronal subtypes: magnocellular neurons which give rise to the rubrospinal tract, and parvocellular neurons which give rise to the rubro-olivary tract. In other species, the parvocellular region composes the rostral 1/3 of the red nucleus, whereas the magnocellular subdivision is located in the caudal 2/3. This raised the possibility that c1ql2 might be specifically expressed in rubrospinal neurons. The anatomical and molecular distinction between rubro-olivary and rubro-spinal projections will be addressed in chapter 4. I first sought to determine whether or not c1ql2 is expressed in rubrospinal neurons.

C1qL2 is expressed in spinal projection neurons within the caudal red nucleus

To determine whether the c1ql2^{+} caudal domain of the red nucleus gives rise to spinal projection neurons, it was necessary to first identify rubrospinal neurons within the red nucleus. To do so, I injected a fluorescent protein-conjugated retrograde tracer, alexa488-CTB, bilaterally into cervical spinal levels C6-C8 in P7

mice (figure 2.3 b). The tracer is taken up by axons terminating in this region, and should identify spinal projection neurons in the midbrain (figure 2.3 c). Analysis of gene expression indicated that all labeled rubrospinal neurons in the caudal red nucleus co-expressed c1ql2 (figure 2.3 f). Although the results shown in Fig. 2.3 represent a single experimental case, the extensive retrograde labeling experiments that will be detailed in chapter 4 confirm and extend these results.

Published images from a C1qL2 BAC transgenic GENSAT GFP reporter line (Gong et al., 2003) demonstrate a projection to the dorsolateral ventral horn at cervical spinal levels. This appears to be highly similar to the projection identified by Kuchler et al using anterograde labeling from the red nucleus (Kuchler et al., 2002), although the exact spinal level of the C1qL2::GFP ventral projections in the GENSAT study was not characterized. Nonetheless, this reproducibility further supports our hypothesis that *c1ql2* is expressed in rubrospinal neurons, and also suggests that neurons within the red nucleus are responsible for the observed descending midbrain projections to lamina IX. I therefore performed preliminary studies aimed at determining whether a transgene driven under the control of the *c1ql2* promoter would allow me to specifically label rubrospinal axons and their projections.

C1ql2 is expressed in an additional population of spinal projection neurons in the midbrain, and a subset of spinal interneurons and dorsal root ganglion neurons

Transgenic mouse technology has greatly advanced the study of neural circuitry by enabling the specific labeling of a restricted population of neurons based on gene expression. I have established that C1qL2 expression in rubrospinal neurons throughout adulthood would classify it as a strong candidate for specifically labeling rubrospinal projections. However, the expression of C1qL2 in other neuronal populations that project to the spinal cord would negate the benefits of generating a transgenic reporter line. Potential confounding neural populations include other descending projection neurons, dorsal root ganglion sensory neurons, and intrinsic spinal neurons.

Although C1qL2 is expressed in the cortex, it is excluded at all timepoints from the layer 5 neurons that give rise to the corticospinal tract (Allen Brain Atlas). C1ql2 is expressed in a restricted number of populations within the midbrain and hindbrain, and I cannot definitively rule out its expression in descending tracts from the hindbrain. However, I have examined its expression in midbrain supraspinal populations. The RNA expression pattern of *c1ql2* demonstrates that it is not expressed in dorsal midbrain populations. C1qL2 expression is also absent from the midline Edinger-Westphal nucleus (Figure 2.3 d). However, the population of C1qL2^{+} neurons located lateral to the periaqueductal gray do partially overlap with the retrogradely labeled spinal projection neurons (figure 2.3 e). The supraspinal population just outside the PAG, which appears to correspond to the interstitial nucleus of Cajal, is C1qL2 negative. A small percentage of the more lateral tegmental spinal projection neurons comprise the C1qL2^{+} supraspinal population.

This location likely contains the deep mesencephalic nucleus. Thus, descending pathways from the DMN represent a potential confounding source of C1qL2+ non-rubrospinal descending projections in C1qL2 reporter lines. Finally, when I examined *c1ql2* RNA expression in the developing spinal cord, I noted labeled populations of ventral interneurons and sensory neurons (Figure 2.3 g, h). To avoid the possibility of a lack of rubrospinal specificity, we decided to pursue instead an intersectional Cre/Lox approach.

Generation of a C1qL2 conditional reporter line

I generated transgenic mice bearing a conditional allele of *c1ql2*, in which Cre-mediated recombination results in the excision of a STOP cassette, and expression of GFP, referred to as *c1ql2::*ϕGFP (figure 2.4 a). To increase the likelihood that GFP would be transported to the axons and terminals, I used a myristoylated version of the protein containing the first 30 amino acids of the MARCKS protein, which promotes targeting to cellular membranes (De Paola et al., 2003). These mice were generated using a modified BAC transgenic strategy (Heintz, 2001). We decided on this approach since the C1qL2 promoter has not been characterized. The BAC approach maintains a large upstream region of the c1ql2 gene, maximizing the likelihood of including essential regulatory regions.

Positive founders were screened for transgene specificity by crossing the line to a ubiquitously-expressed neuronal cre driver (Nestin::Cre), and expression of GFP was compared with endogenous C1qL2 expression. Within the midbrain at P7,

mosaic expression of the reporter was restricted to $C1qL2^+$ cells of the red nucleus (figure 2.4 b-d). There was corresponding expression of mGFP in axons running in the dorsolateral funiculus at cervical spinal levels (figure 2.4 e). I was able to observe a GFP^+ projection into the dorsolateral ventral horn at lower cervical levels that appeared to replicate that seen in previous rubrospinal tracing experiments (Kuchler et al., 2002; McCurdy et al., 1987) (figure 2.4 e, f). This projection terminated in the vicinity of the dorsal-most population of motor neurons, identified by choline acetyltransferase (ChAT) immunoreactivity (Levey et al., 1983). Unfortunately, despite my efforts to maximize reporter expression, the level of mGFP in rubrospinal axons was low, resulting in poor visualization of synaptic boutons even by confocal microscopy. Expression levels were comparable to other reporter lines (Rosa::ϕYFP) that also did not allow robust visualization of synaptic contacts (data not shown). Although the rubrospinal tracing results obtained from the *c1ql2::*ϕGFP transgenic line supports the hypothesis that $c1ql2^+$ neurons in the red nucleus give rise to putative direct projections onto motor neurons, an alternative method that strongly labels synaptic terminals is necessary to further investigate the existence of rubro-motoneuronal synapses in the rodent.

AAV2::synapsin-GFP labeling of descending midbrain projections

The precise analysis of a neural circuit depends upon the ability to strongly visualize the individual components. I therefore decided to use a viral approach to express GFP in rubrospinal neurons under the control of the strong synapsin promoter (AAV2::synapsin-GFP) (figure 2.5a). Recent studies have demonstrated that adeno

associated viral (AAV) vectors containing the cre recombinase gene will stably infect adult hippocampal neurons (Ahmed et al., 2004). I needed to confirm that this approach would be feasible for labeling descending rubrospinal neurons efficiently and robustly.

I first wanted to ensure that AAV vectors would infect rubrospinal neurons. Different AAV serotypes preferentially infect certain populations, and AAV serotypes 2, 6, and 9 have demonstrated the ability to infect neuronal subtypes. To test whether AAV serotype 2 was capable of infecting rubrospinal neurons, I injected AAV2:synapsin GFP unilaterally into the ventral midbrain of a P50 mouse (figure 2.5 a), using stereotaxic coordinates for the red nucleus (Paxinos and Franklin, 2004). Previous studies have indicated that maximal reporter expression is observed two to three weeks after injection of AAV::Cre (Ahmed et al., 2004). Therefore, following a 3-week survival period, I examined the brain for GFP expression. Widespread GFP expression was observed throughout the ventral midbrain, including C1qL2^{+} rubrospinal neurons (Figure 2.5 b). Lastly, I wanted to determine whether stereotaxic viral injections would result in visualization of the rubrospinal tract. Analysis of GFP expression in the cervical spinal cord revealed robust labeling of a descending projection in the dorsolateral funiculus (Figure 2.5 c). A smaller projection was labeled in the ventral funiculus, likely the interstitiospinal tract (figure 2.5 c, asterisk). Rubrospinal axons are clearly visible in the spinal cord even under low magnification (figure 2.5 c).

Determination that projections are arising from the red nucleus

Although this approach lacks the genetic specificity of a rubrospinal-restricted reporter line, the visualization of pre-synaptic terminals of descending spinal inputs is made possible in a way not currently accessible with my C1qL2 reporter strain. Thus, the next question that arises is how to distinguish rubrospinal axons from other descending projections. As a result of the close proximity of the INC, DMN, and EW to the red nucleus, the interstitiospinal tract and the tracts arising from the DMN and the EW are likely co-labeled with the rubrospinal tract.

Neither the interstitiospinal tract, nor the tract from the EW nucleus descend in the dorsolateral funiculus, providing an anatomical distinction from the rubrospinal tract. Labeled projections from the dorsolateral funiculus into the ventral horn can be seen in the two c1ql2 transgenic lines. From my retrograde labeling experiments, I confirmed that c1ql2 is only expressed in the rubrospinal and DMN midbrain supraspinal population. Finally, differential expression of synaptic terminal proteins can be used to distinguish populations of neurons (Betley et al., 2009). Rubrospinal neurons are glutamatergic, therefore I performed an in situ hybridization screen of all three vglut isoforms in the postnatal red nucleus. Rubrospinal neurons express *vglut2*, as do a number of other populations in the ventral midbrain (Figure 2.6 a-d). Unfortunately, *vglut2* is widely expressed throughout the ventral midbrain. If the DMN neurons are a glutamatergic population, then they likely also express this isoform. An analysis of *vglut2* expression in retrogradely-identified supraspinal DMN neurons is necessary to resolve this.

Thus, there is no single defining characteristic to distinguish the rubrospinal projections from other descending fibers originating from neighboring midbrain structures. I therefore decided that in order to classify a labeled axon as "rubrospinal" in origin, it needed to satisfy the following criteria: 1. The projection must arise from the dorsolateral funiculus; 2. Its termination zone must be close to motor pools in the dorsolateral quadrant of the ventral horn, as observed in the GENSAT c1ql2::GFP and in C1qL2::ϕGFP x Nestin::Cre transgenic lines, as well as for projections traced in anterograde labeling studies; and 3. Labeled synaptic boutons must express the synaptic marker, Vglut2. As explained, this criterion does not definitively exclude the possibility that the projections I define as rubrospinal originate from the deep mesencephalic nucleus, and this will be addressed in further detail in the discussion section of this chapter.

Labeling of all spinal motor neurons

Having defined an approach to visualize and define the rubrospinal pre-synaptic component, I then needed to define the labeling strategy for the post-synaptic component of the direct circuit, the motor neuron. In previous anatomical studies of the rubro-motoneuronal circuit, motor neuron labeling was restricted to either a subset of motor neurons identified through a muscle backfill (Kuchler et al., 2002), or limited to only the cell body and proximal dendrites with ChAT immunostaining. To visualize *all* spinal motor neurons, including their extensive dendritic arbors, I crossed a ubiquitous strong reporter line expressing a red fluorescent protein, Rosa::ϕCAGGS-tdtomato, with a motor neuron specific Cre

driver (ChAT::Cre) (figure 2.6 panel e-k). This approach allowed me to definitively address the outstanding question of whether there exists a direct rubro-motoneuronal circuit in rodents.

Postsynaptic targets of the rubrospinal tract

Visualization of the direct rubro-motoneuronal circuit in rodents

To determine whether the rodent rubrospinal tract projects directly onto motor neurons, I injected AAV2::synapsin-GFP into the midbrain of Rosa::ϕCAGGS-tdtomato; $ChAT^{Cre/+}$ adult mice. After a survival period of three weeks, I examined projections in the cervical spinal cord, focusing primarily on C7-T1, the spinal levels where ventral rubrospinal projections are prominent. There was a striking labeled projection to the dorsolateral ventral horn that deviated from the horizontal trajectory of the remainder of the pathway (figure 2.6 f). Low-power imaging confirmed that these projections densely terminated in the vicinity of a dorsolateral population of RFP-labeled motor neurons (figure 2.6 g).

To investigate whether these ventral projections formed synapses onto motor neurons, I used higher (63x) magnification confocal imaging to determine whether the GFP-labeled bouton-like structures were directly apposed to post-synaptic motor neuron membranes. To confirm that these boutons represented synaptic specializations, and to fulfill the criteria that the GFP^+ terminals originate from the red nucleus, I also performed an antibody stain for the rubrospinal terminal marker, vglut2. My results clearly demonstrate that gfp^+ $vglut2^+$ rubrospinal terminals form

direct inputs onto motor neuron cell bodies and proximal dendrites (figure 2.6 h-k). Direct rubro-motoneuronal projections were found in every experiment in which a significant proportion of the C1ql2+ population in the midbrain was successfully infected with AAV (n=10). This determination was made by eye, and a quantitative analysis of the percentage of C1qL2+ neurons infected in each experiment still needs to be undertaken, and correlated with experimental findings. A more detailed quantification of rubro-motoneuronal inputs will be presented in the following chapter.

Restriction of projections onto motor neurons to C7/C8

The dense rubrospinal projections surrounding the dorsolateral population of motor neurons at C7/C8 were not present in the directly surrounding medial or ventral motor neuron populations at those spinal levels (figure 2.6 f). To further characterize the distribution of motor neurons that receive direct rubrospinal input, I analyzed the pattern of ventral horn innervation throughout the cervical enlargement. At neither rostral (C5-C6) nor caudal (T1-T2) spinal levels was a similar density of GFP^+ rubrospinal fibers seen to contact motor neurons (figure 2.7 b, d). Direct $vglut2^+$ projections from the midbrain onto motor neurons – in all likelihood rubro-motoneuronal projections – are therefore strikingly limited to the dorsolateral quadrant of only two segments within the entire cervical enlargement of the ventral horn.

Figure 2.2

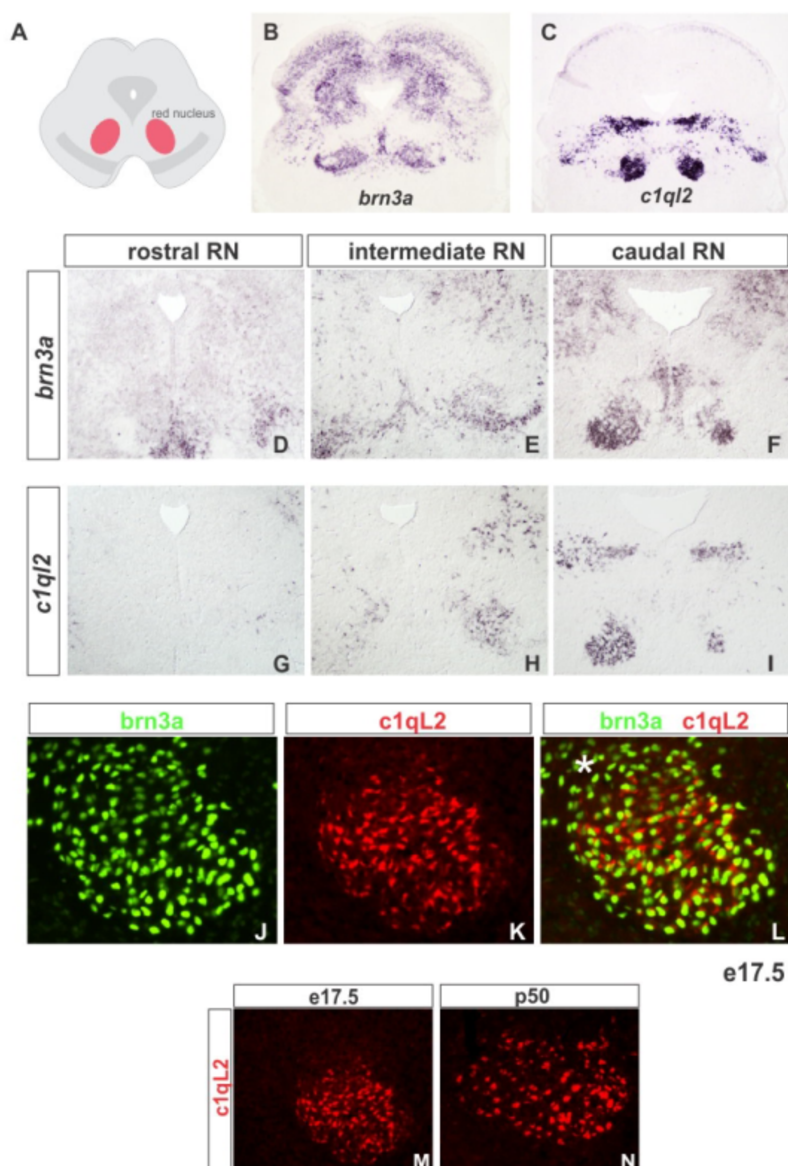


Figure 2.2 Brn3a and C1qL2 expression in the red nucleus The pou transcription factor, *brn3a*, and the complement family member, *c1ql2*, are both expressed in the developing red nucleus at e17.5. A rostro-caudal comparison of the expression domains of these two markers demonstrates that *brn3a* is expressed throughout the red nucleus, whereas *c1ql2* is restricted to the caudal 2/3 of the red nucleus (D-I) at e17.5. At caudal levels, brn3a and c1qL2 are co-expressed in the same population of neurons (J-L) at e17.5. Although Brn3a marks a population of neurons outside the red nucleus (asterisk in L), all C1qL2+ neurons also co-express Brn3a. Brn3a expression is downregulated in the third postnatal week (not shown), whereas C1qL2 levels are maintained through adulthood (N).

Figure 2.3

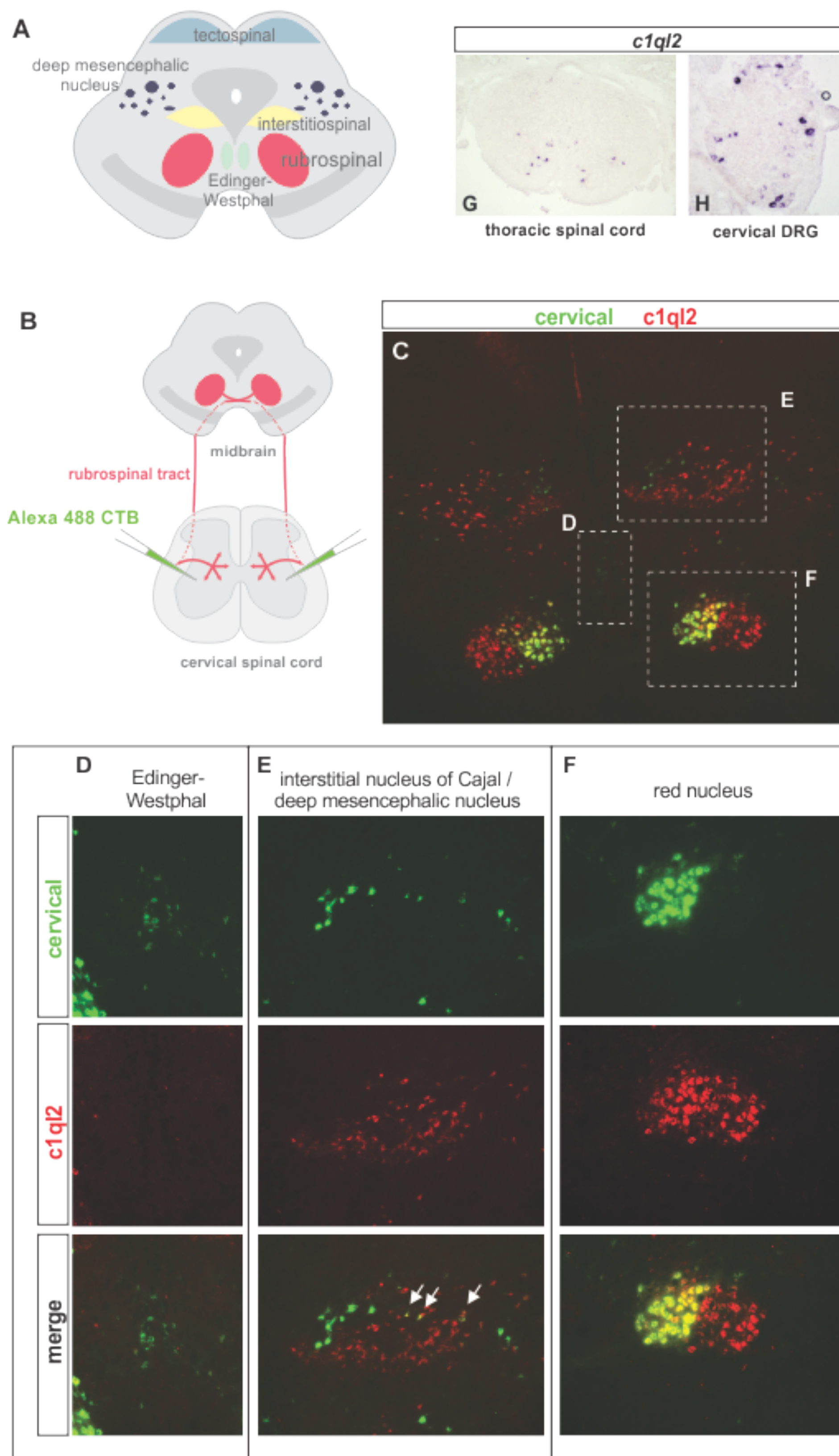


Figure 2.3 C1qL2 expression in spinal projection neurons There are a number of midbrain structures that give rise to a descending spinal projection, including the superior colliculus (tectospinal), interstitial nucleus of Cajal (interstitiospinal), Edinger-Westphal (EW) nucleus, and the deep mesencephalic nucleus (DMN) (A). Bilateral injection of a retrograde tracer, Alexa488-CTB into the cervical spinal cord of p7 mice labeled spinal projection neurons in the midbrain following 3 days of transport (C). Supraspinal neurons were observed in the Edinger-Westphal nucleus (D), the lateral midbrain tegmentum (INC and DMN) (E), and the red nucleus (F). C1qL2 was absentfrom labeled EW neurons, but was observed in a percentage of neurons in the lateral aspect of the midbrain tegmentum, corresponding to the appropriate location of the DMN. All of the spinal projection neurons labeled in the red nucleus were C1qL2+ (F). In addition to its expression in midbrain spinal projection neurons, *c1ql2* expression was also noted in a population of ventral interneurons at all levels of the spinal cord (G), and a subset of dorsal root ganglion sensory neurons at restricted spinal levels (H).

Figure 2.4

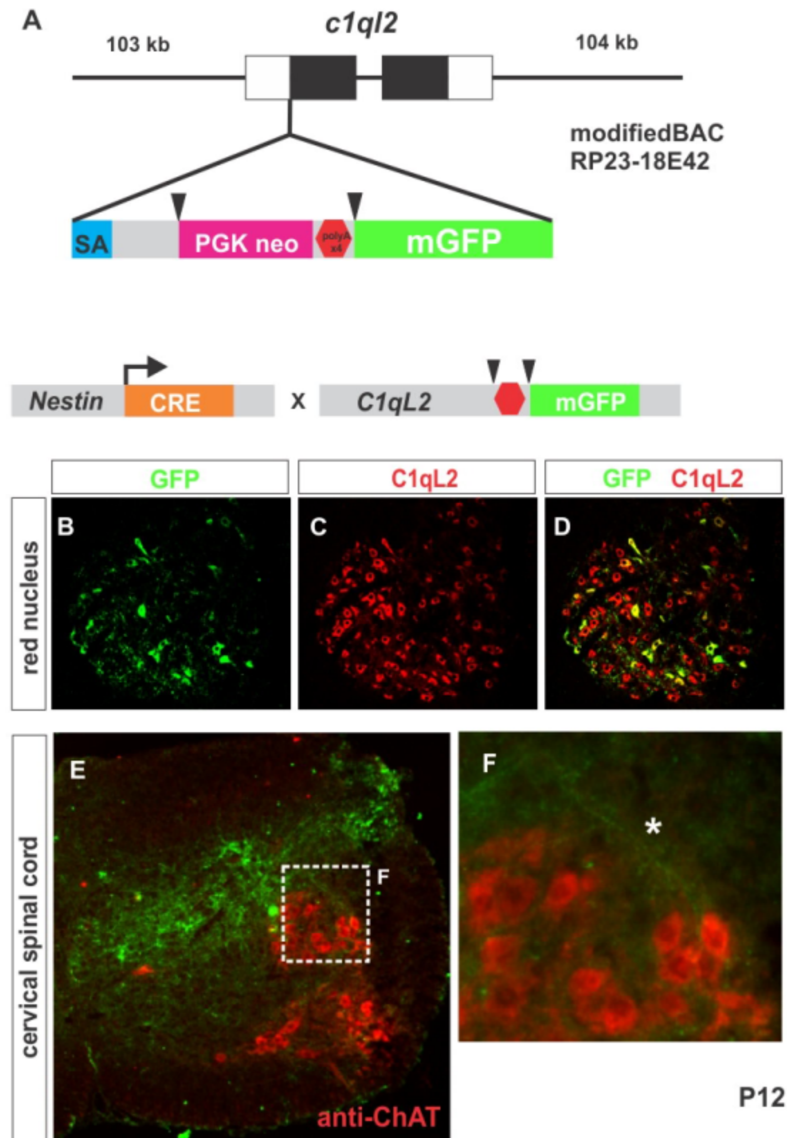


Figure 2.4 Generation of a C1qL2 conditional reporter line A C1qL2 conditional reporter line was generated using a modified BAC approach, replacing the first ten coding amino acids of c1ql2 with myristolated GFP, directly preceded by a polyA STOP signal flanked by LoxP sites (A). Founder lines were characterized by crossing to a neuron-specific Cre driver (Nestin::Cre). Line 14 demonstrated mosaic labeling of the C1qL2 population within the red nucleus (B-D). A spinal tract was labeled in the dorsolateral funiculus of the spinal cord (E), and gave rise to a ventral projection which was clearly observed to approach a dorso-lateral population of ChAT+ motor neurons in the cervical enlargement (F).

Figure 2.5

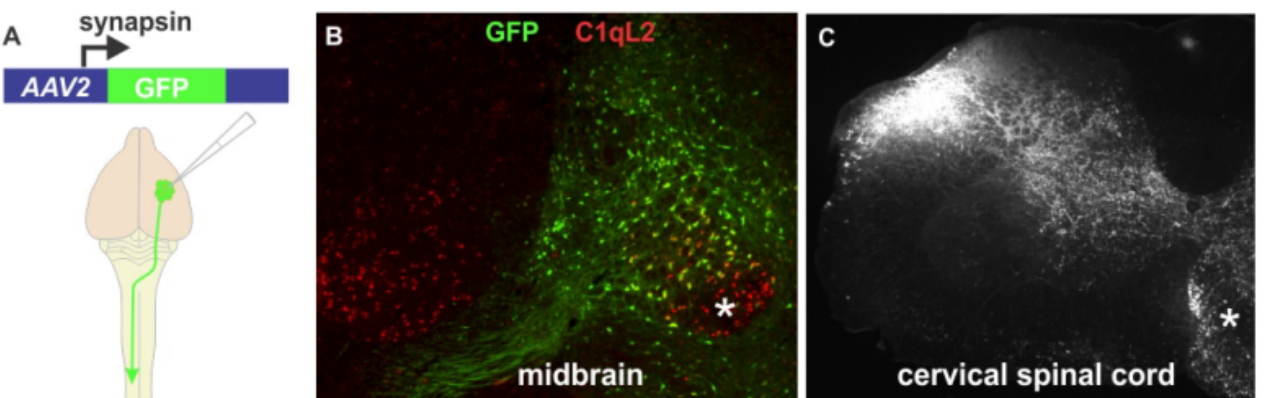


Figure 2.5 Viral labeling of descending projections from the midbrain Stereotaxic injections of 100 uL of AAV2::Synapsin-GFP were made at 3 locations along the rostro-caudal extent of the red nucleus in adult WT mice. The mice were sacrificed two weeks later, and the midbrain and spinal cord examined for GFP expression. C1qL2 positive neurons in the dorsomedial red nucleus were observed to be GFP+ (B). GFP expression was never seen in the ventrolateral C1qL2 population, corresponding to the lumbar-projecting rubrospinal neurons (asterisk in B). A brightly labeled tract was observed in the cervical spinal cord contralateral to the injection side (C), and an additional tract was labeled in the ipsilateral ventral funiculus, corresponding to the interstitiospinal tract (asterisk in C).

Figure 2.6

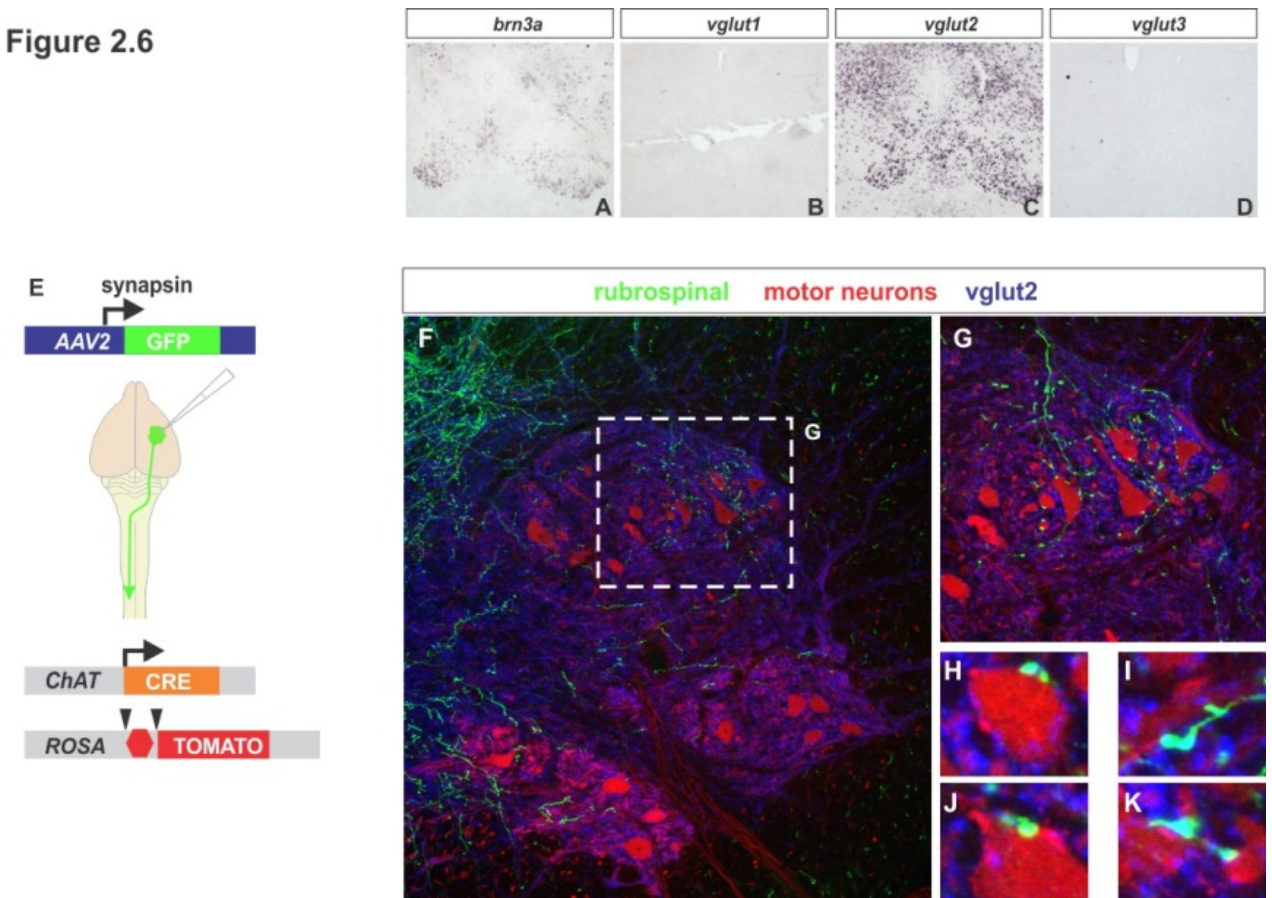


Figure 2.6 Projections from the midbrain form direct synaptic contacts onto motor neurons AAV2::GFP was injected into the ventral midbrain of ChAT Cre/+; Rosa::tomato adult mice, as described. Three weeks post-injection, the spinal cord was sectioned,and labeled projections into the spinal cord were observed in the dorsolateral funiculus. Although the majority of labeled projections were concentrated within the intermediate region of the spinal cord, a small number of GFP+ fibers were observed in the vicinity of dosolateralmotor neurons (F, G). At 63X higher magnification (H-K), GFP+ boutons were observed to contact motor neuron cell bodies (H,J) and proximal dendrites I,K). The GFP+ boutons co-expressed vglut2, the synaptic terminal marker expressed by rubrospinal neurons, as determined by a screen of vglut isoforms in the red nucleus at e17.5 (A-D).

Figure 2.7

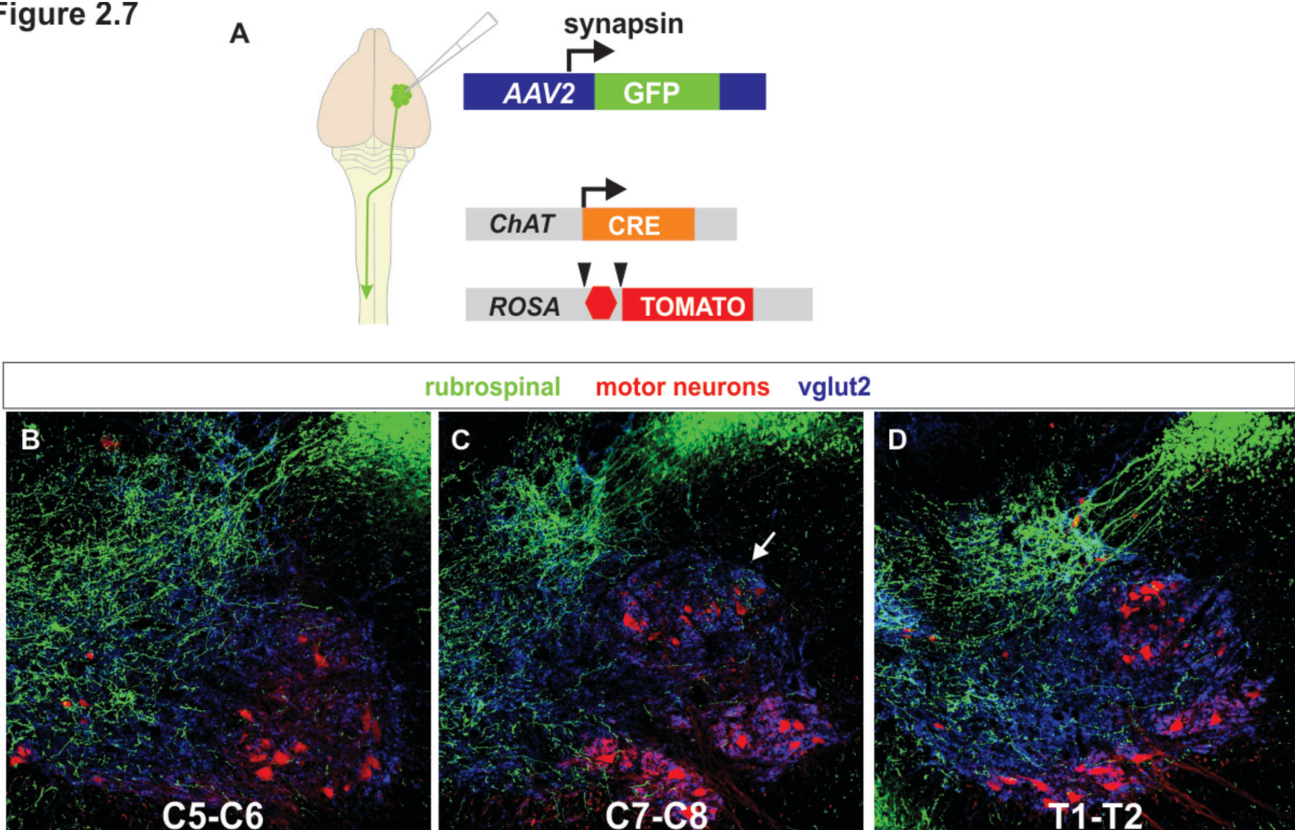


Figure 2.7 Descending rubrospinal* projections onto motor neurons are restricted to C7-C8 To determine whether descending rubrospinal projections onto motor neurons could be observed at any other spinal level, spinal cord sections were examined throughout the cervical enlargement. At rostral (B) and caudal (D) levels, a similar dense projection into the ventral horn was not observed. The arrow in C indicates the population of motor neurons which receive direct rubrospinal input The labeling in the ventromedial spinal cord likely arises from the bilateral interstitiospinal terminations amongst the axial and girdle motor neurons. * additional experiments are required to confirm that these projections do indeed arise from the red nucleus

Figure 2.8

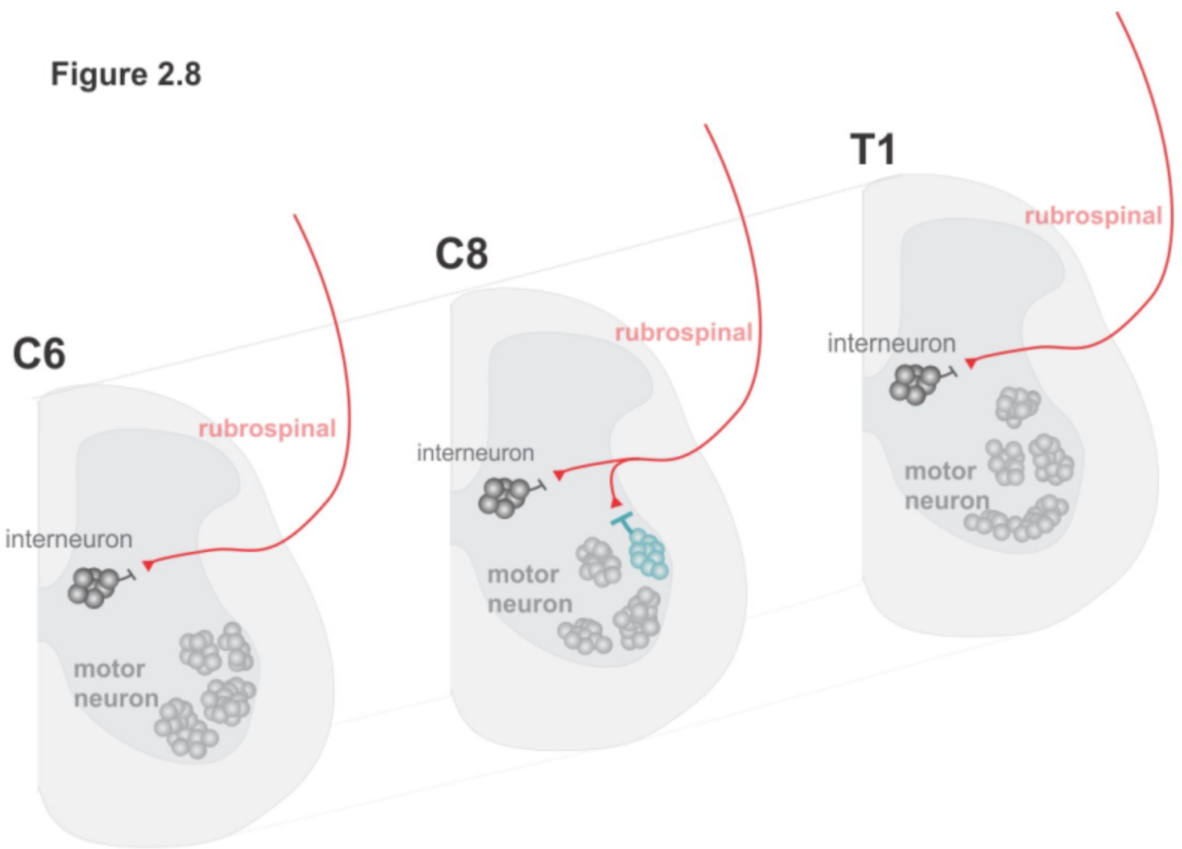


Figure 2.8 The rubrospinal tract projects directly onto a restricted population of motor neurons at C8 Viral labeling of descending rubrospinal projections has confirmed that rubrospinal neurons project directly onto motor neurons in the rodent. These direct projections are limited to a population of dorsolateral motor neurons at the C8 spinal level. The muscle target(s) of these motor neurons remain to be determined.

Discussion

In this chapter, I confirm the existence of a direct circuit arising from the ventral midbrain at the level of the red nucleus onto a restricted population of forelimb motor neurons in the rodent. Although previously published work has indicated that there are close appositions between labeled rubrospinal axons and motor neurons projecting to forelimb muscles, the techniques used to demonstrate this left room for debate as to whether such contacts were synaptic in nature. Here, I demonstrate the existence of rubrospinal synapses onto motor neurons using an established technique of high-magnification confocal imaging combined with co-localization of synaptic terminal markers (Betley et al., 2009).

Developing a method to visualize the rubro-motoneuronal circuit

Previous anatomical studies of the rubrospinal system in rodents have used stereotaxic injections into the red nucleus to incorporate small molecule anterograde tracers into rubrospinal axons and terminals. These experiments have consistently demonstrated the existence of a subset of fibers arising from the dorsolateral funiculus that project into the ventral horn at lower cervical levels. However, the existence of direct synaptic contacts between the rubrospinal tract and forelimb motor neurons in the rodent remained to be definitively demonstrated. To further investigate this circuit, I first needed to establish a technique for visualizing the pre- and post-synaptic components of the putative rubro-motoneuronal circuit.

Viral labeling of the rubrospinal tract

The technique that I have used to efficiently and robustly label descending rubrospinal fibers is to inject a neurotropic viral vector into the midbrain. Adeno-associated viral gene delivery is an emerging tool in the fields of neural circuitry and neurodegeneration and disease (Braz et al., 2002; Hester et al., 2009). A well-characterized strong promoter can be used to drive high levels of reporter protein expression, in this case the promoter of the synapsin gene. Although amplification of the fluorescent protein signal can be accomplished using immunofluorescence, even endogenous fluorescence levels of AAV2::GFP are adequate to visualize rubrospinal synaptic morphology and connectivity.

Although I have demonstrated that viral expression of GFP allows clear visualization of the circuitry between long-range supraspinal motor projections and their spinal neuron targets, this technique unfortunately re-introduces the question of specificity. Although my injections are targeted to the specific coordinates of the red nucleus, viral spread around the injection site is inevitable. In addition, as the population of rubrospinal neurons that project into the ventral horn appears to represent just a small fraction of the overall tract, my intention was to fill the entirety of the red nucleus to ensure labeling of this population. Thus, at this stage, the injections are broader rather than refined. This raises two important questions that must be addressed: 1. How do we confirm that descending projections to the dorsolateral ventral horn originate from the red nucleus? and 2. How do we rule out projections into the ventromedial ventral horn originating from the red nucleus? I examine below the supportive anatomical and molecular evidence.

Evidence in support of direct projections from the midbrain onto motor neurons arising from the red nucleus

I have proposed that the direct projections onto forelimb motor neurons demonstrated following viral injections into the midbrain arise from the red nucleus, despite multiple supraspinal populations being labeled. To this end, I have used the following lines of evidence as support: similarity of projections demonstrated in two C1qL2 reporter lines (C1qL2::GFP Gensat and my C1qL2::ϕGFP), and recapitulation of projections demonstrated in previous rubrospinal anatomical tracing experiments.

From an in situ hybridization screen, I have identified a novel marker of the red nucleus, *c1ql2*, whose restricted expression in the midbrain and spinal cord made it a promising candidate with which to genetically target the red nucleus. Furthermore, cervical spinal cord images from a c1ql2::GFP line (GENSAT) demonstrate a labeled pathway in the dorsolateral funiculus of the spinal cord, whose ventro-lateral projection at cervical levels recapitulates the specific projections seen in the rat, cat, and monkey following an anterograde injection into the red nucleus. The expression of c1ql2 in other spinal projection neurons prompted the generation of a c1ql2 conditional GFP reporter line. Although the low fluorescence levels in descending fibers did not allow for quantitative analysis of rubrospinal projections onto motor neurons, I was able to reproduce the ventrolateral projection seen in the c1ql2::GFP line, and the restriction to the caudal cervical enlargement demonstrated in previous tracing experiments.

I have confirmed that C1qL2 is expressed in anatomically identified rubrospinal neurons within the red nucleus. However, although C1qL2 is absent from the superior colliculus and the Edinger-Westphal nucleus, I observed a small amount of overlap between retrogradely-labeled cervical projection neurons and C1qL2 expression in the mesencephalic tegmentum, likely in the deep mesencephalic nucleus. The DMN contains two populations of spinal projection neurons: a subset that project ipsilaterally in the ventral funiculus, and an additional population that project contralaterally in the ventral part of the lateral funiculus.

The location of a subset of fibers from the deep mesencephalic nucleus descending in the ventral part of the lateral funiculus (Veazey and Severin, 1980a, b) prevents a definitive anatomical distinction of this pathway from the rubrospinal tract. Likewise, although only a small percentage of DMN projection neurons are C1ql2^{+}, we thus cannot rule out that the ventral projection seen in the c1ql2 reporter lines originates from this pathway. Nonetheless, previous tracing experiments from the red nucleus in rats, cats, and monkeys have demonstrated a similar dorsolateral projection to the ventral horn. Therefore, I re-examined the injection sites of previous anatomical tracing studies from the midbrain to determine whether the DMN could have been the source of the observed projections.

Anterograde tracing experiments of the red nucleus in the rat, cat, and monkey label fibers that project ventrally along the lateral aspect of the ventral horn in the cervical enlargement. A closer examination of the area injected in the majority of

these experiments reveals that the tracer is not limited to the extent of the red nucleus, and does appear to include surrounding regions such as the PAG and mesencephalic tegmentum (Holstege, 1987; Holstege et al., 1988). In contrast, a beautiful set of experiments in the cat, in which tracer injections were fully contained within the parvocellular and magnocellular regions of the red nucleus demonstrate that projections into the ventral horn are only seen following an injection into RNm (Pong et al., 2002) (figure 2.9). In addition, a control experiment in the monkey, where the injection site included the majority of the midbrain tegmentum but clearly excluded the red nucleus, did not result in labeling of projections to the ventral horn (Holstege et al., 1988).

Finally, support of an anatomical connection between the rubrospinal tract and distal forelimb motor neurons comes from physiological studies of rubrospinal activity in the cat (Horn et al., 2002). If a stimulating electrode is placed within the confines of the magnocellular red nucleus, it evokes strong facilitation of distal forelimb muscles such as the extensor digitorum communis (EDC), as determined by EMG recordings. The positioning of the stimulating electrode dorsal to the red nucleus, in the PAG or mesencephalic tegmentum, does not result in an increase in muscle activity.

The evidence I have presented strongly supports my conclusion that the demonstrated direct inputs onto a dorsolateral group of motor neurons following midbrain viral injections originate from the red nucleus. Nonetheless, we are

Figure 2.9

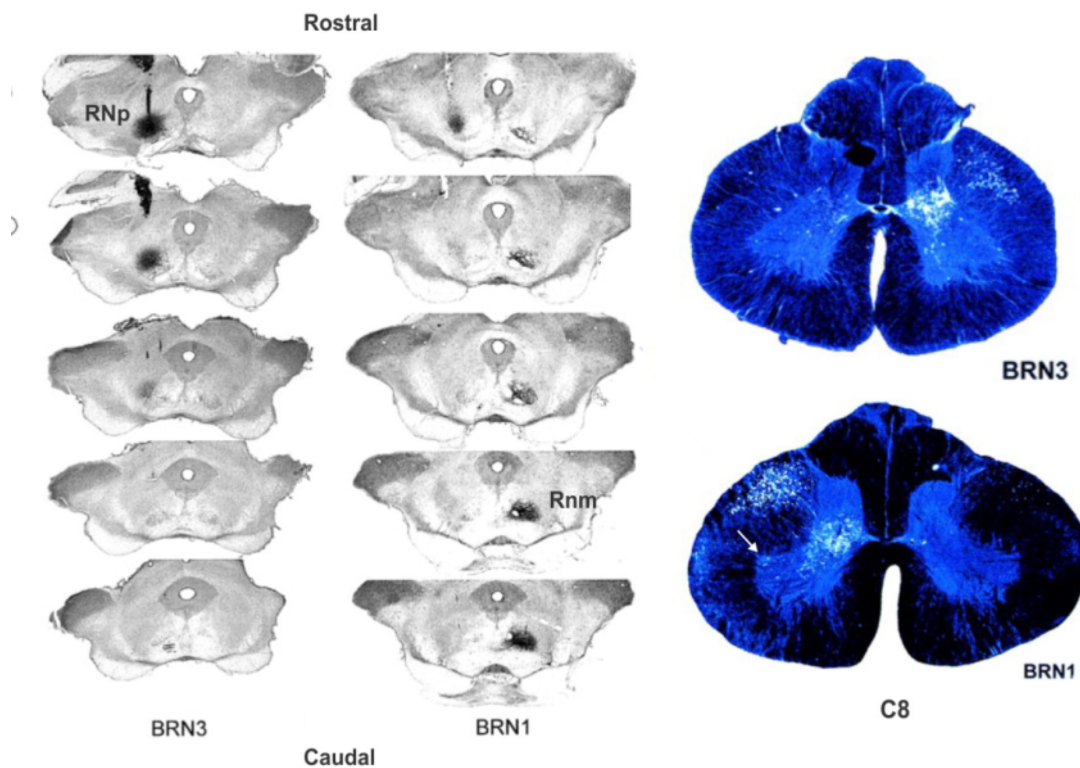


Figure 2.9 Specific injections into the magnocellular red nucleus label descending projections into the ventral horn at C8 in the cat Projections from the rostral, parvocellular red nucleus (Injection Brn3) descend in the dorsolateral funiculus as a component of the rubrospinal tract, but terminate in the intermediate zone of the spinal cord. Specific injections into the caudal, magnocellular red nucleus (injection Brn1) also label the descending rubrospinal tract in the dorsolateral funiculus, and appear to be the origin of the projection into the dorsolateral ventral horn at C8 (arrow in Brn1 spinal cord)

From Pong et al., 2002

undertaking further circuit tracing experiments (discussed in detail in chapter 6), to definitively confirm the identity of midbrain neurons that directly contact forelimb motor neurons.

Projections to the ventromedial spinal cord arise from the midbrain interstitial nucleus of Cajal

The first classification scheme of the descending pathways was based on an anatomical feature, namely their location in either the ventral or the lateral funiculi. Midbrain nuclei that give rise to ipsilateral descending projections located in the ventral spinal funiculus are the interstitial nucleus of Cajal (Nyberg-Hansen, 1966), and the deep mesencephalic nucleus (Veazey and Severin, 1980b). Post-mortem analysis of the midbrain indicates that the above nuclei are likely labeled in my injections, although the lack of defined molecular markers for these populations prevents confirmation.

Anterograde tracing of the interstitiospinal tract via injections that exclude the red nucleus demonstrates a similar medial ventral horn projection as is seen following my injections (Holstege et al., 1988; Holstege and Cowie, 1989). Correspondingly, anterograde injections into the midbrain that are strictly contained within the red nucleus do not indicate a projection running in the ventral funiculus (Horn et al., 2002; Pong et al., 2002). Thus, we can conclude that the labeled fibers in the ventromedial spinal cord likely arise from non-rubrospinal sources. Nonetheless, future experiments should include small, restricted viral injections into the red

nucleus, to definitively rule out the possibility of ventromedial termination zones arising from the red nucleus. In addition, use of the transgenic C1qL2-GFP reporter line should enable me to definitively determine the location of rubrospinal terminations within the ventral spinal cord.

I have demonstrated that neurons within the red nucleus express the vesicular glutamate transporter isoform *vglut2*. As expected, GFP labeled rubrospinal axons co-express vglut2 at putative synapses, not only strengthening the assertion that direct projections onto motor neurons arise from the red nucleus, but importantly, also confirming that 'close appositions' are in fact sites of de novo synaptic contact. I can extend this analysis to investigate the synaptic terminal markers expressed by GFP+ boutons in the ventromedial spinal cord. Motor neurons localized to this region of the ventral horn project to axial muscles, and are intimately involved in postural movements. It is possible that the supraspinal pathways that project onto these motor neurons utilize a distinct neurotransmitter to exert their influence.

Genetic labeling of spinal motor neuron cell bodies and dendrites

One of the limitations encountered in past studies of descending inputs onto motor neurons is the difficulty in labeling motor neuron cell bodies and dendrites. Historically, MNs have been identified through cellular stains by their large cell body size and location within the ventral horn. However, this method is restricted to labeling the MN cell body. Likewise, immunohistochemical stains for motor neuron specific markers like choline acetyltransferase (ChAT) are also restricted to the cell

bodies and proximal-most dendrites, rendering it impossible to definitively rule out descending inputs on more distal dendrites. Individual muscle injections of a retrograde tracer such as CTB can be used to label populations of motor neurons, however, it is expected that only a fraction of motor neurons projecting to a given muscle will be labeled, allowing for the possibility of projections onto unlabeled neurons. Finally, there are certain muscles that are simply not amenable to injection, due to their deep location within the forelimb muscle compartment; this includes the extrinsic thumb extensors and abductors.

Instead, we decided to take advantage of the MN specific expression of ChAT by utilizing it to drive Cre recombinase expression (ChAT::Cre). When this transgenic Cre line is crossed with a ubiquitous conditional reporter line (Rosa::CAGGS-tdtomato), fluorescent protein expression allows visualization of the MN cell body and the entirety of its dendritic arbor. In the absence of direct inputs from the red nucleus onto spinal motor neurons, this approach would have provided definitive confirmation that I was not missing projections onto a population of unlabeled motor neurons. Nonetheless, it is important to note that in this chapter and throughout this thesis, I have restricted my analysis of rubrospinal projections onto motor neurons largely to cell bodies. This will be addressed in further detail in the following chapter.

Motor neuron targets in the spinal cord

Although the identification of specific motor neuron pools that receive direct input will provide us with insight into the rationale underlying descending control of motor output, we have already taken a significant step forward in unambiguously confirming monosynaptic rubrospinal projections onto cervical motor neurons in the rodent. The existence of direct descending projections onto motor neurons in the rodent has always been a contentious subject, with neuroanatomists reserving the direct circuit for species whose digit movements reflected the precision and skill that this circuit is thought to provide. The existence of direct projections in the rodent raises a number of questions: is the sole purpose of direct projections onto motor neurons to execute fractionated muscle movements? If projections onto motor neurons do encode individual movements as suggested, could the rubrospinal circuit have evolved to fulfill a functionally distinct or complementary role to the corticospinal tract? Finally, a suggestion that is not mutually exclusive with either of these interpretations is that the rodent is in fact capable of a higher degree of fractionated movement than previously thought.

It is impossible to attempt an answer to the first two questions without a more complete understanding of the identity of the MN targets receiving rubrospinal input. I am now in a position where I am well-equipped to develop an anatomical correlate of the lesion and electrophysiological studies that have so far provided us with the majority of our insight into the contribution of descending pathways to the execution of a motor behavior. The complimentary anatomical specificity of rubrospinal projections onto individual motor pools, to be investigated in the following chapter,

will provide further insight into exactly how the rubrospinal tract modulates and manipulates motor output.

Chapter 3: The rubrospinal tract specifically targets the extensor digitorum motor pool

Control of individual muscles requires the ability of a given supraspinal center to regulate the activity of the corresponding motor pools. My data show that the rubrospinal tract is potentially capable of directly modulating the activity of a subset of motor neurons projecting to forelimb muscles. However the specific muscle target(s) of these motor neurons remains to be identified. In this chapter, I undertake a motor pool-focused analysis of the rubro-motoneuronal circuit.

Introduction

The circuit tracing strategies outlined in the previous chapter have provided definitive evidence that in rodents, there is a population of dorsolateral motor neurons in the cervical spinal cord that receives a dense direct synaptic input from supraspinal centers in the midbrain, likely the red nucleus. The existence of this specialized rubrospinal circuit providing direct access to motor neurons in rodents, once thought to be restricted to species that exhibit high levels of individual digit dexterity, raises the question as to its functional role in motor control. I will first review what is known about the role of rubromotoneuronal projections in other species.

Rubrospinal projections onto individual motor pools in rat, cat, monkey

Previous studies have concluded that descending projections from the red nucleus in the cat and monkey appear to contact distal forelimb motor neurons.

However, this statement is based on the observation that the termination zone of rubrospinal neurons lies within a lateral compartment of the ventral horn, and that this same compartment contains motor neurons projecting to the predicted limb target (Holstege, 1987; Holstege et al., 1988). In a study by McCurdy et al. (1987), motor neurons projecting to forelimb digit extensor and flexor muscles were identified on one side of the spinal cord by muscle backfills, and their location was compared to the site of dense rubrospinal terminals on the other side of the spinal cord (McCurdy et al., 1987; Robinson et al., 1987). From these experiments, the authors consistently conclude that rubrospinal neurons contact motor neurons innervating distal forelimb muscles, yet no distinction between specific motor pools has been noted.

Recent anatomical tracing experiments in the rat are the first to demonstrate labeled rubrospinal boutons that appear to contact retrogradely identified motor neurons in the same preparation (Kuchler et al., 2002). However, the authors divide the forelimb into three segments: proximal (biceps, triceps), intermediate (forearm, including wrist flexors/extensors and extrinsic digit muscles) and distal (intrinsic hand muscles), and inject multiple muscles within a given segment. While the authors report projections onto intermediate and distal muscle motor pools, individual muscle specificity within these categories is not considered.

The physiological confirmation of short-latency projections onto motor neurons in the rat was demonstrated by EMG recordings from intermediate forelimb muscles, but individual muscle specificity was not investigated (Kuchler et al., 2002).

Spike-triggered averaging of EMG recordings from individual muscles following stimulation of individual red nucleus neurons in the monkey have demonstrated a clear preference for facilitation of distal forelimb wrist and digit extensor muscles (Mewes and Cheney, 1991). However, this technique does not definitively distinguish between a monosynaptic vs. polysynaptic effect. In the cat, a more detailed evaluation of monosynaptic rubrospinal inputs onto motor neurons has been performed (Fujito and Aoki, 1995; Fujito et al., 1991). Stimulation of the rubrospinal tract resulted in EPSPs recorded intracellularly from forelimb motor neurons that had been identified by antidromic nerve stimulation. Monosynaptically excited motor neurons were located at C8-T1, and contributed to either the radial or ulnar nerve. The radial nerve innervates multiple proximal and distal extensor muscles in the forelimb, including the triceps, and all extrinsic wrist and digit extensors. Likewise, the ulnar nerve innervates multiple flexor muscles of the forelimb, including extrinsic wrist and digit flexors, and the intrinsic ventral footpad muscles.

Thus, although the existence of direct rubrospinal projections onto motor neurons has now been confirmed in the rat, cat, and monkey, in none of these species has the question of the specificity of rubrospinal inputs onto individual motor pools been addressed. The remarkable similarity of the rubrospinal projection to the ventral horn between species raises the possibility that the functional role of the direct circuit may be conserved throughout evolution. Thus, an investigation of the motor pool-specific organization of the circuit in rodents may provide insight into the supraspinal control of motor behavior in higher species.

Organization of forelimb motor pools in the cervical enlargement

The rostro-caudal distribution of limb-innervating motor neurons within the spinal cord mirrors the proximo-distal axis within the limb (Hollyday and Jacobson, 1990; Sterling and Kuypers, 1967). Thus, motor pools innervating limb "girdle" muscles (physically located outside the forelimb, but with an insertion onto the forelimb) and proximal limb muscles are found rostrally within the cervical enlargement, whereas motor pools corresponding to distal muscles are located at the caudal end. This longitudinal distribution suggests that rubrospinal projections into the ventral horn, seen only at C7-T1, are likely to contact muscles of the distal limb. This would fit well with the perceived role in manual grasping and dexterity. However, within each spinal segment, multiple pools are localized at different dorsoventral and mediolateral positions, so this information is not *per se* sufficient to identify the specific motor pools that are targeted.

In their original study of the cat brachial motor plexus, Sterling and Kuypers performed various nerve lesions affecting the innervation of flexor and extensor limb muscles, and came to the conclusion that extensor motor neurons of the forelimb are aligned together, but occupy a ventrolateral position compared to flexor motor neurons, thought to be the dorsal-most population within the ventral horn (Sterling and Kuypers, 1967). Fritz and colleagues expanded on this general organization by mapping out the distribution of motor neurons supplying individual distal forelimb nerve branches (Fritz et al., 1986a; Fritz et al., 1986b). The idea of columnar organization has been expanded by studies that map out the location of individual

motor pools, and detailed transverse maps of individual wing muscle motor pools have been compiled in the chick (Hollyday, 1980; Hollyday and Jacobson, 1990) and forelimb motor pools in the cat and monkey (Holstege, 1991; Jenny and Inukai, 1983) (figure 3.1a).

The distribution of individual forelimb motor pools in the rat has also been mapped out, and a number of motor pools have been found to be localized within the dorsolateral region of the ventral horn in the caudal cervical enlargement (McKenna et al., 2000) (Figure 3.1b). However, the level of resolution provided is insufficient to analyze the distribution of motor pools in relation to one another. Nonetheless, this motor pool map provides an excellent foundation with which to base further studies of individual forelimb motor pool organization.

The rubrospinal projection to the ventral horn demonstrated in chapter 2 suggests that direct motor neuron targets might be more restricted than previously thought. Rather than contacting *all* the intermediate and distal limb muscles, the rubrospinal tract might selectively innervate a restricted subset of motor neurons in the dorsolateral most region of the ventral horn at spinal levels C7-T1. To investigate this, I have first mapped out in detail the distribution of forelimb motor pools projecting to intermediate and distal forelimb muscles, and correlated these findings with a study of motor pool specificity within the direct rubro-motoneuronal circuit.

Results

Mapping forelimb motor pools involved in skilled reaching

I have demonstrated that the rubrospinal tract projects directly onto a restricted population of motor neurons in the dorsolateral ventral horn at cervical levels C7-C8. Rubrospinal terminations in the vicinity of motor neurons appear to be restricted to these spinal levels. To characterize the specificity within the direct rubrospinal circuit in rodents, I need to identify the muscle targets of motor nuclei receiving monosynaptic inputs.

The location and identity of motor neurons in lamina IX varies based on spinal level. Previous studies mapping the distribution of forelimb motor pools in the adult rat have identified a number of forelimb motor pools located at C7-C8 (McKenna et al., 2000). To generate a detailed positional map of forelimb motor pools in the cervical enlargement, I injected a fluorescent conjugated tracer, cholera toxin B subunit (CTB) into individual forelimb muscles of P30 mice. I focused on the motor pools projecting to the following wrist and digit forelimb muscles: extensor carpi radialis (ECR), extensor carpi ulnaris (ECU), flexor carpi radialis (FCR), flexor carpi ulnaris (FCU), extensor digitorum (including extensor digiti minimi) (ED), flexor digitorum (FD), and ventral footpad (intrinsic thenar and hypothenar muscles) (VF). I also included the triceps (TRI), a proximal limb muscle, in my analysis, as previous studies have indicated that the TRI motor pool is included with intermediate and distal forelimb motor pools at caudal cervical levels (McKenna et al., 2000; Vrieseling and Arber, 2006).

For each individual muscle, I mapped out the transverse position of its corresponding CTB-labeled motor pool by comparison with a ChAT antibody labeling all motor neurons present at a given cervical level (figure 3.2 a-f). Motor neurons corresponding to wrist muscles of the radial side (ECR, FCR) were positioned in the dorsolateral population of motor neurons. However, these were restricted to cervical levels C5-C6, and were therefore located too rostral to be contacted by the dense ventral rubrospinal termination zone at C7-C8 (figure 3.2 a, b). Likewise, the motor pool(s) innervating muscles of the ventral footpad were located dorsolaterally, but at levels too caudal (T1) to be contacted (figure 3.2 g). The TRI motor pool, while located at the appropriate spinal level (C7-C8), was positioned among the ventrolateral population of motor neurons (figure 3.2 f). Flexor muscles innervating the ulnar wrist and digits (FCU and flexor digitorum) comprised the dorso-medial population of motor neurons at C7-C8 (figure 3.2 d, e). The motor pool corresponding to the extrinsic digit extensor muscle, the extensor digitorum (ED), was also located in the dorsal population of motor neurons at this level, but occupied the lateral side (figure 3.2 c). For all muscles, retrograde labeling experiments were performed a minimum of three times to ensure reproducibility. In each case, restriction of fluorescence to individual muscles was confirmed by post-mortem dissection to ensure accuracy and specificity of the injection.

The GFP-labeled rubrospinal axons in C1qL2 reporter mice and AAV2::synapsin-GFP midbrain injections form direct inputs to dorsolateral motor neurons at C7-C8. Therefore, of the forelimb motor pools that I have mapped out,

only the extensor digitorum pool appears to be located in an appropriate position to receive direct rubrospinal input (figure 3.2 c). However, in order to support this hypothesis, and to improve on the resolution of the studies reported in the literature, it was necessary to visualize descending fibers and identified motor pools in the same sections of spinal cord.

Direct projections from the red nucleus are restricted to the extensor digitorum motor neurons

The extensor digitorum muscle is an extrinsic hand muscle that simultaneously extends and separates digits 2-5. Its most prominent role during a skilled reach to grasp movement appears to be for opening the digits in preparation for the arpeggio and subsequent object grasp (Whishaw et al., 1992). Given that lesions of the rubrospinal tract result in a significant impairment during arpeggio (Whishaw et al., 1998), I wanted to examine direct connectivity between the red nucleus and the extensor digitorum motor pool. Therefore, I retrogradely labeled the extensor digitorum motor pool with Alexa555 conjugated CTB in an adult mouse in which the descending rubrospinal tract had been labeled with an AAV2::GFP viral injection into the midbrain three weeks prior (figure 3.3 a, b). Under low magnification, CTB-labeled ED motor neuron cell bodies were found to be located precisely in the dense termination zone of the descending rubrospinal fibers (figure 3.3 c). Indeed, the ED motor pool occupies slightly different ventral coordinates at different positions along its rostro-caudal extent, and the ventral-projecting rubrospinal fibers alter their trajectory to match this positional shift (data not shown).

This provides further evidence that the apposition of the terminals and ED motor neurons is not simply coincidental and that the rubrospinal tract specifically targets this particular motor pool.

Although I have previously confirmed that a population of motor neurons in the dorsolateral ventral horn receive synaptic inputs from descending midbrain projections, there remains the possibility that direct inputs might be targeted to a neighboring motor pool located in close proximity to the ED motor neurons. There are a small number of forelimb muscles that are inaccessible for injection, as they are located in the deeper compartments of the forearm. These include the extrinsic muscles controlling thenar and hypothenar movements (McKenna et al., 2000). Likewise, direct contacts may instead be onto interneurons located in the vicinity of the motor pools. For example, Renshaw cells that give rise to recurrent projections onto motor neurons are located close to the motor nuclei (Alvarez et al., 2005). To address this, I used high power confocal imaging to visualize synaptic connections between rubrospinal axons and ED motor neurons. I found a significant number of GFP^+ $vglut2^+$ rubrospinal boutons directly apposed to CTB+ extensor digitorum motor neurons (Figure 3.3 panel d, e). Each labeled ED motor neuron received on average 1-2 rubrospinal synaptic inputs (168 boutons/100 ED motor neurons, N=3) (figure 3.5 g). Rubrospinal inputs were located on both the ED motor neuron cell body and proximal dendrites (figure 3.3 e). As CTB is accumulated in the soma and proximal dendrites, the possibility remains that direct inputs are also found on unlabeled distal dendrites.

Do all ED motor neurons receive direct rubrospinal input?

The extensor digitorum motor pool contains motor neurons that innervate two components of the ED muscle: the extensor digitorum proper, which controls digits 2-5, and the extensor digiti minimi, which specifically innervates digits 4 and 5. Thus, the question arises as to whether all motor neurons within the ED motor pool are under monosynaptic rubrospinal control. Of the 100 ED motor neurons that I analyzed, I found direct rubrospinal inputs onto 57%. This number is likely an underestimate, as there might be additional rubrospinal inputs onto unlabeled distal dendrites, and the viral injection into the midbrain might not result in the labeling of every rubro-motoneuronal cell. The percentage of ED motor neurons receiving direct innervation varied from 42% to 91% in three separate experiments.

If there are indeed ED motor neurons that lack direct rubrospinal input, it is possible that the rubrospinal tract preferentially innervates either the extensor digitorum or the extensor digitorum minimi. Unfortunately, I was unable to restrict my injections to just one component of the extensor digitorum, and therefore cannot address this question. However, I was able to address an alternate possibility: that the rubrospinal tract distinguishes between alpha and gamma motor neurons within the ED pool.

Both alpha and gamma motor neurons within the extensor digitorum pool receive direct rubrospinal input

Alpha motor neurons innervate the extrafusal muscle fibers and cause muscle contraction, whereas the smaller, specialized gamma motor neurons innervate intrafusal muscle fibers and regulate muscle spindle sensitivity (Hunt and Paintal, 1958). To assess whether gamma motor neurons might comprise the population of ED motor neurons that lack direct rubrospinal input, I took advantage of a recent study demonstrating that alpha and gamma motor neurons can be distinguished based on their differential expression of the nuclear marker NeuN (Shneider et al., 2009). Based on this criterion, I examined rubrospinal inputs onto alpha and gamma motor neurons, and found that both populations received direct rubrospinal input (figure 3.4 b-e), indicating that gamma motor neurons do not themselves account for the population of non-innervated ED motor neurons.

Other considerations make it possible that there are no ED motor neurons that lack direct rubrospinal input. Rubrospinal labeling through midbrain injections is inherently variable. Although in each experiment, the injection site is confirmed post-mortem to include the c1ql2+ red nucleus, the completeness of labeling is never 100% (data not shown). We are in the process of correlating the percentage of C1qL2 infected neurons in the red nucleus with the percentage of ED motor neurons that receive direct inputs. Complete labeling of the rubrospinal tract using a genetic approach may provide further insight.

The extensor digitorum motor pool receives the majority of direct rubrospinal input

The emergence of direct descending inputs onto motor neurons has been postulated to reflect an increase in the supraspinal control of distal muscles. The extensor digitorum muscle certainly fulfills these requirements as an extrinsic digit extensor. To assess whether other forelimb muscles that control digit movements receive a similar amount of direct rubrospinal input, I quantified the rubrospinal projection onto the flexor digitorum and ventral footpad motor pools (figure 3.5 b, c). The flexor digitorum, also an extrinsic hand muscle, is an antagonist of the ED; when activated, it results in the flexion of digits 2-5. The ventral footpad contains intrinsic hand muscles involved in flexion, abduction, and adduction of the digits. I found that the flexor digitorum motor pool received negligible input from the rubrospinal tract (1 bouton on 40 FD motor neurons, N=2) (figure 3.5 e), whereas the ventral footpad motor neurons received minimal input (8 boutons on 39 VF motor neurons, N=3) (figure 3.5 f). These results indicate that rather than controlling individual distal forelimb movements, the direct rubrospinal circuit in rodents is focused on the activation of simultaneous digit extension, demonstrating a previously unknown specialization of this particular motor control system.

As mentioned in the prior chapter, I have focused my analysis on direct projections from the red nucleus specifically onto motor neuron cell bodies and proximal dendrites, as the distal dendrites cannot be visualized to the same extent by the CTB tracer. Therefore, it remains a possibility that there are monosynaptic contacts between rubrospinal neurons and flexor digitorum motor neurons that are restricted to the more distal regions of the dendritic arbor. A more rigorous

evaluation of rubrospinal projections onto distal dendrites of both the extensor digitorum motor pool and other forelimb motor pools remains to be undertaken.

Although the tendons of the extensor digitorum insert onto the proximal and distal phalanges, and its primary function is to extend and separate the digits, this muscle is also considered a secondary wrist extensor. It is possible that the rubrospinal tract preferentially contacts intermediate and distal forelimb extensors. However, as I have already demonstrated, the radial wrist extensor, ECR, is located at spinal levels C5-C6, rostral to the ventral rubrospinal projection (Figure 3.2 b). Nonetheless, a quantification of rubrospinal synaptic inputs onto this population is in progress. Specific backfills of the ulnar wrist extensor, the ECU, have proved difficult and so the location of this pool is still undetermined. The ECR and FCR are both located at C5-C6, whereas the FCU is further caudal, at C7-C8. Therefore, I would predict that the ECU is also located at C7-C8, and would be the more probable recipient of direct rubrospinal input. Rubrospinal innervation of the ECR and ECU needs to be investigated in further detail.

Specificity of afferent inputs onto extensor vs. flexor digitorum motor neurons

The precise matching of pre-motor inputs onto their appropriate motor neuron target is essential for the regulated flow of information through the motor system. Such specificity is excellently demonstrated in the monosynaptic sensory-motor circuit, where Ia proprioceptors must contact their homonymous motor pools. However, the restriction of projections onto a single or extremely limited number of

motor pools – such as that demonstrated here for the rubrospinal pathway - is rarely observed. The presence and absence of rubrospinal inputs onto the extensor and flexor digitorum, respectively, is particularly striking, given that these two muscles comprise a muscle pair that perform antagonistic movements around the same joint. To determine whether the rubrospinal distinction between these two pools was unique, I compared other pre-motor inputs onto ED and FD motor neurons.

Both the ED and FD motor pools lack direct corticospinal input

To investigate whether there is a similar specificity in projections from the motor cortex onto spinal motor pools, I used a genetic approach to label the descending corticospinal tract (Bareyre et al., 2005). A cortex specific cre driver (Emx1::Cre) crossed to a strong conditional reporter line (Rosa::ϕtdtomato) will label the corticospinal tract in its entirety, including the minor components that give rise to corticomotoneuronal connections (Bareyre et al., 2005). I injected AAV2::synapsin-GFP into the ventral midbrain of these mice, thus allowing a direct comparison of the descending projections from the two significant motor regions involved in the control of voluntary skilled movement. Both the corticospinal and rubrospinal projections appear to converge within the intermediate laminae of the spinal cord, presumably on interneurons mediating pre-motor spinal circuits (Figure 3.6 a, b). However, unlike the dense rubrospinal projection to the dorsolateral motor neurons, corticospinal inputs appear to be excluded from the ventral horn (Figure 3.6 b).

Motor neurons projecting to intermediate and distal forelimb muscles are located across the rostro-caudal range from C5-T1. To rule out the possibility that the corticospinal tract might specifically target other forelimb motor pools, I examined CST projections to lamina IX between C5 andT1, and determined that the corticospinal tract does not send a dense projection to any motor pools within the cervical enlargement (Figure 3.6 c-e). It is possible that the corticospinal tract may project diffusely onto motor neurons, and a more rigorous analysis is necessary to properly quantify the extent of the cortico-motoneuronal projection. Indeed, other groups have reported that direct cortical projections onto cervical motor neurons are present, but lack the motor pool specificity of the rubrospinal tract (John Martin, Silvia Arber, personal communication). My data confirm that there is not a dense CST termination zone onto forelimb motor pools that parallels the rubrospinal projection observed onto ED motor neurons.

The ED and FD motor pools both receive monosynaptic Ia sensory inputs

Group Ia proprioceptive sensory neurons convey information about muscle position back to homonymous and synergistic motor neurons through either mono- or poly-synaptic pathways. Although certain motor pools, such as that of the cutaneous maximus muscle, lack monosynaptic Ia input (Vrieseling and Arber, 2006), the majority of motor neurons receive monosynaptic sensory input, forming the basis of the spinal stretch reflex. Nonetheless, the presence of monosynaptic sensory inputs has never been specifically addressed in extensor or flexor digitorum motor neurons.

Group Ia proprioceptors are responsible for almost all vglut1$^+$ terminals onto motor neurons, with descending CST inputs thought to add an additional small percentage (J. Nicholas Betley, Julia Kaltschmidt). As expected, given the absence of *vglut1* in the red nucleus, GFP$^+$ and vglut1$^+$ boutons comprised non-overlapping populations of synaptic inputs onto ED motor neuron cell bodies and proximal dendrites (figure 3.7 b, c). Synaptic imaging of CTB-labeled ED and FD motor neurons revealed that both motor pools received comparable numbers of vglut1$^+$ proprioceptive inputs (figure 3.7 b-e, N=2).

Cholinergic modulatory input

Motor neurons also receive modulatory input from cholinergic spinal interneurons (Zagoraiou et al., 2009). These so-called "c-bouton" inputs are responsible for modulating motor neuron excitability. In the lumbar spinal cord, c-bouton input onto hindlimb motor pools decreases along the proximo-distal limb axis. It is unknown whether c-bouton input onto forelimb motor neurons follows a similar rule. To investigate whether cholinergic input differs between ED and FD motor pools, which innervate muscles with the same proximo-distal address in the forelimb, I examined the number of c-bouton inputs on identified motor neurons. Genetic labeling of cholinergic neurons using a ChAT:: Cre driver labels both motor neurons and the cholinergic interneuron population that gives rise to C-bouton inputs. C-boutons on motor neurons are easily recognizable by their unique morphology. My results indicate that extensor and flexor digitorum motor pools receive a qualitatively comparable level of c-bouton input (figure 3.8, N=1). Therefore, I can conclude that

the motor pool specificity exhibited by the rubrospinal tract is not replicated by any other modulatory input that I have investigated.

Figure 3.1

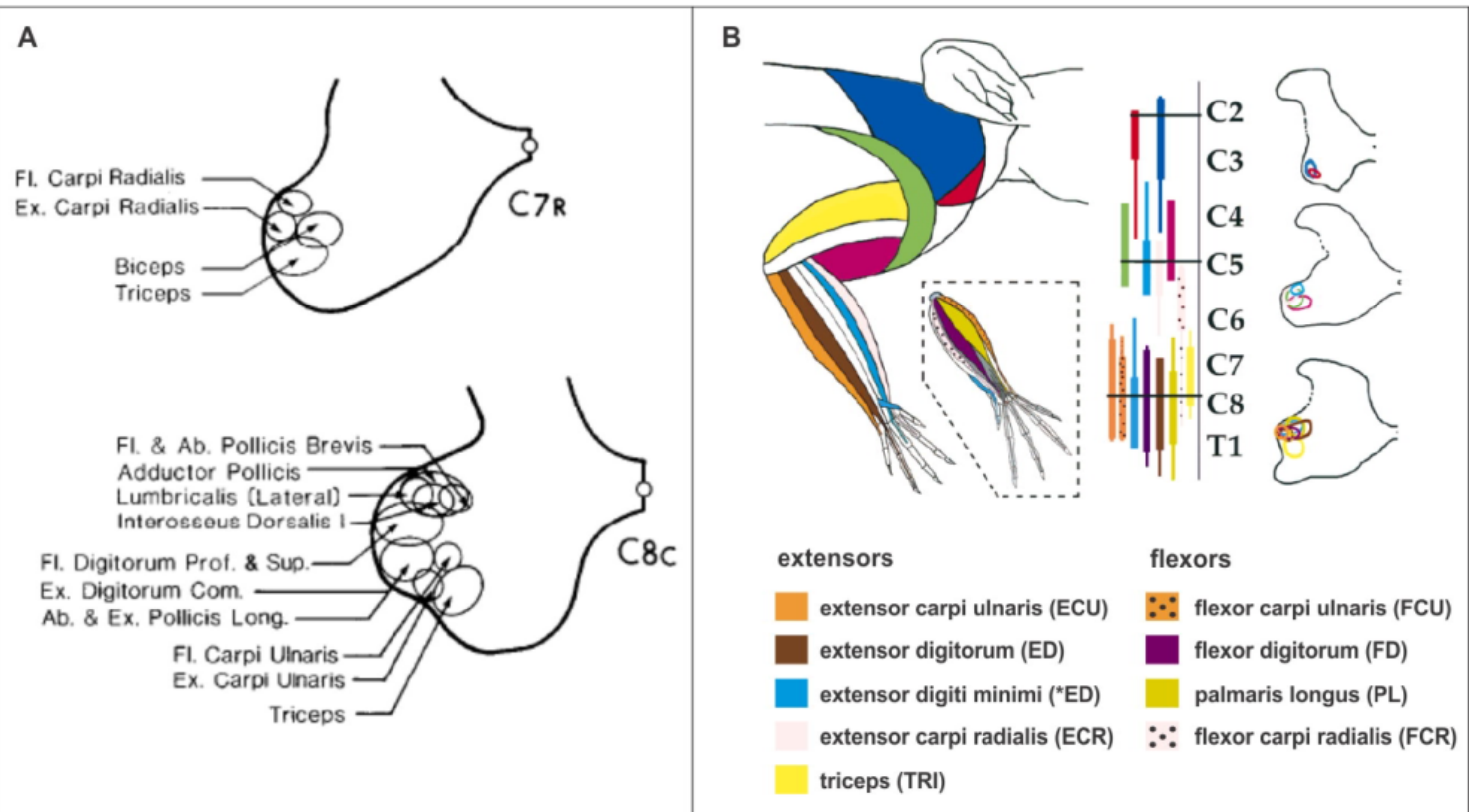


Figure 3.1 Organization of motor pools projecting to forelimb muscles The distribution of motor neurons projecting to individual muscles of the forelimb has been mapped out in the monkey (A) and rat (B). Individual muscle backfills have retrogradely identified the cohort of motor neurons that project to that muscle. In the monkey, motor pools innervating the intrinsic hand muscles are located in the dorsal-most position in the ventral horn. The extrinsic flexor muscles are generally located dorsal and medial to their extensor counterparts (A). The pool distribution has also been mapped out in the adult rat, however, the spatial distribution of motor pools in a transverse plane has not been as well characterized (B).

From Jenny et al., 1983 (A) and McKenna et al., 2000 (B)

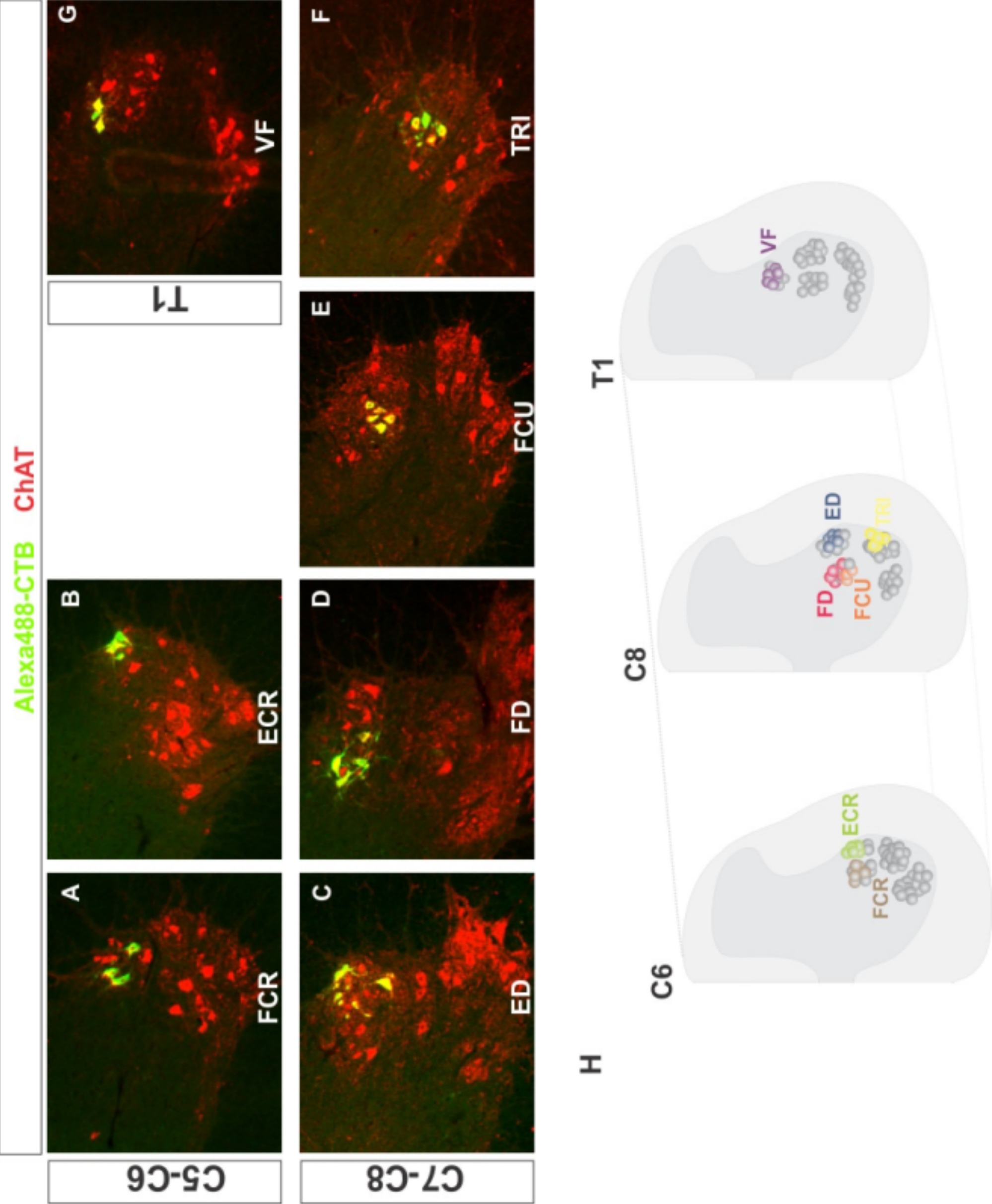
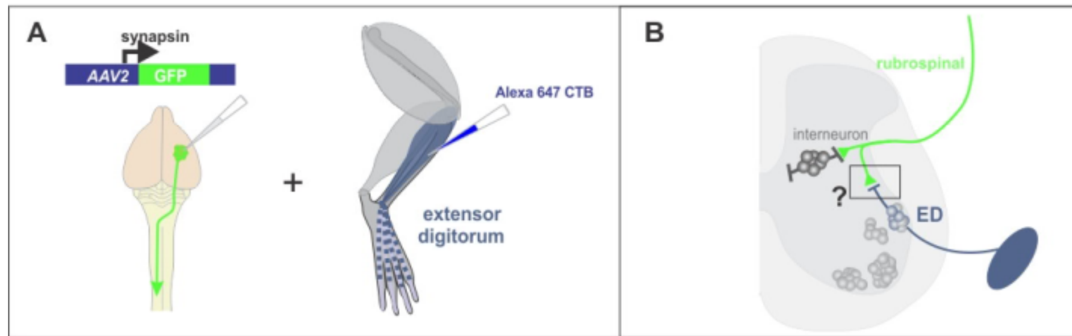
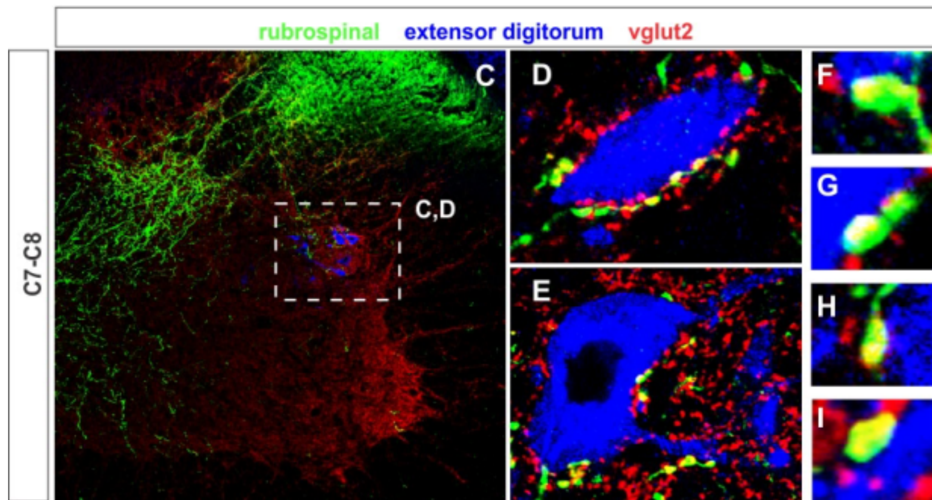


Figure 3.2

Figure 3.2 Motor pool distribution of forelimb muscles in the adult mouse To determine the spatial relationship of forelimb motor pools within the cervical enlargement of the adult mouse, fluorescent protein conjugated cholera toxin B subunit (CTB) was injected intramuscularly into identified distal forelimb muscles. After three days, the animal was sacrificed, and the pattern of labeled motor neurons determined throughout the cervical enlargement. The location of all motor neurons at a given spinal level was determined by anti-ChAT immunostaining. Although the majority of distal muscle motor pools were located at C7-C8, motor neurons controlling radial wrist movements (ECR, FCR) were located at C5-C6. Flexor motor neurons were consistently found medial to extensor motor neurons, however, a dorsal flexor bias was not observed (C, D). Intrinsic footpad muscles were located in the caudal end of the cervical enlargement, and were further dorsal than any other motor pools (G). A schematic of motor pool distribution at rostro-caudal levels of the cervical enlargement is illustrated in H.

Figure 3.3





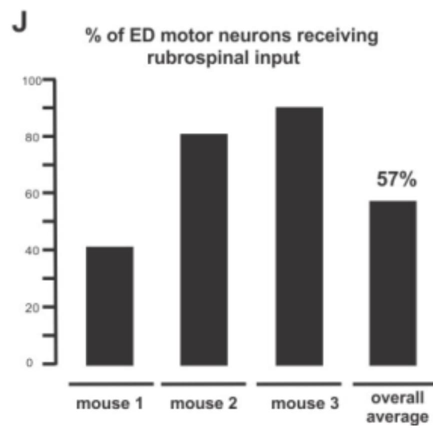


Figure 3.3 The extensor digitorum motor pool receives direct inputs from the red nucleus
The location of the ED motor pool in the dorsolateral ventral horn at C8 suggests that it might be the target of ventral rubrospinal fibers (B). Descending fibers were labeled by a viral injection of AAV2::GFP into the midbrain as described previously. Three days prior to sacrifice, the extensor digitorum muscle was injected with a retrograde tracer, Alexa657-CTB. Rubrospinal fibers at C7-C8 appeared to terminate in the vicinity of the labeled ED motor pool (C). 63X confocal imaging demonstrated that GFP+ terminals made direct appositions onto ED motor neuron cell bodies and proximal dendrites (D,E). These boutons co-expressed the rubrospinal synaptic terminal marker, vglut2 (F-I). The percentage of ED motor neurons receiving at least one synaptic input from the rubrospinal tract varies by experiment, with an overall average of 57% (J).

Figure 3.4

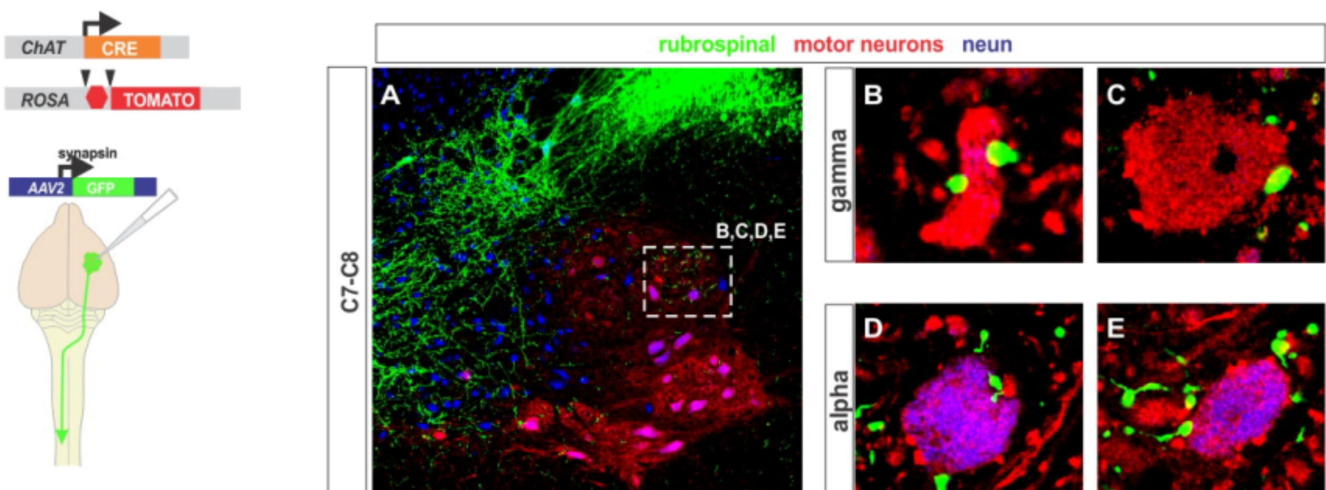


Figure 3.4 Alpha and gamma ED motor neurons receive rubrospinal inputs To determine whether the rubrospinal inputs are restricted to a class of motor neuron, alpha and gamma motor neurons in the dorsolateral ventral horn at C8 were identified by their differential expression of the nuclear marker, NeuN. In this experiment, all motor neurons were labeled in a Chat::Cre; Rosa::ΦTomato mouse, and descending rubrospinal fibers were labeled by an AAV2::GFP injection into the ventral midbrain (A). GFP+ rubrospinal terminals were observed on both NeuN- gamma motor neurons (B,C) and NeuN+ alpha motor neurons (D,E).

Figure 3.5

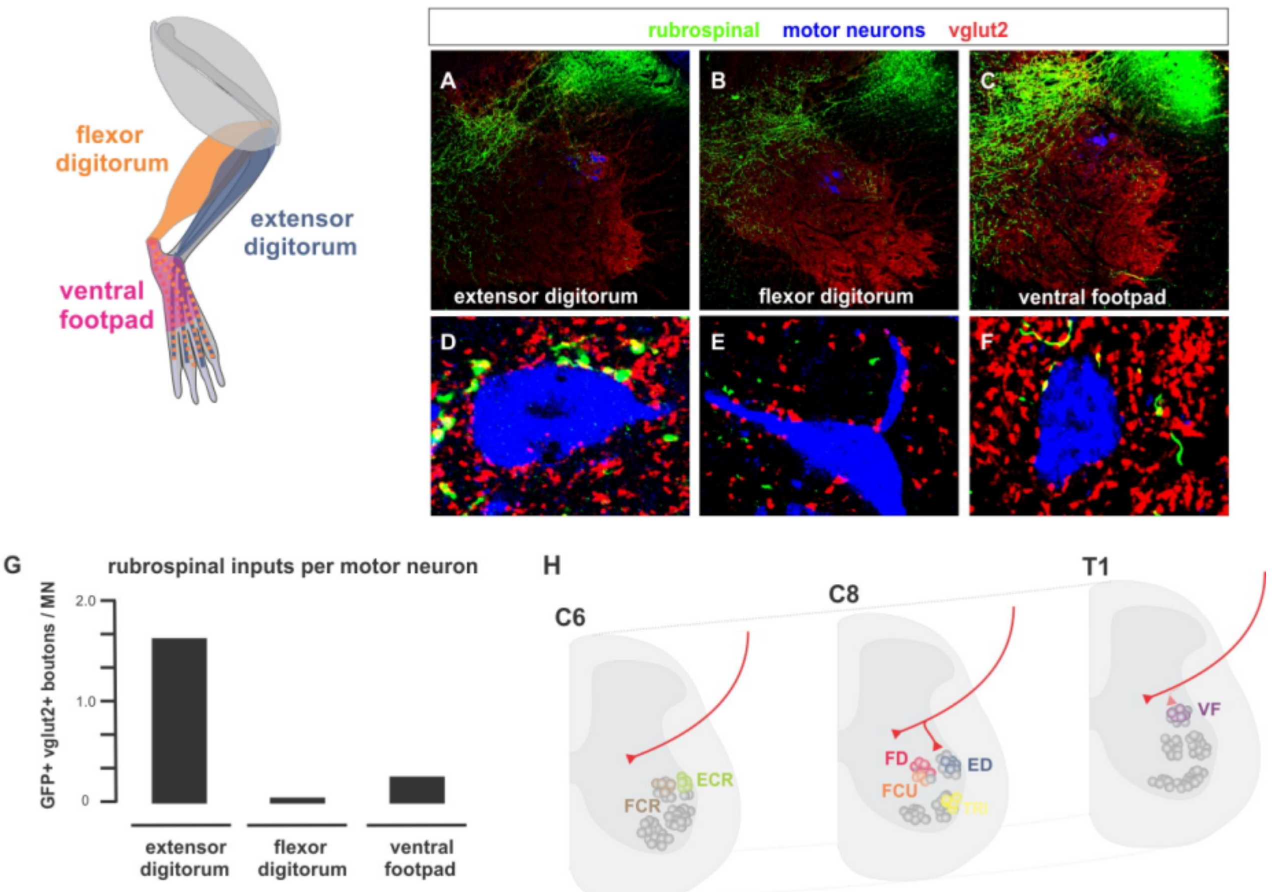
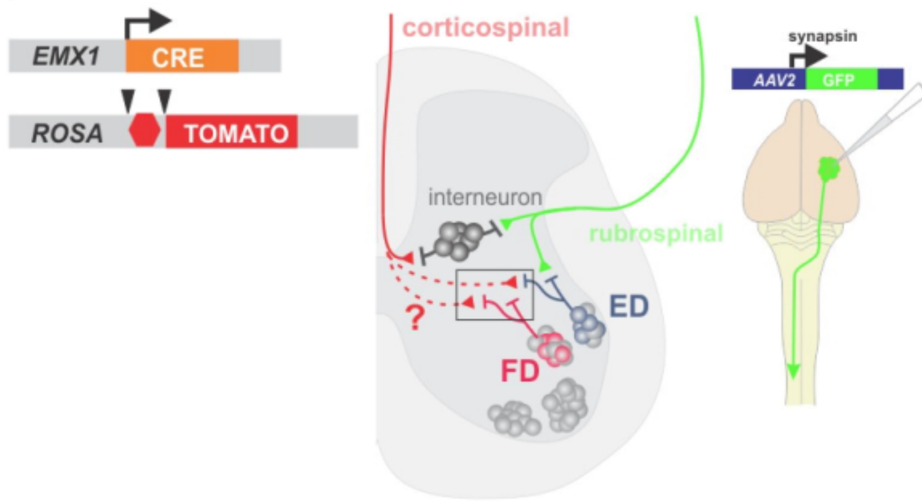
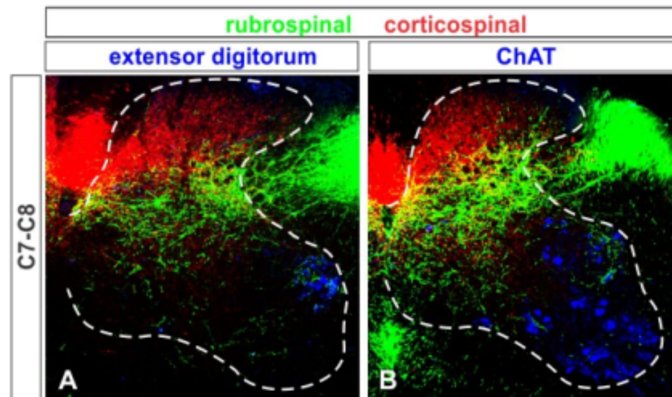


Figure 3.5 Extrinsic digit flexor motor neurons do not receive direct rubrospinal input The extent of rubrospinal inputs onto forelimb digit muscle motor neurons was investigated by retrogradely labeling flexor digitorum and ventral footpad motor neurons in mice in which the rubrospinal tract was labeled. Low magnification images indicate that the flexor digitorum motor pool is located medial to the ventral rubrospinal termination zone (B) whereas the ventral footpad motor pool is dorsomedial (C). High magnification imaging reveals that flexor digitorum motor neurons (E) are devoid of rubrospinal input (1 bouton on 30 MNs, N=2 mice) whereas ventral footpad motor neurons (F) receive a small amount of rubrospinal input (8 boutons on 39 MNs, N=3 mice), but significantly less than ED motor neurons (168 boutons / 100 MNs, N=3 mice) (G). The relative distribution of rubrospinal inputs onto forelimb motor pools is illustrated in H.

Figure 3.6





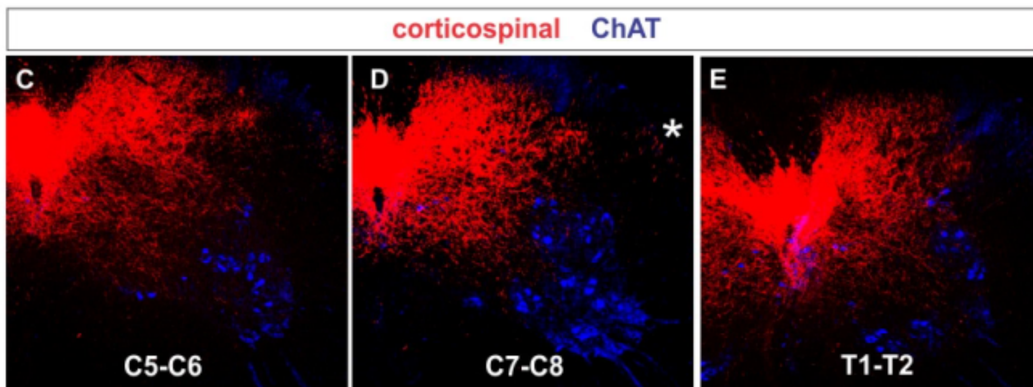


Figure 3.6 The corticospinal tract does not appear to target a specific population of motor neurons To determine if the corticospinal tract projects directly onto a restricted population of motor neurons in the rodent, descending corticospinal projections into the ventral horn were investigated. The corticospinal tract was genetically labeled by inducing reporter expression in corticospinal neurons of a conditional reporter line (rosa::tomato) using a cortex-restricted cre driver (emx1::cre). Midbrain injection of AAV2::GFP enabled a direct comparison with rubrospinal projections in the same animal (A,B). Although rubrospinal fibers are clearly observed amongst the ED motor neurons, corticospinal fibers do not appear to invade the ventral horn (B). A rostro-caudal examination of corticospinal terminations throughout the cervical enlargement demonstrates that corticospinal fibers do not appear to target a specific pool of motor neurons (C-E), although this remains to be investigated in further detail. The asterisk in D indicates labeled corticospinal axons in the dorsolateral funiculus, demonstrating that the minor corticospinal tracts, thought to be the origin of direct projections onto lumbar motor neurons in the rodent, are labeled in this preparation.

Figure 3.7

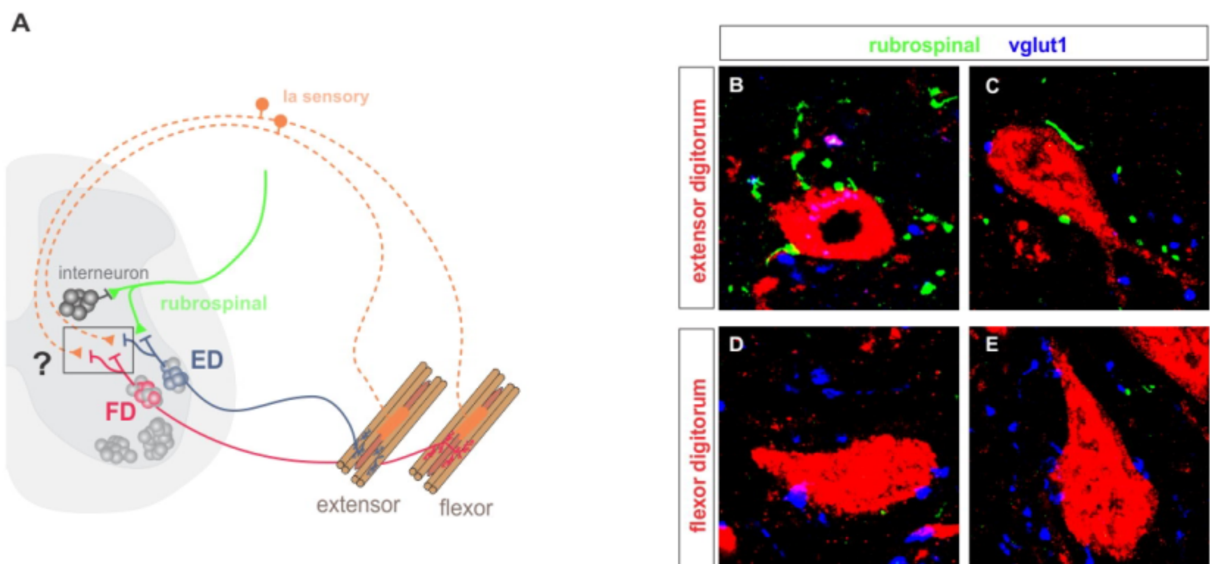


Figure 3.7 Extensor and flexor digitorum motor neurons both receive monosynaptic sensory input To determine whether other motor neuron modulatory circuits preferentially target a specific pool of motor neurons, the existence of monosynaptic sensory feedback onto ED and FD motor neurons was examined. Sensory terminals express the synaptic terminal marker vglut1. Both ED and FD motor neurons, identified through intramuscular injections, receive a large number of vglut1+ inputs (B-E). The ED motor neurons also receive GFP+ rubrospinal inputs that are separate from the vglut1+ inputs (B,C).

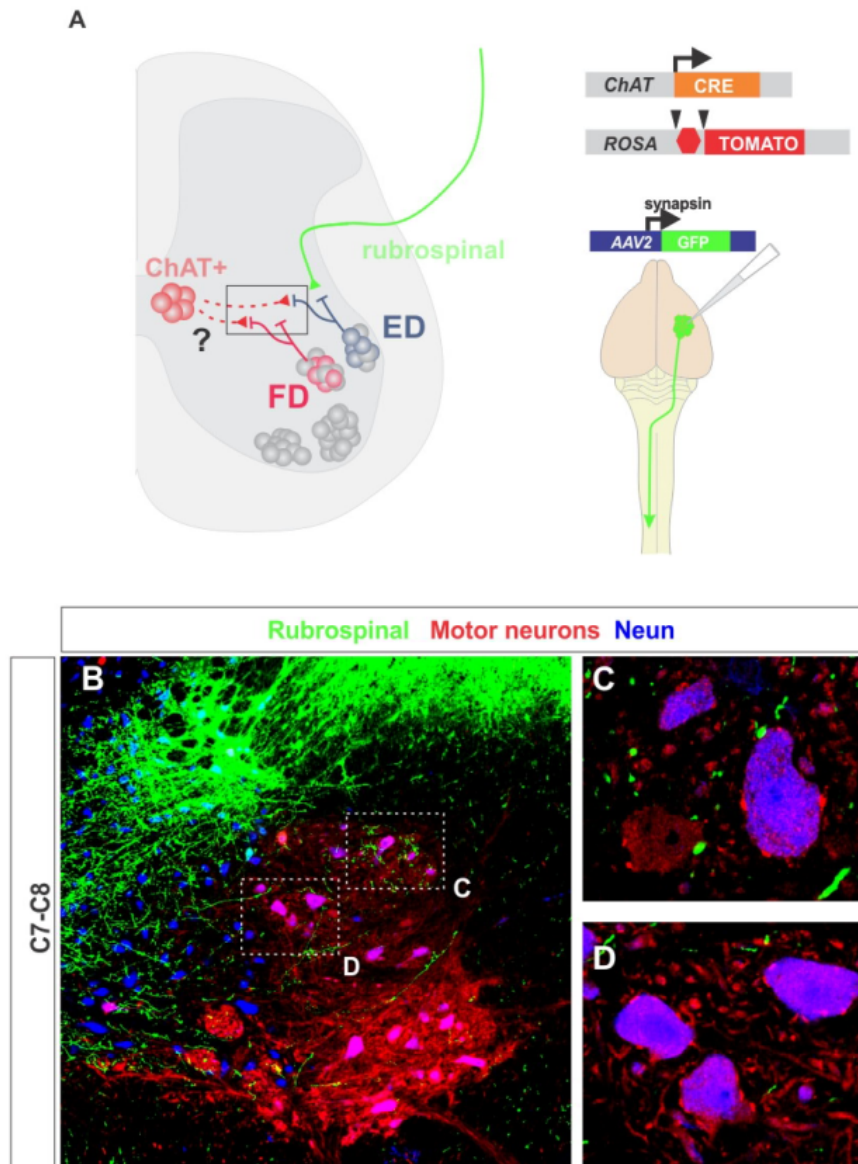


Figure 3.8 Extensor and flexor digitorum motor neurons both receive cholinergic modulatory input Cholinergic inputs onto motor neurons can be identified using a ChAT::Cre driver to label all cholinergic spinal neurons, including the interneuron population that gives rise to C-bouton inputs onto motor neurons. Cholinergic inputs are clearly distinguishable on the motor neuron cell body by their characteristic size, despite both expressing the same fluorescent reporter protein (C,D). Both dorsomedial (flexor) and dorsolateral (extensor) motor neurons receive similar levels of cholinergic input (C,D). The motor pool identity of these motor neurons has not been determined, although the spinal level, location within the ventral horn, and density of rubrospinal inputs suggests that the motor neurons in C belong to the ED motor pool, whereas those in D belong to distal forelimb flexor motor pools.

Discussion

Previous anatomical tracing studies in the rodent and cat have suggested that the rubrospinal tract projects onto intermediate and distal forelimb muscle MNs . In this chapter, I combine anterograde rubrospinal tracing and retrograde muscle backfills to characterize motor neuron targets of the rubrospinal tract, using confocal imaging of synaptic proteins to definitively identify synaptic contacts. With this technique, I have demonstrated an unprecedented level of motor pool specificity in the monosynaptic circuit of a major descending motor pathway.

Comparison with previous rubrospinal tracing studies in the rat

The Schwab lab provided the most detailed study of motor pool innervation by descending rubrospinal fibers in the rodent to date (Kuchler et al., 2002). In this study, anterograde tracing of the rubrospinal tract is combined with muscle backfills to determine the muscle targets of direct rubrospinal input. However, the authors did not inject individual muscles; instead, they grouped the forelimb into three segments: proximal (biceps, triceps), intermediate (forearm including extrinsic digit muscles), and distal (intrinsic hand). They counted, on average, 250 rubrospinal contacts total on intermediate motor neurons (370 labeled; 0.67 boutons/MN average), and 25 contacts on distal motor neurons (139 labeled; 0.18 boutons/MN), per animal. The relative distribution of rubrospinal terminations on intermediate and distal motor neurons correlates well with my findings. Extensor digitorum motor neurons receive, on average, 1.68 inputs/MN, whereas the ventral footpad (comprising the entirety of the 'distal' muscle group considered by Kuchler et al.) receives 0.2 inputs/MN. The

'intermediate' muscle group of Kuchler et al. includes ED motor neurons, and other forelimb motor pools that do not receive rubrospinal input, likely explaining the lower average input/MN.

The quanitification of rubro-motoneuronal connections from the Kuchler et al. study includes both axo-somatic and axo-dendritic projections onto motor neurons although it is unclear whether the entirety of the motor neuron dendritic arbor was visualized. Although I have confirmed that rubro-motoneuronal connections are present on both the somata and proximal dendrites, the full extent of the dendritic distribution of rubro-motoneuronal synapses remains unknown. Furthermore, I cannot definitively rule out the possibility of rubrospinal projections being present on distal dendrites of other motor neuron pools, such as the FD motor neurons, as I have focused my analysis on the cell bodies and proximal dendrites. A more detailed analysis of projections onto forelimb motor neuron pools needs to be undertaken, using a labeling method that will strongly illuminate the entire dendritic arbor. Viral labeling techniques of motor neurons via intramuscular injections have been developed, and will provide an efficient way to accomplish this (Towne et al., 2011). Nonetheless, it remains evident that the dense focus of rubromotoneuronal synapses in the mouse are uniquely distributed on ED motor neurons.

Do direct projections onto motor neurons originate from axon collaterals?

A key question that emerges from our results is whether the rubrospinal axons that are observed projecting into the ventral horn and forming direct connections onto

motor neurons represent collaterals from RN neurons projecting onto interneurons, or whether they in fact represent a unique population of rubrospinal neurons that singularly specialize in the direct activation of motor neurons. Delineation between these two possibilities would provide further insight into the functional significance of the rubrospinal tract, and the underlying mechanisms by which it achieves these goals. Additional experiments focusing on the connectivity of individual rubrospinal neurons (either anatomically or physiologically) will be necessary to differentiate between these two possibilities.

Motor pool specificity of the rubrospinal tract in other species

Given what we now know about the specificity of rubrospinal projections in the rodent, is it possible that other species demonstrate a similar level of specificity? Anterograde tracing of the rubrospinal tract has been reported in both cats and monkeys. Unfortunately, as with previous rubrospinal tracing in the rodent, a precise quantification of rubrospinal projections onto defined motor neuron pools was not undertaken. Nonetheless, the rubrospinal projection is qualitatively similar between rodents and cats, with the majority of projections onto interneurons and an additional projection into the dorsolateral region of the ventral horn, at spinal levels C8-T1 (Holstege, 1987).

In fact, an anatomical study of the cat rubrospinal projection onto motor neurons (McCurdy et al., 1987) suggests that rubrospinal projections into the ventral horn specifically target motor neurons responsible for distal digit movements

(McCurdy et al., 1987). The authors correlate the location of rubrospinal fibers on one side of the spinal cord with retrogradely labeled motor pools on the contralateral side, and find a strong similarity between the rubrospinal termination zone and the distribution of motor neurons innervating the extensor digitorum communis (EDC) and extensor digitorum longus (EDL). However, they also postulate that the area of overlap includes that of the palmaris longus (PL) and other distal flexor motor neurons. My results indicate that distal flexor motor neurons are located in a dorsomedial population, and do not receive inputs from the rubrospinal tract, at least in mouse, with the caveats that were mentioned previously. Detailed visualization of rubrospinal inputs onto specific motor pools in the cat would be necessary to resolve this issue. Likewise, in the monkey, rubrospinal projections into the dorsolateral aspect of the ventral horn are present in the cervical enlargement (Holstege et al., 1988; Ralston et al., 1988). Precise anatomical tracing is required to determine which pools receive direct rubrospinal input. Nonetheless, the most parsimonious conclusion would be that the rubrospinal specificity demonstrated in the rodent may hold true across species. Perhaps understanding the contribution and relevance of this muscle during a skilled movement could shed some light onto why the motor system has evolved to include such specialized supraspinal control of this particular muscle.

Extensor digitorum activity is maximal prior to object grasp

In the rodent, as in humans, the extensor digitorum muscle is an extrinsic forelimb digit muscle, originating from the humerus and attaching to the phalangeal joints of the medial and distal digits. It is responsible for concurrent extension and

separation of digits 2-5. Still frame analysis of forelimb movement during the execution of a skilled reach-to-grasp task indicates that just prior to the object grasp, the forelimb pauses directly above the object and the rat appears to extend and separate all of its digits just prior to lowering its paw onto the object during the stereotyped arpeggio movement (figure 1.6b). In fact, detailed measurements of digit flexion/extension and separation during the entirety of the reach-to-grasp task indicate that the digits only become fully extended and separated in the very last stages of the forelimb advance and continue in this position through the pronation of the arpeggio movement (Figure 3.9 a, c). What is the purpose of extending and separating the digits prior to subsequent digit manipulation and object grasping? As discussed previously, the temporal pattern of digit positioning during a skilled movement is highly conserved between rodents and humans, despite the obvious disparity in fine movement capacity (figure 3.9 b, d).

When the hand is in its normal resting position, the digits are in a semi-flexed position, suggesting that the resting properties of the flexor muscles outweigh the extensor muscles. One can imagine that from this initial resting position, the extent and range of digit movement would be limited and even small movements might require a greater degree of exertion. Thus, the role of the digit extensor muscles could be to provide the tonic support necessary for subsequent fractionated digit movements required during skilled movements.

The red nucleus provides the 'framework' support necessary for individual digit movement

Whishaw and Gorny have previously suggested that the role of the red nucleus is to provide the support framework necessary for individual movements, which they refer to as the 'framework' hypothesis (Whishaw and Gorny, 1996). They postulate that the rubrospinal tract might be responsible for the initial 'whole hand' movements that provide the substrate against which further individual digit movements can then be layered.

One of the unparalleled aspects of studying the monkey system is that it affords the opportunity to record neuronal activity in vivo during various behavioral tasks. Thus, it is possible to monitor RN neuronal activity over the course of a skilled movement, and correlate it with muscle activity. These experiments have focused on the activity of RN neurons during motor tasks involving proximal vs. distal forelimb muscle activity, and have also included a detailed examination of RN discharge during 'whole hand' vs. fractionated digit movements in the context of hand preshaping.

RN activity in the monkey correlates with metacarpi-phalangeal (MCP) extension

In an interesting set of experiments measuring RN activity during a reaching task, RN forelimb neuron discharge was significantly higher during all aspects of the movement when the task specifically included a hand component(van Kan and McCurdy, 2001). This study indicates that although the RN likely modulates activity

Figure 3.9

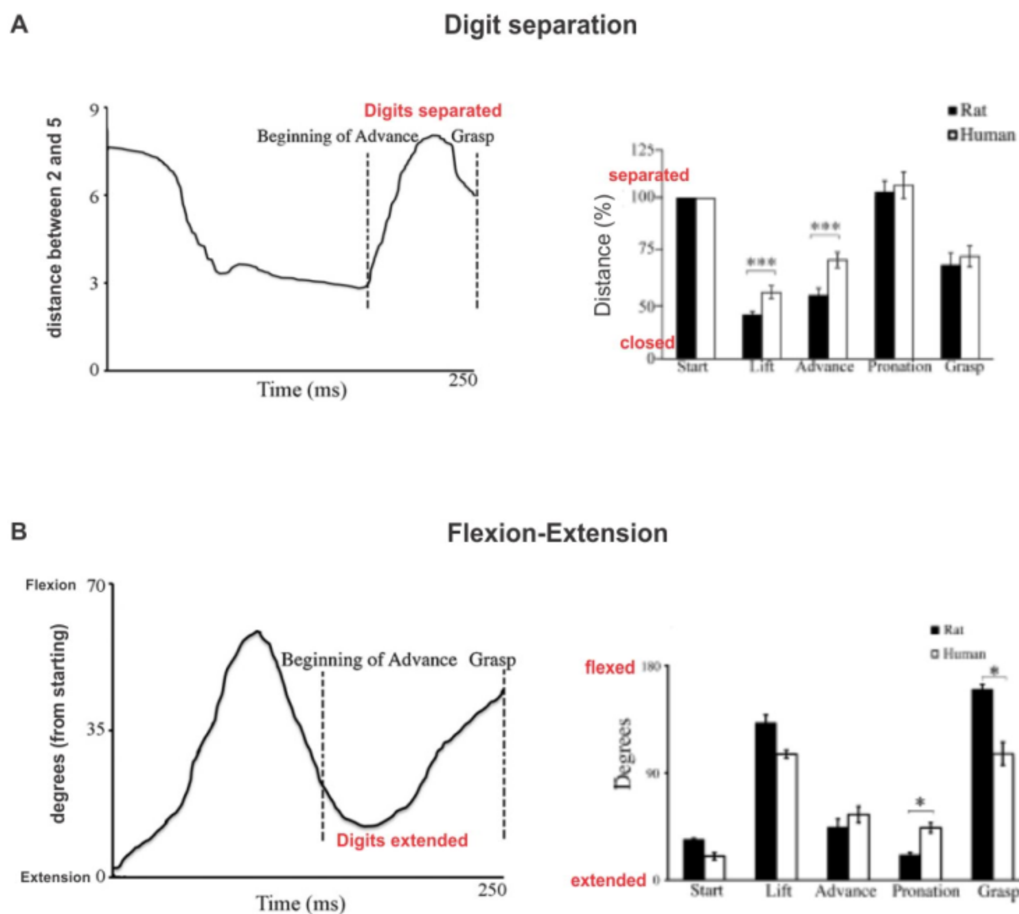


Figure 3.9 In a rat skiilled reach-to-grasp movement, the digits are extended and separated during arpeggio Digit separation (A, B) and extension (C, D) has been plotted over the course of a skilled movement. Digit separation is measured as the distance between digits 2 and 5. Although the hand is closed during lift and the beginning of the advance stage, just prior to object grasp, the digits become fully separated (A). A comparison between rats and humans indicates that the position of the digits is conserved during a skilled movement (B). Digit extension is measured as the change in angle between the digit tips and the wrist, with the paw starting in a fully extended position (0 degree angle). The paw is initially advanced towards the object in a flexed position and just prior to the grasp phase, indicating that the digits are fully extended prior to and during the arpeggio (pronation) movement. Once again, a comparison between rats and humans confirms that digit flexion/extension during a skilled movement is similar at each stage of the movement.

From Sacrey et al., 2009

in both proximal and distal muscle MNs, either indirectly or directly, its predominant role is in the execution of reach-to-grasp tasks, which is consistent with its role in the rodent. Furthermore, if RN activity is correlated with movement at various proximo-distal joints in the arm (shoulder, elbow, wrist, and metacarpi-phalangeal MCP), increased bursts of RN activity precede extension at the MCP joint, and correlated with duration, velocity, and amplitude of the movement. RN activity did not correlate with activity at the more proximal joints (van Kan and McCurdy, 2001). Although extension at the MCP joint in monkeys could reflect activity in either intrinsic or extrinsic digit extensor muscles, this study supports a conserved preferential role for the RN in extending the digits.

In fact, given the emphatic predominance of RN discharge during distal limb movements, a follow up study was conducted to test the hypothesis that the role of the RN was to specify the timing and quality of MCP extension during hand preshaping rather than in the execution of the actual grasp (Van Kan and McCurdy, 2002). RN activity was recorded during two reach-to-grasp tasks. The first task required independent use of the thumb and forefinger, whereas the second task utilized a 'whole hand' grasp with simultaneous finger movement. Single unit recordings of RN neurons indicated that the majority of neurons were active during both tasks, regardless of the digit manipulation required for object grasp. Furthermore, temporal analysis indicated that RN activity was most closely phase-locked to extension at the MCP joint. Finally, if the monkey is trained to complete a task requiring a thumb

movement, the red nucleus is inactive if the rest of the hand is already provided with an inanimate source of support (Gibson et al., 1985a).

Thus, it appears that in the monkey, commands from the red nucleus are tightly correlated with the digit extension that occurs just prior to object manipulation. Although this is referred to as hand "preshaping" in the monkey, one can imagine that it plays a similar role to the digit extension seen in rodents just prior to the arpeggio movement. Furthermore, the red nucleus remains active while the digits are extended, regardless of the individual or combined digit flexion that is layered onto this baseline hand position, providing support for the 'framework' hypothesis of RN involvement across species.

Red nucleus lesion studies suggest a specific role in digit extension and separation

The idea that direct descending projections onto motor neurons reflect a mechanism for fine control of individual distal forelimb muscles has been strengthened by lesion studies of the major supraspinal pathways. In rodents, the deficits seen after lesioning the descending pathways are not prohibitively severe; the rat is still able to complete a skilled task, but components of the movement are significantly affected. For example, after a pyramidal tract lesion, the guidance of the limb to the target is altered (Whishaw et al., 1998). In the case of a rubrospinal lesion, there is a mild impairment of limb guidance, but the major deficit observed is during the arpeggio movement (Whishaw et al., 1998; Whishaw et al., 1990). If the arpeggio movement is scored on 3 point scale, with 2 points for present, 1 point for

recognizable but flawed, and 0 points for absent, a significant deficit is seen in the contralateral limb following a specific lesion of the red nucleus (Figure 3.9). In comparison, arpeggio is fully maintained in mice with pyramidal tract lesions. Given the established connection between the execution of arpeggio and the ability to extend and separate the digits, it follows that loss of rubrospinal inputs to the extensor digitorum motor pool might be responsible for the loss of arpeggio seen following a rubrospinal lesion.

To further define the defect in distal limb movement responsible for the lack of arpeggio following a supraspinal lesion, still-frame video images were used to capture hand position before, during, and after the object grasp (Whishaw et al., 1998). There was no discernable difference between wild type and CST-lesioned animals. In the RN lesion group, however, the digits are never fully extended prior to object grasp. In fact, rather than lowering the fully extended paw and grasping the object before then retrieving its hand, the rat simply lowers the semi-flexed paw and grasps the object during the limb retrieval phase (Figure 3.9). I have established that the extensor digitorum motor pool receives a preferential direct input from the red nucleus, and this appears to be directly reflected in the functional loss of digit extension following a RN lesion.

The striking effect on digit extension seen following the removal of red nucleus inputs to the spinal cord certainly raises the question as to the significance of direct supraspinal inputs onto motor neurons. The vast majority of rubrospinal axons

feed into interneuron circuits within the spinal cord, where they are integrated with other incoming motor and sensory commands. Given the established widespread connections from these previously identified interneurons (Jankowska, 1988), one can imagine that the removal of descending input would indirectly affect the activity of multiple populations of motor neurons. However, although there is a mild impairment of other aspects of limb movement, the most severe impairment appears to involve a muscle whose activity is directly modulated by the red nucleus.

At this point, further experiments are required to definitively ascertain that the digit extension phenotype is a result of the removal of direct rubrospinal inputs to ED motor neurons, and will be discussed in greater detail in the general discussion chapter. However, given the highly selective loss of function that can be specifically correlated with ED function, and the demonstrated direct circuit from the red nucleus to the ED motor pool, it remains a strong possibility.

Rubrospinal projections onto other distal forelimb extensor muscles

Although the rubrospinal tract sends a dense projection to the extensor digitorum motor pool, I have not ruled out the possibility that it also projects to synergistic forelimb extensor muscles. The extrinsic thumb muscles, including the extensor pollicis longus and brevis, and the abductor pollicis longus, are located beneath the other forelimb extensor muscles, and have not been injected in this study. Although the primary function of the extensor digitorum is to extend the digits, it also acts concurrently as a secondary wrist extensor. Rubrospinal projections onto the two

primary wrist extensors, the ECR and the ECU, must be examined, although the rostral location of the ECR at C5-C6 does not support a hypothesis that it receives direct rubrospinal input. Direct projections onto wrist extensor motor pools would not conflict with the proposed role of the red nucleus in providing tonic whole hand support for further digit movements.

How are individual distal digit movements encoded?

One of the key points of the "framework" hypothesis is that two systems are required to execute a skilled movement: the rubrospinal tract provides tonic support while an alternative source, purported to be the corticospinal tract, simultaneously activates individual muscles. The key function of the direct projections from the RN to the extensor digitorum motor pool appears to be the establishment of an appropriate hand position from which skilled, fractionated digit movements can then be undertaken. If direct input from supraspinal motor centers is involved in distal limb movements, one would expect other distal limb muscles, particularly digit muscles such as the extrinsic digit flexors (flexor digitorum) and the intrinsic footpad muscles, to also receive direct supraspinal input. However, I have demonstrated in this chapter that rather unexpectedly, the cervical motor neurons that receive the majority of direct input from the rubrospinal tract are those innervating the extensor digitorum muscle. The flexor digitorum (FD) and ventral footpad (VF) motor pools receive minimal levels of direct rubrospinal input. I thus raise the possibility that other descending pathways might provide direct modulatory input to other forelimb MNs.

In the rodent, the two main supraspinal motor regions involved in the execution of skilled voluntary movement are the red nucleus and the motor cortex. In fact, it is the corticospinal tract which appears to provide the majority of direct projections onto motor neurons thought to be responsible for the increased dexterity seen in primates and humans (Lemon, 2008), and would thus be the most likely candidate to provide direct input responsible for further fractionated digit movements. In fact, one could imagine that the precise activation of the muscles responsible for digit flexion (i.e. the flexor digitorum and the intrinsic footpad muscles), and thus object grasp, would play a greater role in the execution of a skilled movement.

Although recent studies have indicated that the rodent corticospinal tract provides direct inputs to lumbar motorneurons, the anatomy of its projections to the cervical spinal cord have yet to be determined. Surprisingly, I found that none of the forelimb digit muscle MNs (ED, FD, VF) appeared to receive direct corticospinal input. Although it is still possible that there is a minor CST input to these muscles, it does not resemble the dense, targeted rubrospinal projection to the extensor digitorum motor pool. If direct inputs from the corticospinal tract are not providing the driving force to activate the remaining digit muscles, the question arises as to what is. Two potential explanations are that there is an alternative source of direct descending input in the rodent, or that the forelimb digit MNs, with the exception of the ED pool and a subset of intrinsic hand motor neurons, are not under direct supraspinal control.

Conclusions

In this discussion, I have detailed a number of experiments in the monkey providing support for the hypothesis that the rubrospinal tract provides the tonic whole-hand support required for fine distal movement, which correlates with the anatomical specificity of rubrospinal projections onto extensor digitorum motor neurons in the rat. Furthermore, I have reviewed studies of previous anatomical tracing of the rubrospinal tract in the cat that suggest a similar motor pool specificity to that seen in my results. Thus, it remains a strong possibility that the rubrospinal tract maintains a conserved evolutionary role in forelimb movement across species. One can imagine a scenario in which the 'framework' hypothesis of rubrospinal function is maintained, but the increase in dexterity could reflect an expansion of the corticospinal projections onto individual digit muscle MNs. It will be interesting to follow whether the functional distribution between the rubrospinal tract and other descending systems is conserved between species.

Chapter 4: Anatomical and molecular characterization of neuronal populations within the red nucleus

Introduction

The establishment of direct rubrospinal input onto a restricted pool of motor neurons represents an unprecedented level of specificity within a descending motor pathway. In order for this circuit to be established, axons must follow a precise long-range trajectory while faced with multiple choice points. Given the conserved location of the extensor digitorum motor pool at C7-C8, the small cohort of rubro-motoneuronal axons must be able recognize the exact spinal level at which to terminate. Upon entering the cellular region of the spinal cord, they must then project ventrally, instead of following a horizontal trajectory into the intermediate zone of the spinal cord along with the majority of rubrospinal axons. Finally, a specific post-synaptic motor neuron target must be selected from multiple motor pools located at a similar laminar level of the spinal cord. How is this accomplished? To begin to address these questions, we first need to establish a basic understanding of the organization of neuronal populations within the red nucleus.

Functional domains within the red nucleus

The red nucleus is divided into two functionally separate motor systems: the rubro-olivary circuit and the rubro-spinal circuit. Historically, the rostral, parvocellular red nucleus was thought to be the origin of fibers projecting to the inferior olive, whereas the caudal, magnocellular red nucleus projecting to other

regions of the brainstem and the spinal cord. However, this distinction appears to be somewhat inaccurate. Recent studies in the cat have demonstrated the presence of spinal projection neurons at rostral levels of the red nucleus, in the ventrolateral half of the parvocellular red nucleus (Pong et al., 2002). Likewise, in the rat, anatomical tracing studies have identified the presence of spinal projection neurons throughout the rostro-caudal extent of the red nucleus (Shieh et al., 1983). These include neurons projecting to cervical and lumbar spinal cord. Do the rostral and caudal rubro-spinal neurons represent a single cohesive population?

Anatomical tracing of the two populations in the cat has focused on projections in the cervical spinal cord. While the termination zones seem largely overlapping, the authors suggest a topographic organization with the rostral neurons projecting to upper cervical levels and the caudal neurons projecting to lower cervical levels. However, the presence of lumbar projection neurons in rostral areas of the red nucleus in the rat suggests that the rostro-caudal distribution of rubrospinal neurons within the red nucleus is not simply a representation of their spinal termination level. In my initial studies of gene expression within the red nucleus, I have identified two genetic markers: Brn3a, and a novel red nucleus marker, C1ql2. Unexpectedly, the expression of these two genes delineated two populations within the red nucleus: a rostral Brn3a^{+} population, and a caudal Brn3a^{+} C1qL2^{+} population (Figure 2.1 panels d-i). Correlation of gene expression in spinal projection neurons may provide insight into the relationship between rubrospinal neurons throughout the red nucleus.

Somatotopic organization of rubrospinal projections

The central nervous system must maintain the organization of information within neural circuits as it progresses through various processing steps in various brain regions. A common strategy is to physically arrange afferent or efferent projections in a topographic manner, reflective of the information type within a given system. Previous tracing experiments in the rat and cat have demonstrated that rubrospinal neurons are organized in a topographic manner within the red nucleus, representing their projections to different rostro-caudal levels of the spinal cord (Holstege, 1987; Shieh et al., 1983). Projections to the cervical spinal cord are located in a dorsomedial "forelimb" half of the red nucleus, and lumbar projecting neurons located in a ventrolateral "hindlimb" region. Within the rostral red nucleus, the distribution of cervical and lumbar projection neurons is unclear. In addition, the location of rubrospinal neurons that project to non-limb levels within this organizational layout are as of yet undefined.

The establishment of topography within a circuit can be accomplished through a variety of molecular mechanisms. We are just beginning to understand the complex interactions of intrinsic and extrinsic molecular programs that contribute to the motor pool-muscle connectivity seen in the spinal cord (refer to chapter 1). The early establishment of neuronal heterogeneity is usually dependent on the establishment of transcriptional programs in subsets of neurons within a population, which then control the expression of molecular 'effectors' that play a role in the patterning of specific connections within a circuit. Although the Pou transcription factor Brn3a is

expressed throughout the entire red nucleus from early development onward, transcription factors restricted to topographic domains within the red nucleus have not been identified. Likewise, the expression of molecules that are intimately involved in circuit formation, such as axon guidance receptors or mediators of cell-cell adhesion, has not been investigated within the red nucleus. Although atlases of gene expression are available, such as the Allen brain atlas, a stronger understanding of the somatotopic divisions within the red nucleus is necessary to interpret gene expression profiles.

The identification of neurons based on their post-synaptic target innervation is an important consideration in neural tracing studies. Small molecule tracers are available that can be taken up by synaptic terminals and then transported to the cell body in a retrograde direction. This technique has been used previously to identify rubrospinal neurons terminating in the cervical or lumbar spinal cord. Paraformaldehyde-fixable tracers are now available, which allows the co-detection of retrogradely identified neurons with the immunodetection of gene expression. Likewise, fluorescent-protein conjugated tracers are also available with different spectral properties, enabling the direct comparison of populations terminating in different regions in the same preparation.

Subpopulations of rubrospinal neurons

At a given spinal level, axons of the rubrospinal tract are capable of forming synapses on various classes of spinal neurons. In order for the rubrospinal system to

accurately contribute to the execution of a motor behavior, one can image that the choice of synaptic target must be highly regulated. Not only is there a general choice between interneuron and motor neuron targets, but within those broad classes, as we and other labs have demonstrated (Jankowska, 1988), further subclass specific targets are then selected. This raises the question as to how the choice of post-synaptic intra-spinal target is molecularly determined during development. To address this, we must evaluate the molecular heterogeneity of rubrospinal neurons within the larger forelimb and hindlimb domains.

Identifying molecular correlates of rubrospinal populations

Although candidate screens can be used to investigate the expression profiles of a limited set of genes, a complete analysis of gene expression within a population requires the use of a high-content method such as microarray analysis. The sensitivity of this technique depends on the precision with which distinct neuronal populations can be identified and isolated. Fluorescence activated cell sorting (FACS) is commonly used to fractionate cellular subtypes, but this requires the directed expression of a fluorescent protein in neuronal subpopulations through transgenic or other techniques. An alternate technique that facilitates the precise isolation of anatomical subdivisions of complex tissues is laser capture microdissection (LCM) (Emmert-Buck et al., 1996). This technique has the resolution to isolate individual neurons, and has been demonstrated to preserve RNA quality such that gene expression can be assessed (Kamme et al., 2004).

I have explored the specificity of the rubrospinal tract onto its post-synaptic spinal targets, and will now extend that exploration to include an evaluation of the level of heterogeneity within the pre-synaptic components of the circuit. In this chapter, I will investigate the somatotopic organization and molecular composition of rubrospinal neurons within the red nucleus, with the intention of providing insight into the molecular mechanisms by which specificity is established within the developing rubrospinal circuit. Furthermore, identifying molecular correlates of rubrospinal functional subpopulations will enable future experiments aimed at genetically accessing and exploring individual rubrospinal circuits within the greater organization of the rubrospinal circuit.

Results

Anatomical and molecular identification of subdivisions within the red nucleus

Retrograde labeling from the spinal cord (cervical, lumbar, thoracic)

In the rodent, the rubrospinal tract projects along the length of the spinal cord. To identify topographic subdivisions of the red nucleus based on spinal projection level, I injected fluorescent protein conjugated CTB into the intermediate laminae of the spinal cord of mice. The tracer is taken up primarily by nerve terminals at that spinal level and transported back to the cell body. By injecting tracers at different spinal levels, I began to map out the somatotopic organization within the red nucleus based on target region (figure 4.1a).

A previous study in the rat demonstrated that rubrospinal neurons projecting to the cervical spinal cord are located in the dorsomedial aspect of the red nucleus, whereas those projecting to lumbar levels occupy the ventrolateral half (Shieh et al., 1983). To confirm these results, I injected rhodamine-dextan into cervical levels C6-C8 and fluorescein-dextran into lumbar levels L1-L5 of adult animals. As the majority of rubrospinal neurons terminate in the intermediate region of the spinal cord, I measured the diameter of the isolated spinal cord, and used stereotaxic measurements to inject the tracer at the appropriate depth (figure 4.1 b, c). My experiments reproducibly demonstrate that rubrospinal neurons projecting to cervical levels of the spinal cord are located in the dorsomedial half of the red nucleus, whereas neurons projecting to lumbar levels are located in the ventrolateral half (figure 4.1 d). These neurons all express the red nucleus marker C1ql2 (figure 4.1 e-h). I cannot assess the co-localization of brn3a in these neurons, since Brn3a expression in the red nucleus turns off around postnatal week 3.

Although the retrograde tracer will be taken up primarily by neurons that terminate at that spinal level, one concern is that axons that pass through rostral levels on their way to more caudal destinations will also take up the tracer. This possibility is increased if there is damage to the spinal cord during the surgery or the needle injection. I have carefully examined my results to address this concern. Although en passant axons do take up the tracer (i.e. lumbar neurons following a cervical injection), the levels are low enough to clearly distinguish this population from the rubrospinal neurons that take up higher levels of the tracer through their synaptic

terminals. Therefore, neurons with low fluorescence intensity were not included in the analysis above.

To determine whether this topographic organization is established earlier in development, I performed similar experiments in P4-P7 animals. Rather than injecting conjugated dextran, I injected Alexa555-CTB into C6-C8, and Alexa488-CTB into L1-L5, as CTB appears to transport more efficiently in younger animals. Even at this earlier timepoint, there is a clear segregation of cervical- and lumbar-projecting rubrospinal neurons within the red nucleus (figure 4.2 panel b). These neurons co-express the caudal red nucleus combination of c1ql2 and brn3a (figure 4.2 c, d). My results indicate that a discrete topographic organization within the red nucleus is present from the earliest time at which projections to the spinal cord can be traced.

Although a clear topographic organization of rubrospinal projections to the cervical and lumbar limb-controlling spinal levels has been established, it is unclear where projections to non-limb levels would be located. To investigate whether thoracic rubrospinal neurons are contained within a distinct domain of the red nucleus, I injected Alexa488-CTB into the mid-thoracic spinal cord of p7 mice (figure 4.2 e). Thoracic neurons were located along a diagonal line directly between the cervical and lumbar subdivisions of the red nucleus (Figure 4.2 f).

Rubrospinal projections from the rostral red nucleus

During the course of the retrograde labeling studies, I also noticed a significant spinal projection arising from the lateral border of the rostral red nucleus, which included projections to the cervical and lumbar spinal cord (figure 4.3 a). Interestingly, the rostral population of rubrospinal neurons does not maintain the strict cervical and lumbar segregation observed in the caudal red nucleus (figure 4.3 c). In addition to this apparent anatomical intermingling, I also observed a number of neurons that sent projections to both cervical and lumbar spinal cord, as demonstrated by their co-expression of the two fluorescent tracers (figure 4.3 arrows in a, b). Once again, the question arises as to whether these double-labeled neurons might be an artifact due to en-passant labeling of axons in passing. But if this were the case, I would expect to see a contamination of double-labeled cells in the caudal RN population as well. Instead, I observed a strict segregation of cervical and lumbar populations in the caudal RN of the same experiments excluding this technical artifact as an explanation for the double-labeled cells. The organizational differences between the rostral and caudal rubrospinal neurons suggest that there might be two functionally distinct populations within the red nucleus. Further establishment of molecular differences would support this hypothesis.

Expression of brn3a and c1ql2 delineates two distinct rubrospinal domains

My initial studies of gene expression in the developing red nucleus identified two molecularly distinct populations of neurons: a Brn3a^{+} population in the rostral red nucleus, and a Brn3a^{+} C1qL2^{+} population in the caudal red nucleus (see figure

2.1). To determine whether the rostral and caudal spinal projecting populations map to these two molecular domains, I examined Brn3a and C1qL2 expression in retrogradely identified rubrospinal neurons throughout the red nucleus. At rostral levels, the rubrospinal domain contains intermingled cervical, lumbar, and dual-projection neurons expressing Brn3a, but not C1qL2 (figure 4.3 d-e and i-j). At intermediate levels, the rubrospinal domain is now seen at the ventrolateral pole of the red nucleus, and the segregation between the cervical and lumbar projection neurons is becoming clearer, although double-labeled neurons are still present at this level. C1qL2 expression begins to emerge at intermediate levels of the red nucleus, but is restricted to the dorsomedial red nucleus (refer to figure 2.1 e, h). The intermediate plane of section in figure 4.3 panel k appears to be rostral to the emergence of c1ql2 expression. Despite the apparent segregation of cervical and lumbar neurons at intermediate levels, these rubrospinal neurons are Brn3a^{+}, C1qL2^{-} (figure 4.3 f-g, k-l). Finally, the caudal red nucleus contains a rigorous topographic organization of cervical and lumbar projection neurons. These neurons co-express Brn3a and C1qL2 (figure 4.3 g-h, m-n).

Magnocellular and parvocellular regions within the red nucleus (size, density)

In the rat, cat, and monkey, the parvocellular region is located in the rostral 1/3 of the red nucleus and gives rise to projections to the inferior olive. Cell bodies within the RNp are significantly smaller on average than those within the more caudal RNm, and appear to be more clustered, resulting in a higher cellular density (Burman et al., 2000; Reid et al., 1975). I have determined that the rodent red nucleus can be

divided into two molecularly distinct domains along its rostro-caudal extent. I therefore wanted to investigate whether these two populations could also be distinguished based on their cellular size or density. There was no significant difference in nuclear size between Brn3a single positive rostral neurons and Brn3a+ C1qL2+ double positive caudal neurons (258 +/- 102 vs. 271 +/- 115, figure 4.4 e). The cellular density appears to be increased in the Brn3a+ C1qL2+ caudal red nucleus as compared to the Brn3a+ rostral red nucleus (257 cells/100,000 pixel area vs. 173 cells/100,000 pixel area), indicating that this measure also does not support a distinction of parvocellular vs. magnocellular (figure 4.4f).

Although the rostral, parvocellular red nucleus in the rat is thought to contain neurons of smaller cell body size (Reid et al., 1975), I have not found evidence of this in my measurements of nuclear size. It is possible that nuclear size is not an accurate representation of neuronal size, and this needs to be investigated further using a cellular stain such as Neurotrace to mark neuronal cell bodies. The main functional distinction of the RNp is that it sends an ipsilateral projection to the inferior olive. To determine whether the brn3a single positive domain in the rostral RN represents the origin of rubro-olivary neurons, I attempted to inject a retrograde tracer into the inferior olive. Unfortunately, due to the location of the inferior olive in the hindbrain, this surgical procedure proved unfeasible. It therefore remains unclear whether brn3a and c1ql2 delineate RNp and RNm in the rodent.

Isolation and genetic profiling of rubrospinal subpopulations

By this stage, I had identified three anatomically distinct populations of rubrospinal neurons within the red nucleus: a rostral population containing intermingled cervical and lumbar projection neurons, and two strictly segregated caudal populations projecting to the cervical and lumbar spinal cord, respectively. Although I had molecularly distinguished the rostral and caudal rubrospinal populations by the differential expression of C1qL2, the question remained as to how these two populations of rubrospinal neurons are molecularly specified during development of the red nucleus, resulting in their rostro-caudal segregation, and differing somatotopy. Within the caudal RN, how is the strict segregation between the cervical and lumbar domains established? Finally, within the cervical domain, how is the choice of interneuron or motor neuron target determined by individual rubrospinal neurons? The answers to these questions are likely contained within the unique molecular profiles of anatomical and functional subpopulations of rubrospinal neurons.

Laser capture microdissection of rubrospinal subpopulations

I used laser capture microdissection (LCM) to isolate subpopulations of red nucleus neurons. To maximize the chances of identifying genes which specify neuronal identity and genes responsible for establishing the specific topographic circuitry seen in spinal projection neurons, I collected the tissue at embryonic day 17.5, when rubrospinal axons are likely en route towards their target region. In the rat, the developing rubrospinal tract reaches the lumbar spinal cord by P0 (Lakke and Marani, 1991; Shieh et al., 1983). The experimental design is based on the premise

that rubrospinal subpopulation identity emerges early in development, rather than following a period of cell body migration and/or reorganization, as suggested by retrograde labeling experiments in neonatal rats that demonstrate a topographic organization by P2 (Shieh et al., 1983).

The large, Nissl-heavy neurons of the red nucleus are easily identifiable in fresh frozen e17.5 midbrain coronal sections by a cresyl violet stain (Figure 4.5 b, f). Retrograde labeling to definitively identify the cervical and lumbar subdivisions is impossible at this age, as rubrospinal fibers likely have not reached lumbar levels of the spinal cord. Therefore, I relied on my knowledge of the anatomical distributions of rubrospinal subpopulations acquired from retrograde labeling experiments in postnatal animals. There was a risk that this approach could increase cross-contamination between cervical and lumbar populations. To avoid further overlap, I left a small amount of tissue remaining between the cervical and lumbar populations (figure 4.5 j, asterisk). In addition, I mapped out the expression of brn3a and c1ql2 on serial sections to aid in the identification of the rostral vs. caudal divisions (figure 4.5 c-d, g-h). The three populations collected by LCM were the rostral Brn3a single-positive population (figure 4.5 e), and the caudal brn3a$^+$ c1ql2$^+$ dorsomedial (cervical) and ventrolateral (lumbar) populations (figure 4.5 i).

Quantitative PCR / RNA quality

For each rubrospinal population, a single sample required collecting and pooling tissue from 3 animals to ensure enough material for microarray analysis. The

quality and quantity of RNA was then determined on a bioanalyzer, and two samples for each subpopulation were found to be excellent for further analysis (figure 4.7 k, l). To ensure that the appropriate subpopulations had been collected, quantitative polymerase chain reaction (qPCR) analysis of control genes was performed on the cDNA samples prior to RNA synthesis and microarray analysis. Brn3a levels were found to be comparable between all 3 samples, whereas C1qL2 levels were significantly higher in cervical and lumbar samples as expected (figure 4.5 m). I decided to proceed with the microarray analysis of duplicate instead of triplicate samples, with the knowledge that the number of false positive results would increase and would require a more time-intensive validation step.

Microarray analysis

Duplicate samples from each subpopulation were subjected to microarray gene analysis on Affymetrix gene chips. Clustering analysis indicates that the cervical and lumbar samples are more closely related to one another in terms of gene expression, as expected (figure 4.6 a). As an internal control, C1qL2 expression was significantly upregulated in the cervical (11.44 fold) and lumbar (10.29 fold) caudal subpopulations whereas Brn3a levels did not differ significantly between rostral, cervical, and lumbar populations (figure 4.6 b and c). Unfortunately, as only two samples were used per condition, the majority of statistical analyses were unavailable. Even in the Bayesian t-test analysis performed, the p-values were quite high (figure 4.6 e). Nonetheless, a number of genes were demonstrated to be significantly upregulated in each condition (figure 4.6 e, only C vs. L are listed), and I chose an

initial, biased set to validate expression patterns. However, the majority of the gene sets have not been validated to date. The log values of the fold change of all genes that will be discussed further are illustrated, along with their RNA patterns in figure 4.7.

Initial in situ hybridization screen to validate microarray candidates

In situ hybridization of mRNA expression in e17.5 red nucleus was used to confirm expression of candidate genes in indicated subpopulations. Given the high number of differentially expressed genes between the three populations, a biased candidate approach was employed. I initially screened forty genes that met the following criteria: 1. Upregulated in a single population (focused on cervical or lumbar) 2. Either a transcription factor, potential molecular determinant of circuitry, or otherwise interesting gene (e.g. a subpopulation marker as suggested by Allen brain atlas expression pattern) 3. Did not appear to be a false positive based on Allen Brain Atlas expression or gene identity (i.e. dopaminergic neuron marker contaminants in the lumbar population). I confirmed by RNA expression that a percentage of the candidate genes demonstrated subpopulation specificity in the developing red nucleus when compared with the comprehensive red nucleus marker, *brn3a* (figure 4.7). Transcription factors *tshz3* and *mafb*, and the axon guidance molecule *kitl* (kit ligand, stem cell factor) were expressed in the dorsomedial half of the caudal domain. A chemokine, fam19a4, was restricted to the ventrolateral half of the caudal domain. Although the demonstrated gene expression patterns were strongly suggestive of specific cervical and lumbar markers, these domains have not

been characterized at e17.5. Finally, an orphan g-protein coupled receptor, *gpr8*8, and a chemokine, *cxcl13* (not shown), were expressed in just a small subset of neurons in the caudal red nucleus. The cervical or lumbar restriction of these genes was unclear.

Molecular analysis of gene expression within defined subpopulations

To definitively confirm that the candidate markers validated by RNA expression were expressed in anatomically defined subpopulations, I examined gene expression within retrogradely-labeled cervical and lumbar domains of the red nucleus. The expression patterns of some candidate genes confirmed their original assignment. However, other genes demonstrated more complex patterns of expression.

Tshz3 and mafB are markers for the caudal cervical population

I injected Alexa488-CTB into the lower cervical spinal cord of P7 animals to identify cervical projection neurons within the red nucleus. Overlay of candidate gene RNA expression on fluorescent labeled cervical neurons demonstrated the restriction of a transcription factor, *tshz3*, to the caudal cervical domain (figure 4.8 a-c). Although I have not definitively confirmed the expression of an additional transcription factor, mafB, in anatomically defined cervical neurons, its expression overlaps with that of tshz3 in the caudal RN at e175 (figure 4.8 k-m).

Fam19a4 is expressed in the lumbar subdivision

Lumbar spinal neurons are restricted to the ventrolateral half of the caudal red nucleus. The only gene validated to have this expression pattern was the uncharacterized chemokine, fam19a4. RNA expression confirmed that fam19a4 was expressed in all retrogradely identified caudal rubrospinal neurons projecting to lumbar spinal levels (Figure 4.8 d-f). Double fluorescent in situ hybridization (FISH) demonstrated the distribution of *tshz3* and *fam19a4* in the caudal RN (figure 4.8 j).

KitL is expressed in the caudal cervical population and rostral rubrospinal neurons

An additional gene indicated by microarray analysis as a candidate for differential expression in the cervical subnucleus of the caudal RN was kitL. Although I have not correlated *kitl* expression with retrograde labeling to confirm its expression in cervical neurons, I have compared its RNA expression at e17.5 with that of *tshz3* and *mafB*, two confirmed cervical markers (figure 4.9 a-l). Unlike the other cervical markers whose expression was restricted to the c1ql2^{+} intermediate and caudal RN, *kitl* was expressed in a subset of neurons within the lateral aspect of the rostral RN (figure 4.9 arrows in b, h). Apart from brn3a, kitL is the only gene that I have identified with expression in both the rostral and the caudal red nucleus. Its restriction to the cervical domain of the caudal RN raised the possibility that kitL might be a specific marker for all cervical projection neurons throughout the red nucleus. However, correlation of *kitl* expression in retrogradely-labeled cervical neurons in the rostral RN indicates that *kitl* is expressed in all rubrospinal neurons in the rostral RN, including lumbar projection neurons (figure 4.9 m-x).

Cxcl13 and gpr88 are expressed in subpopulations across the cervical and lumbar subnuclei

The expression patterns of two genes, *cxcl13* and *gpr88,* suggested that rather than marking a somatotopic domain, they are expressed in a smaller subset of neurons within a domain. Retrograde labeling confirmed that a chemokine, *cxcl13*, is expressed in a subset of neurons across throughout both the cervical and lumbar domains (Figure 4.8 g-i). Although I did not correlate the expression pattern of *gpr88* with retrogradely labeled neurons, both its expression pattern and the labeling seen in a gpr88-GFP Gensat transgenic line (not shown) suggests that gpr88 is also a subpopulation marker across the cervical and lumbar domains. The expression pattern of these two genes suggests the presence of rubrospinal subsets within the framework of a larger somatotopic organization. I have demonstrated that rubrospinal neurons projecting to a given spinal level share aspects of a common molecular identity. One would predict that the same might hold true for rubrospinal neurons targeting a specific subtype of neurons throughout the spinal cord.

The thoracic population expresses the same molecular markers as cervical neurons

I have determined that thoracic-projecting rubrospinal neurons comprise their own sub-nucleus, located between the cervical and lumbar domains. To determine whether thoracic rubrospinal neurons share aspects of molecular identity with cervical or lumbar rubrospinal neurons, I examined whether thoracic neurons express any of the identified domain markers. I found that retrogradely labeled thoracic neurons express the same molecular markers as cervical neurons (*tshz3*) whereas lumbar

markers such as *fam19a4* are excluded (figure 4.10 b-g). It is possible that there is an as-yet-unidentified thoracic specific marker that distinguishes thoracic from cervical rubrospinal neurons. Unfortunately, since I excluded the region between the cervical and lumbar domains from collection during LCM to prevent cervical-lumbar cross contamination, it is unlikely that a thoracic-specific gene would have been identified in either the cervical or lumbar data sets.

There are still a number of genes identified in the microarray whose expression patterns must be examined. Nonetheless, these initial results demonstrate a level of molecular heterogeneity within the red nucleus that was perhaps expected, but has not been previously investigated. It now remains to correlate molecular expression patterns with the functional establishment of rubrospinal circuit specificity fundamental to its role in motor behavior.

Analysis of Tshz3 loss of function in the cervical subnucleus of the red nucleus

One of the primary goals of undertaking a microarray analysis of gene expression was to identify genes that play a role in the establishment of somatotopy within the rubrospinal pathway. I have identified a transcription factor, *tshz3* as a specific marker of cervical projection neurons in the caudal red nucleus. Interestingly, this gene is a homolog of the *drosophila* teashirt gene, a zinc finger transcription factor that is involved in the antero-posterior patterning of trunk identity along with Hox genes. To determine the role of *tshz3* in the specification of cervical

projection neurons, I acquired mice in which the coding sequence for *tshz3* was replaced with the gene for β-galactosidase, kindly shared with us by the Fasano lab (Caubit et al., 2010).

Tshz3 is not required for survival of rubrospinal neurons

To determine whether *tshz3* is required for the generation and differentiation of neurons in the cervical subnucleus, I first examined β-galactosidase expression in Tshz3$^{lacz/lacz}$ e14.5 midbrains. I found that β-galactosidase was localized to the dorsomedial population of neurons in the red nucleus, and replicated the expression of tshz3 in a tshz3$^{lacZ/+}$ red nucleus (figure 4.12 a-f). My results demonstrate that cervical rubrospinal neurons are generated in apparently normal numbers and survive to e14.5 in the absence of tshz3. The segregation of the tshz3^{+} population within the dorsomedial half of the red nucleus suggests that topographic subdivisions are established early in development. The loss of another transcription factor, *brn3a*, resulted in the death of red nucleus neurons around e18.5, indicating that *brn3a* is required for late survival of these neurons (Xiang et al., 1996). β-galactosidase expression at e18.5 demonstrates that *tshz3* is not required for late survival of cervical RN neurons (figure 4.12 g-l). However since tshz3$^{lacZ/lacZ}$ mice die at birth, later timepoints could not be analyzed. I can conclude that *tshz3* is not required for the early or late survival of cervical caudal RN neurons.

Molecular role for tshz3 in the establishment of specific projections to the cervical spinal cord

Although inactivation of tshz3 does not affect survival, it may function to modulate downstream gene regulatory networks within the cervical red nucleus. To explore this possibility, I examined gene expression in the cervical red nucleus of e17.5 tshz3 mutants, where residual β-galactosidase expression identifies the *tshz3*+ neurons. Expression of brn3a remained unchanged in the absence of tshz3 (figure 4.13 h, p). Interestingly, I found that C1qL2 protein expression is significantly decreased in the cervical subpopulation in the absence of tshz3 (11.55 mean pixels/area in $Tshz3^{+/+}$ vs. 2.47 in $Tshz3^{lacZ/lacZ}$, p value = .00001). In contrast, C1qL2 expression is maintained at normal levels in ventrolateral neurons (16.98 in $Tshz3^{+/+}$ vs. 14.05 in $Tshz3^{lacZ/lacZ}$, p value not significant), indicating the presence of distinct regulatory pathways maintaining the expression of c1ql2 in cervical vs. lumbar domains. It remains to be seen whether other cervically-restricted transcription factors such as *mafb* retain their expression in the absence of *tshz3*. Likewise, it is possible that genes normally expressed in the lumbar domain, such as *fam19a4,* may expand their expression domain into the dorsomedial red nucleus, especially if tshz3 is responsible for the suppression of lumbar cell fate in cervical neurons.

Figure 4.1

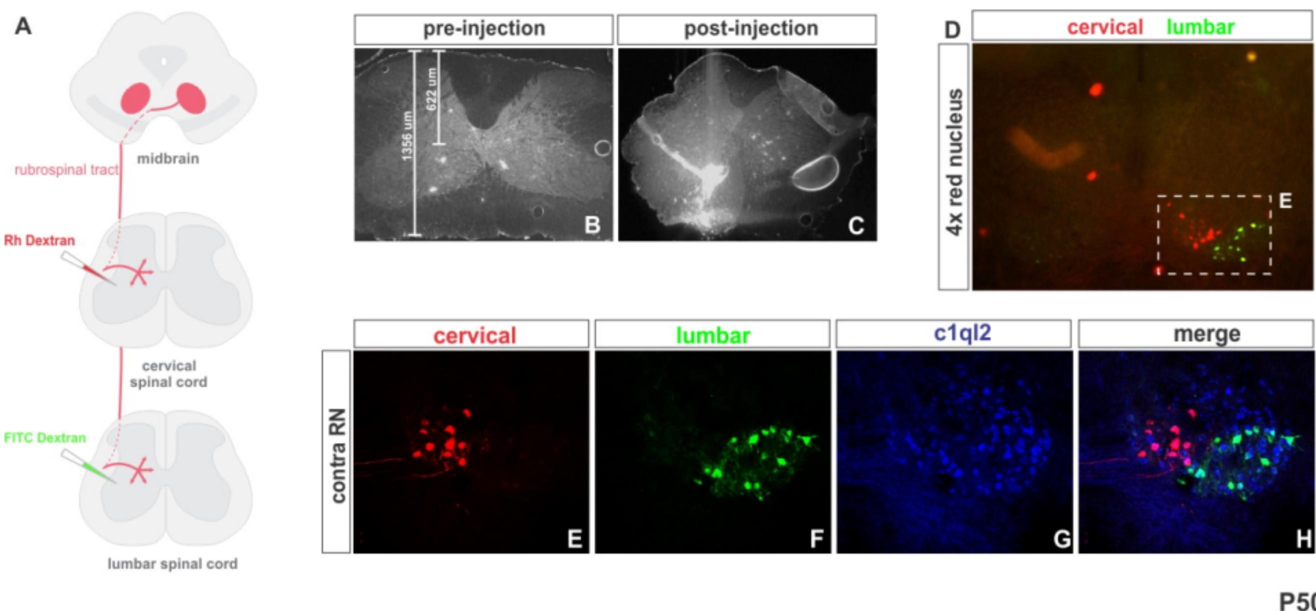


Figure 4.1 Somatotopic organization of rubrospinal neurons in the adult RN To determine the location of rubrospinal neurons projecting to the cervical and lumbar spinal cord, fluorescent protein-conjugated dextrans were injected into the intermediate region of the spinal cord of P50 mice at cervical and lumbar levels (A), based on spinal cord depth measurements (B). Post-mortem examination of the injection site indicates that the tracer is concentrated within the intermediate and ventral regions of the spinal cord (C). Following 7-10 days transport, fluorescent protein labeled rubrospinal neurons can be observed in the contralateral red nucleus (D). Neurons projecting to the cervical spinal cord are located in the dorsomedial RN (E) whereas those projecting to lumbar levels are located in the ventrolateral RN (F). Both populations are C1qL2+ (G,H).

Figure 4.2

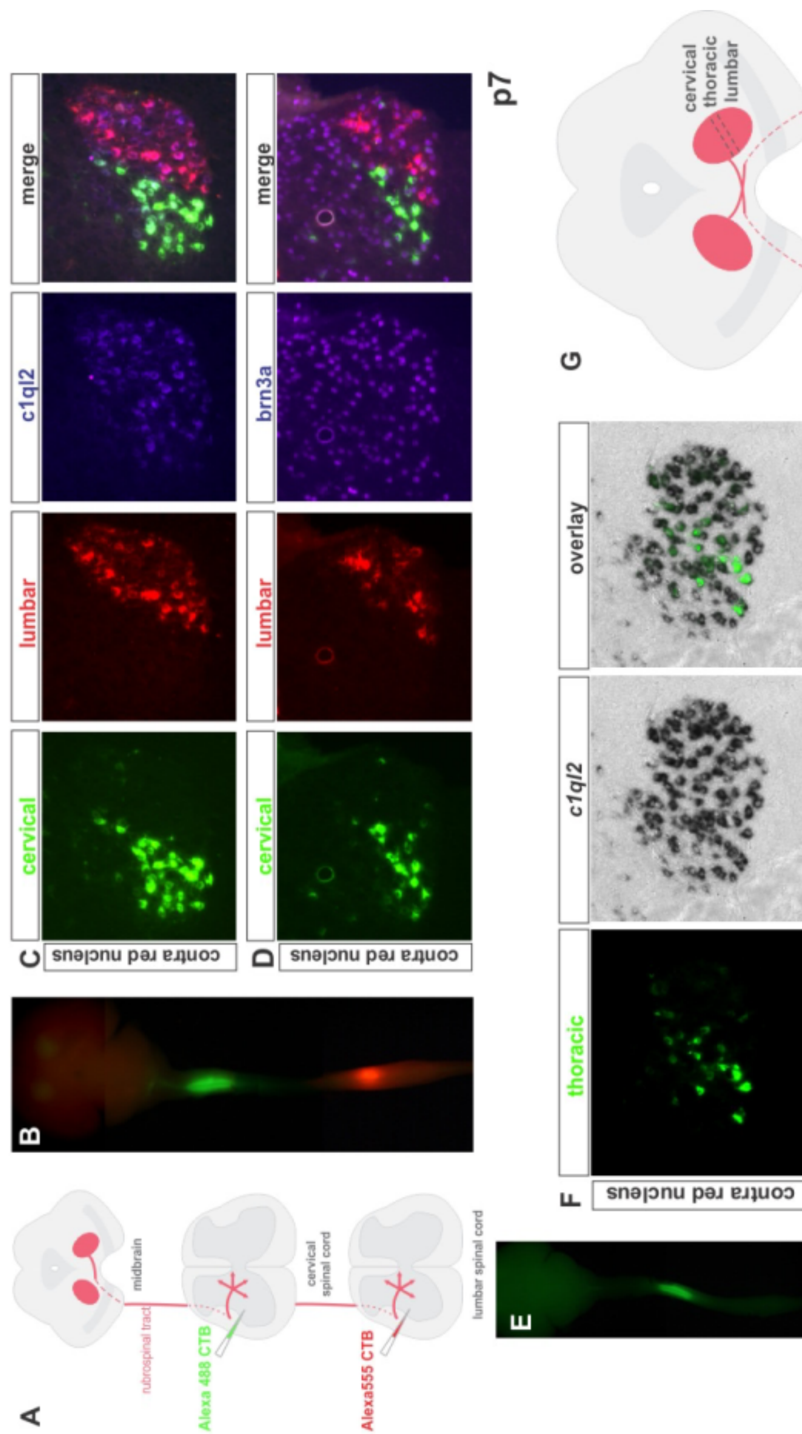
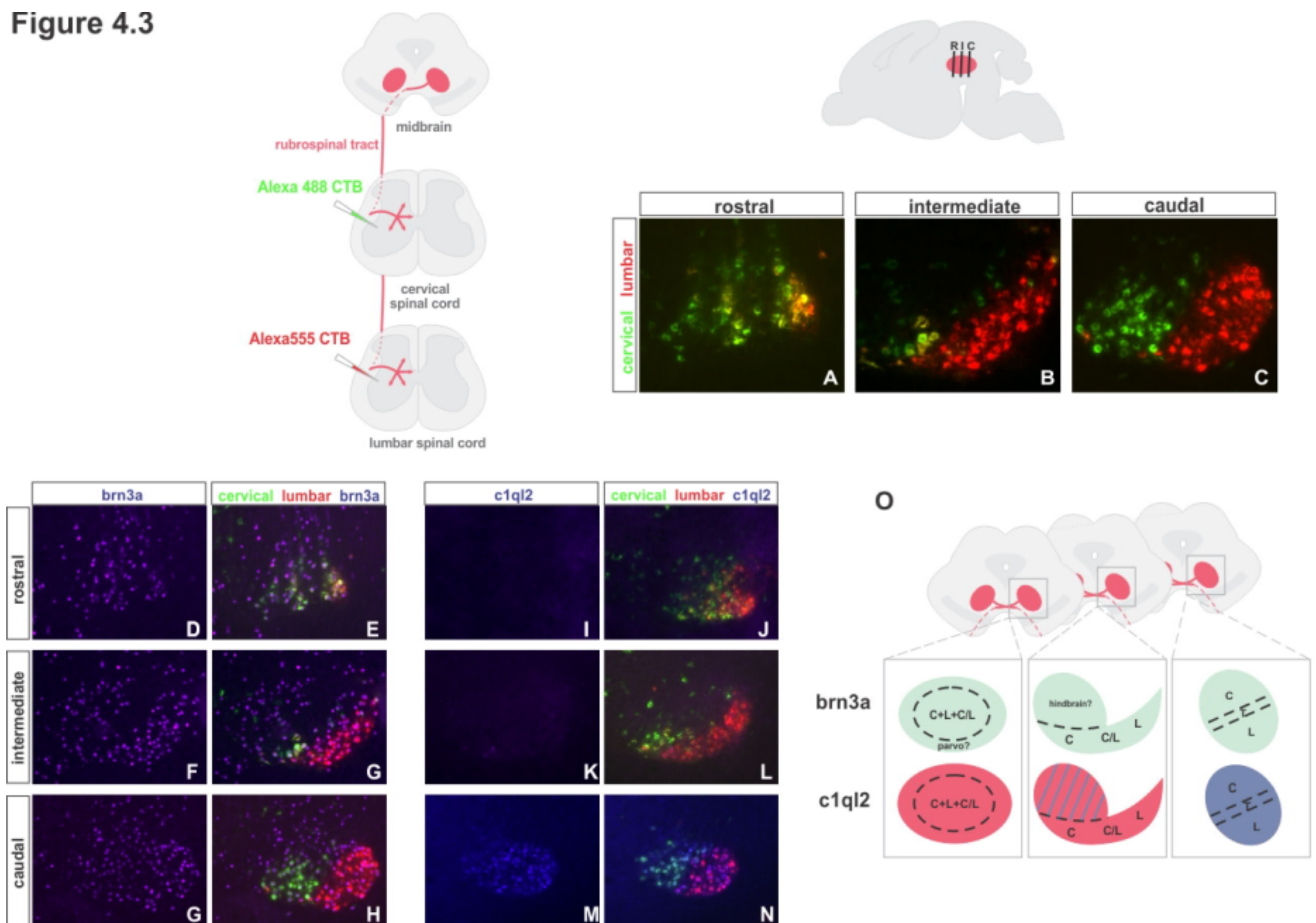


Figure 4.2 Somatotopic organization of rubrospinal neurons in p7 RN To determine whether topographic organization emerges early in development, fluorescent protein-conjugated CTB was injected into the cervical and lumbar spinal cord of P7 mice (B). Following 3 days of transport, labeled neurons were observed within the contralateral RN. Distinct cervical and lumbar domains were present within the red nucleus (C,D). Both cervical and lumbar neurons co-expressed the red nucleus markers C1qL2 (C) and Brn3a (D). Thoracic-projecting rubrospinal neurons are located between the cervical and lumbar domains of the RN (E,F). The schematic in G illustrates the relative distribution of cervical, thoracic, and lumbar projection neurons within the red nucleus.

Figure 4.3



P7

Figure 4.3 Rostro-caudal distribution of spinal projection neurons within the RN To determine whether spinal projection neurons are present within the parvocellular RN, the location of anatomically identified rubrospinal neurons was examined throughout the rostro-caudal extent of the RN. At rostral levels, cervical and lumbar projection neurons are present, but intermingled with one another (A), whereas at caudal levels, the two populations are strictly segregated (C). In addition, the rostral RN contains a population of neurons which project to both cervical and lumbar spinal cord (arrows in A,B). Dual-projecting neurons are never observed at caudal levels. To examine whether the rostral and caudal populations are molecularly distinguishable, the expression of Brn3a and C1qL2 was examined. All spinal neurons within the RN express Brn3a (D-H), whereas only caudal rubrospinal neurons express c1qL2 (I-N). Anatomical and molecular organization along the rostro-caudal axis of the RN is illustrated in the diagram in O. All experiments in figure 4.3 were performed at P7.

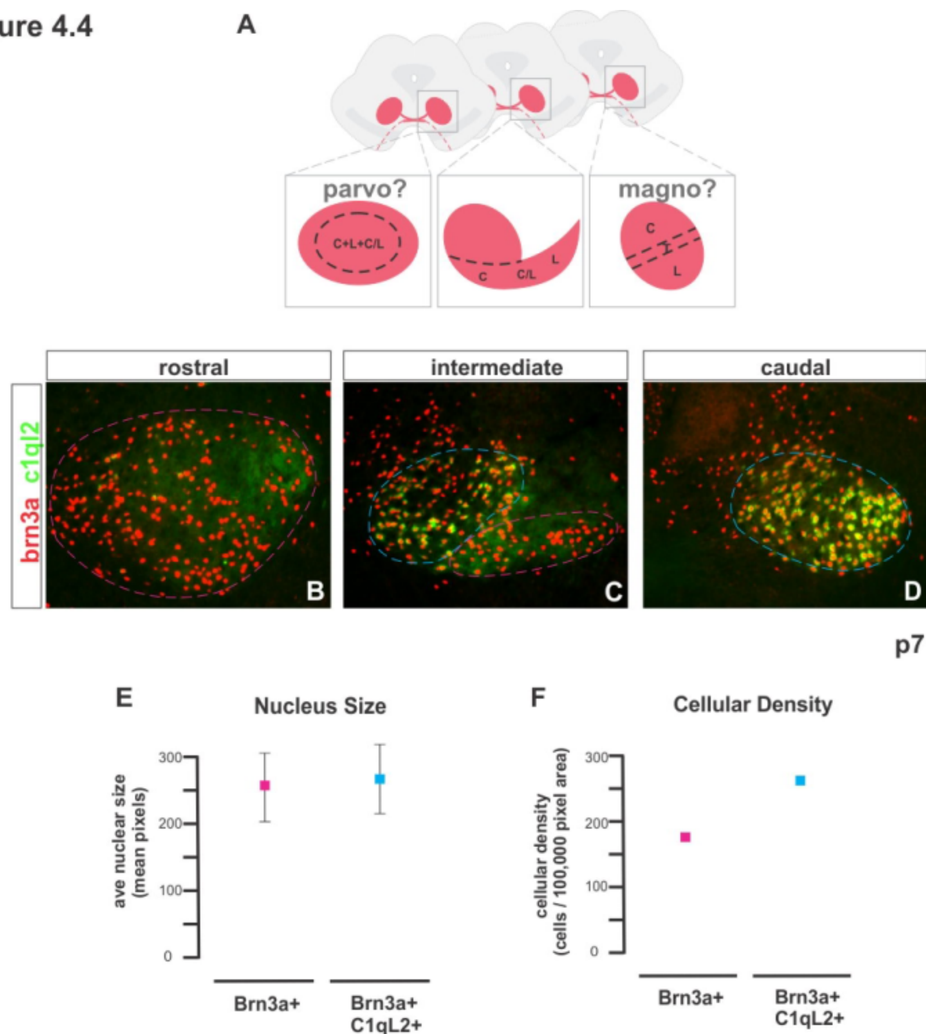


Figure 4.4 Parvocellular and magnocellular distribution is not apparent within the RN The parvocellular red nucleus is characterized by smaller size neurons that are distinguishable from the larger spinal-projecting neurons in the magnocellular RN. To determine whether Brn3a and C1qL2 expression delineates RNp and RNm, neuronal size and density was compared between the Brn3a+ rostral RN (A) and the Brn3a+ C1qL2+ caudal RN (C). At intermediate levels, both molecular populations are present (B). A comparison of nuclear size (determined by brn3a expression), demonstrated no significant difference between the Brn3a+ and the Brn3a+ C1qL2+ populations (E). Cellular density (mean cells/100,000 pixels) appears to be higher in the caudal red nucleus (F), however both E and F must be repeated for statistical significance.

Figure 4.5

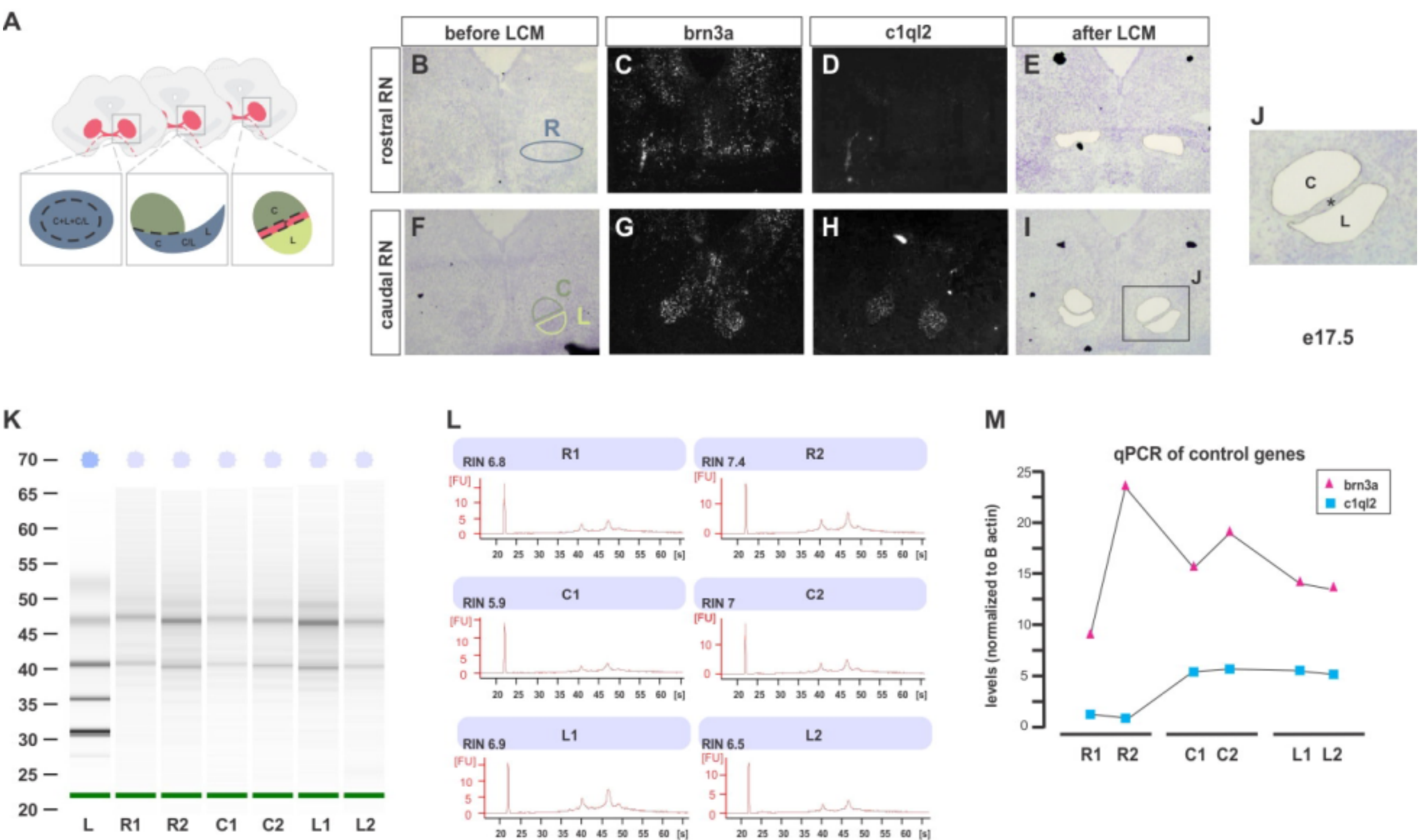
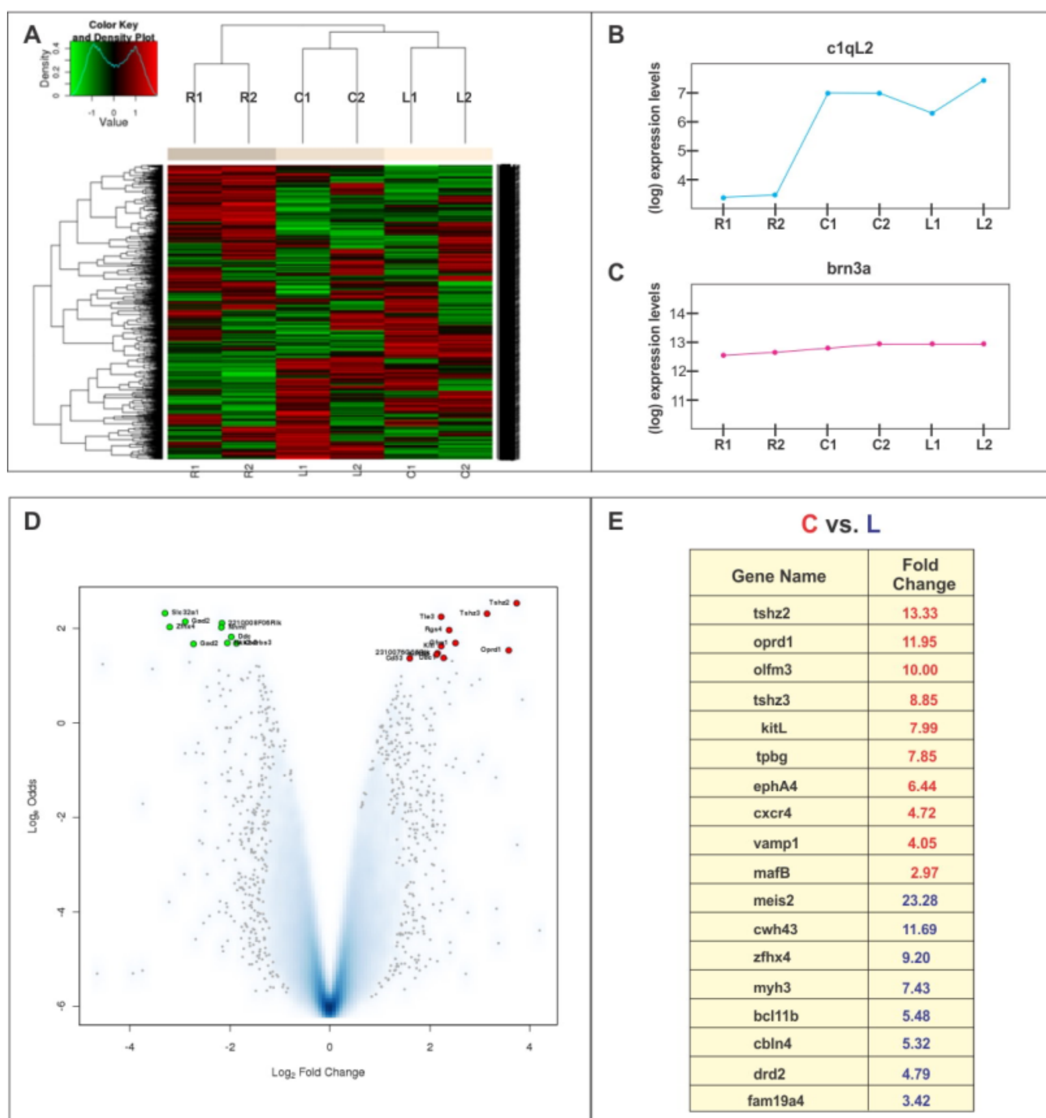


Figure 4.5 Isolation and genetic profiling of rubrospinal subpopulations E17.5 fresh frozen midbrains were sectioned at 10 uM, and the location of the red nucleus visualized with a cresyl violet stain (B,F). Every third section was collected on a reference slide, which was fixed and stained for Brn3a and C1qL2 expression (C,D,G,H). Laser capture microdissection was used to isolate three populations: the Brn3a+ rostral population (R), and the cervical and lumbar domains of the Brn3a+ C1qL2+ caudal RN (E,I). To prevent cross-contamination of the cervical and lumbar populations, a small region was left between the two (asterisk in J). Tissue from three animals was pooled for each sample, and the RNA purified. Bioanalysis indicated that the RNA was of adequate quality and quantity (K,L) to procede with microarray analysis. Quantitative PCR indicated that c1ql2 levels were enriched in cervical and lumbar samples, whereas Brn3a levels remained constant between rostral, cervical, and lumbar, confirming that the appropriate tissue regions had been microdissected (M).

Figure 4.6



Gene Name	Fold Change
tshz2	13.33
oprd1	11.95
olfm3	10.00
tshz3	8.85
kitL	7.99
tpbg	7.85
ephA4	6.44
cxcr4	4.72
vamp1	4.05
mafB	2.97
meis2	23.28
cwh43	11.69
zfhx4	9.20
myh3	7.43
bcl11b	5.48
cbln4	5.32
drd2	4.79
fam19a4	3.42

Figure 4.6 Microarray analysis of gene expression in rubrospinal subpopulations Two samples from each subpopulation were subjected to Affymetrix gene chip analysis. A heat map display (A) indicates that the cervical and lumbar samples display the highest similarity with each other. As an internal control, Brn3a levels do not change between rostral, cervical, and lumbar (B), wherease C1qL2 is upregulated in cervical and lumbar samples (C). Select upregulated genes from the cervical vs. lumbar comparison are shown in D and E. Differentially regulated genes from the rostral samples are not indicated in this figure.

Figure 4.7

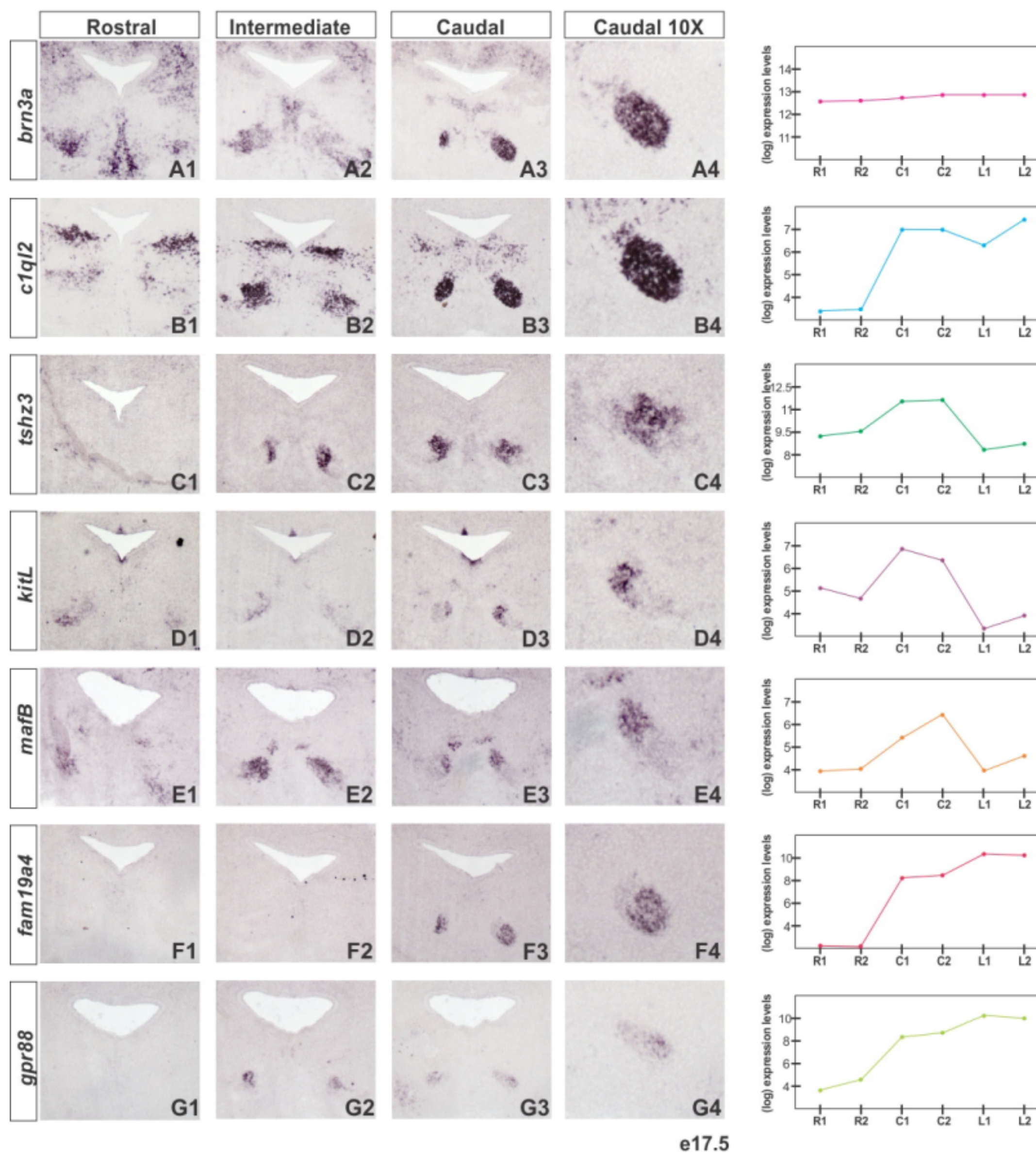
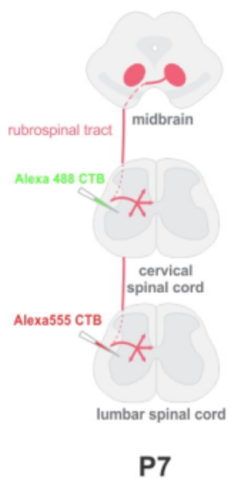


Figure 4.7 Validation of microarray candidate gene expression The expression of the following candidate genes was confirmed by in situ hybridization, and comparison of gene expression with *brn3a* and *c1ql2* at e17.5: *tshz3* (cervical), *kitl* (cervical), *mafb* (cervical), *fam19a4* (lumbar), and *gpr88* (cervical/lumbar)

Figure 4.8



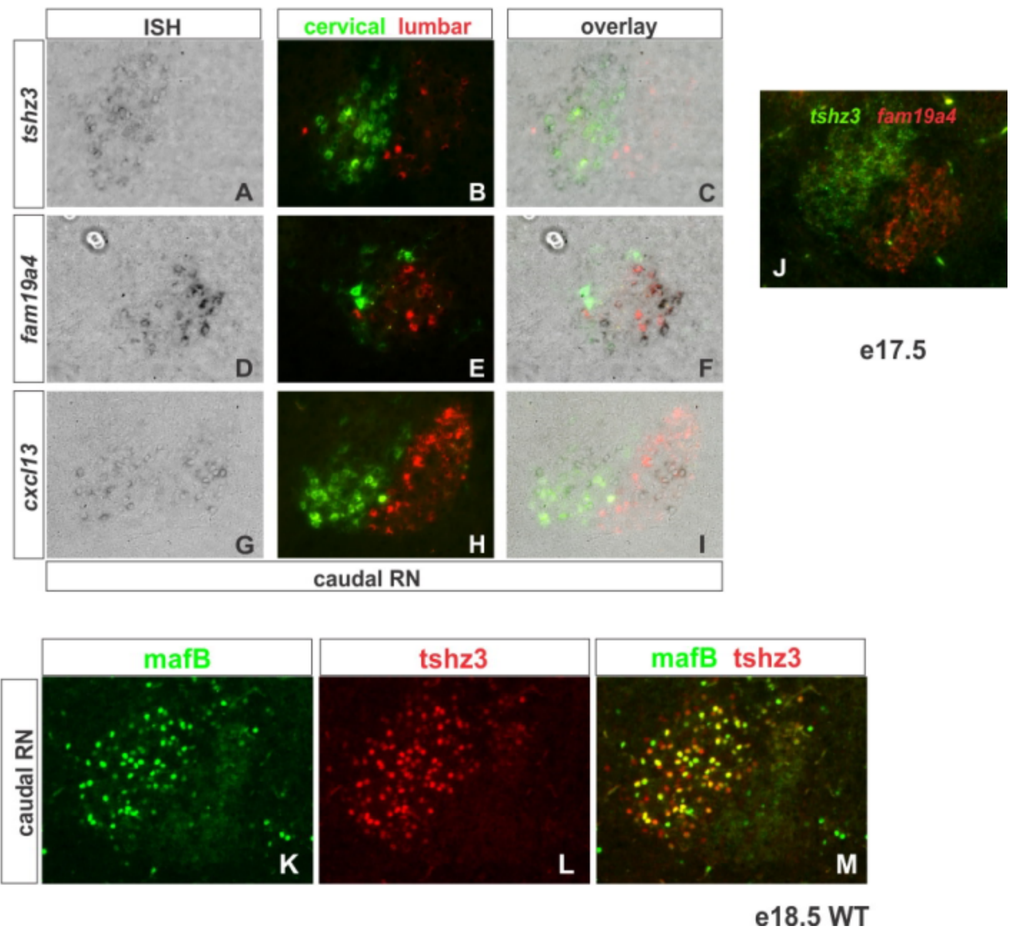
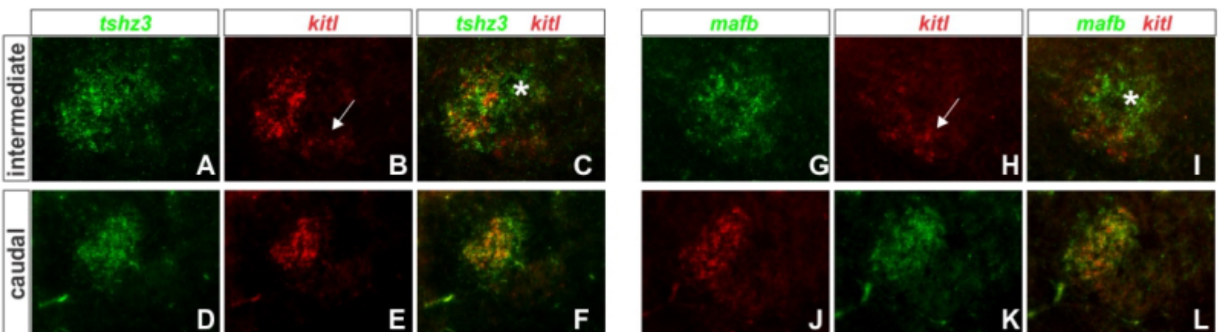
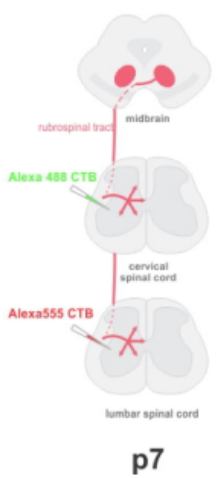


Figure 4.8 Correlation of gene expression with anatomically identified rubrospinal subpopulations Expression of subpopulation candidate markers was examined in retrogradely identified cervical and lumbar rubrospinal neurons at P7. Fluorescent images were taken, followed by in situ hybridization analysis of candidate RNA expression on the same sections. ISH images were then overlaid onto the fluorescent images. *Tshz3* expression is restricted to cervical rubrospinal neurons within the caudal RN (A-C), whereas *fam19a4* is expressed in lumbar projection neurons (D-F). *Cxcl13* appears to be expressed in a subpopulation of neurons across both the cervical and lumbar domains (G-I). Double fluorescent in situ hybridization confirms the dorsomedial and ventrolateral expression of *tshz3* and *fam19a4*, respectively (J). MafB and Tshz3 are transcription factors identified in the microarray as being upregulated in the cervical domain. Antibody staining confirms that they are expressed in largely overlapping populations within the dorsomedial RN (K-M).

Figure 4.9





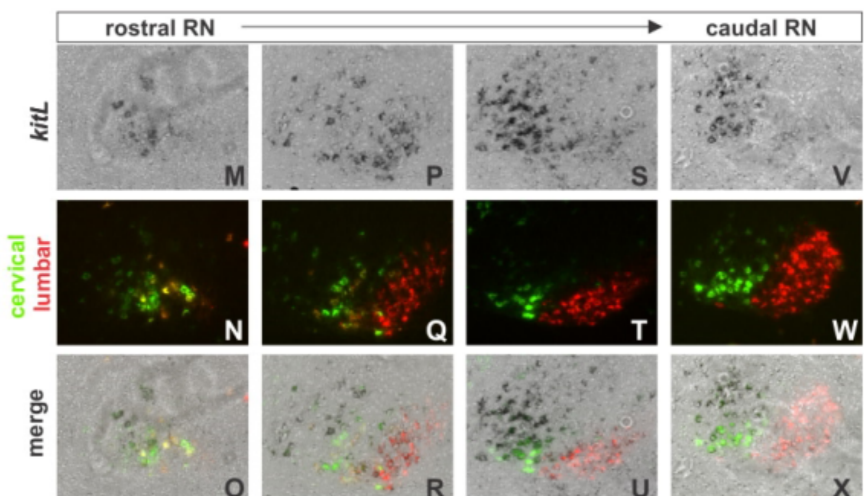


Figure 4.9 Analysis of gene expression in cervical-projecting rubrospinal neurons Tshz3, kitL, and mafB were identified in the microarray as being upregulated in the caudal cervical domain. Tshz3 and mafB appear to be expressed in an overlapping population of neurons in the dorsomedial RN at intermediate and caudal levels, but excluded from the rostral and ventrolateral intermediate domains. Comparison of *kitl* with *tshz3* and *mafb* expression by double FISH indicates that *kitl* is expressed in an additional population of neurons in the lateral RN at intermediate levels (arrow in B, H). Furthermore, *kitl* expression appears to be restricted within the dorsomedial domain at intermediate levels, compared to *tshz3*/*mafB* (asterisks in C,I). At caudal levels however, *kitl* expression overlaps with that of *tshz3* and *mafB* (D-F, J-L). Kitl expression was then evaluated in spinal projection neurons. In the rostral RN, cervical, lumbar, and dual-projection neurons express *kitl* (M-R), whereas at caudal levels, *kitl* appears to be downregulated in the lumbar population (S-X). *Kitl* is the first identified gene to be expressed in subpopulations of rubrospinal neurons in both the rostral and caudal RN.

Figure 4.10

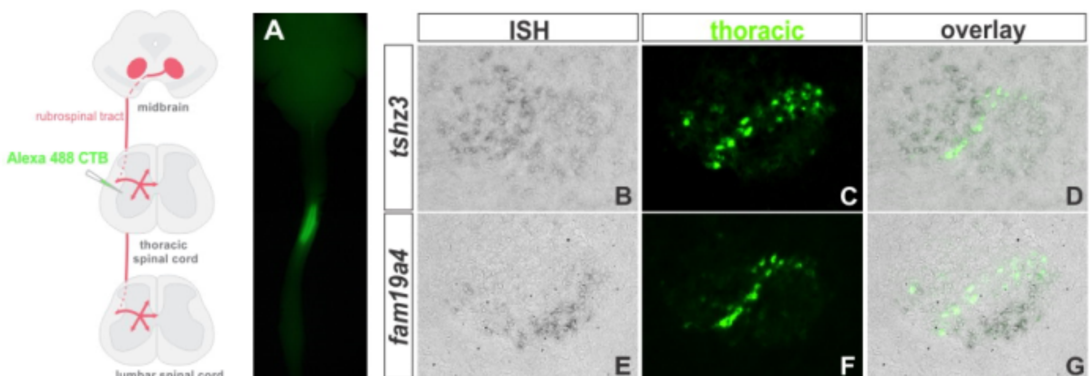


Figure 4.10 Thoracic rubrospinal neurons express *tshz3* but not *fam19a4* To determine whether gene expression in thoracic neurons overlaps with either the cervical or lumbar population, expression of the cervical marker, *tshz3*, and the lumbar marker, *fam19a4*, was examined in retrogradely labeled thoracic neurons. RNA expression revealed that thoracic neurons expressed *tshz3* (B-D) but not *fam19a4* (E-G) demonstrating molecular similarity between cervical and thoracic populations.

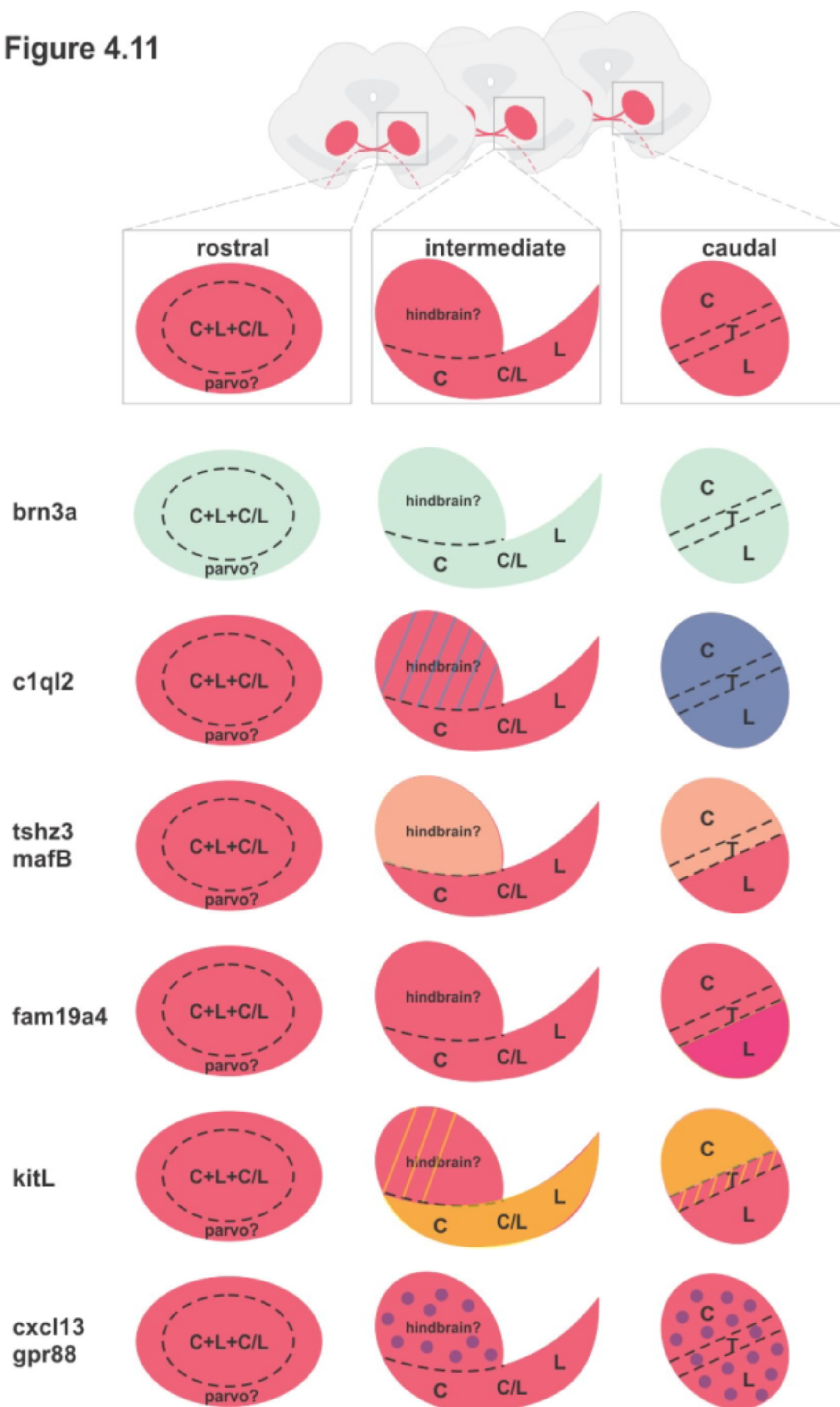


Figure 4.11 Summary of gene expression in rubrospinal subpopulation

Figure 4.12

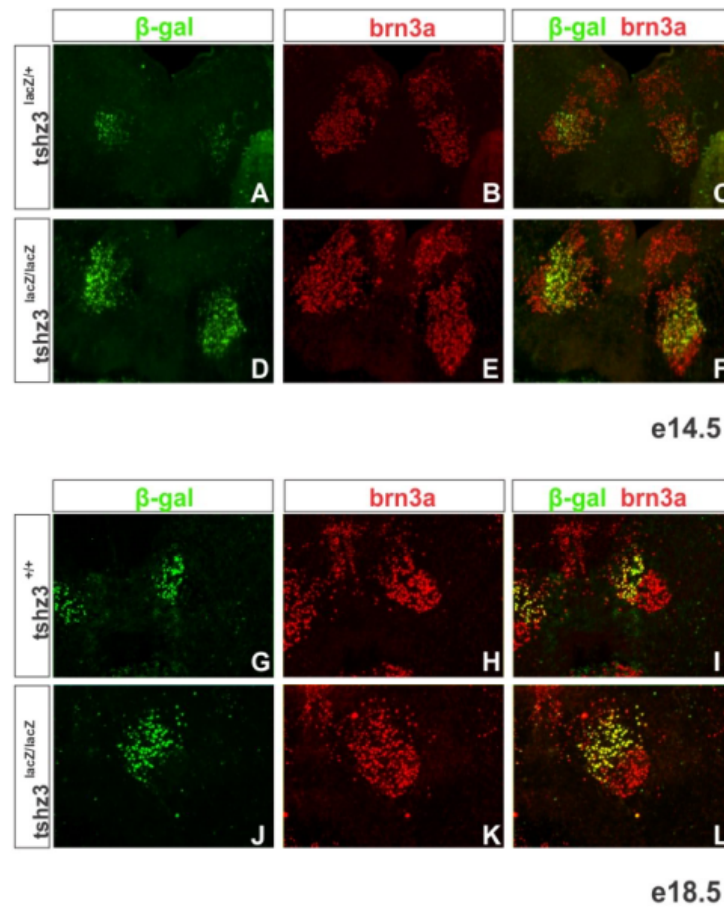
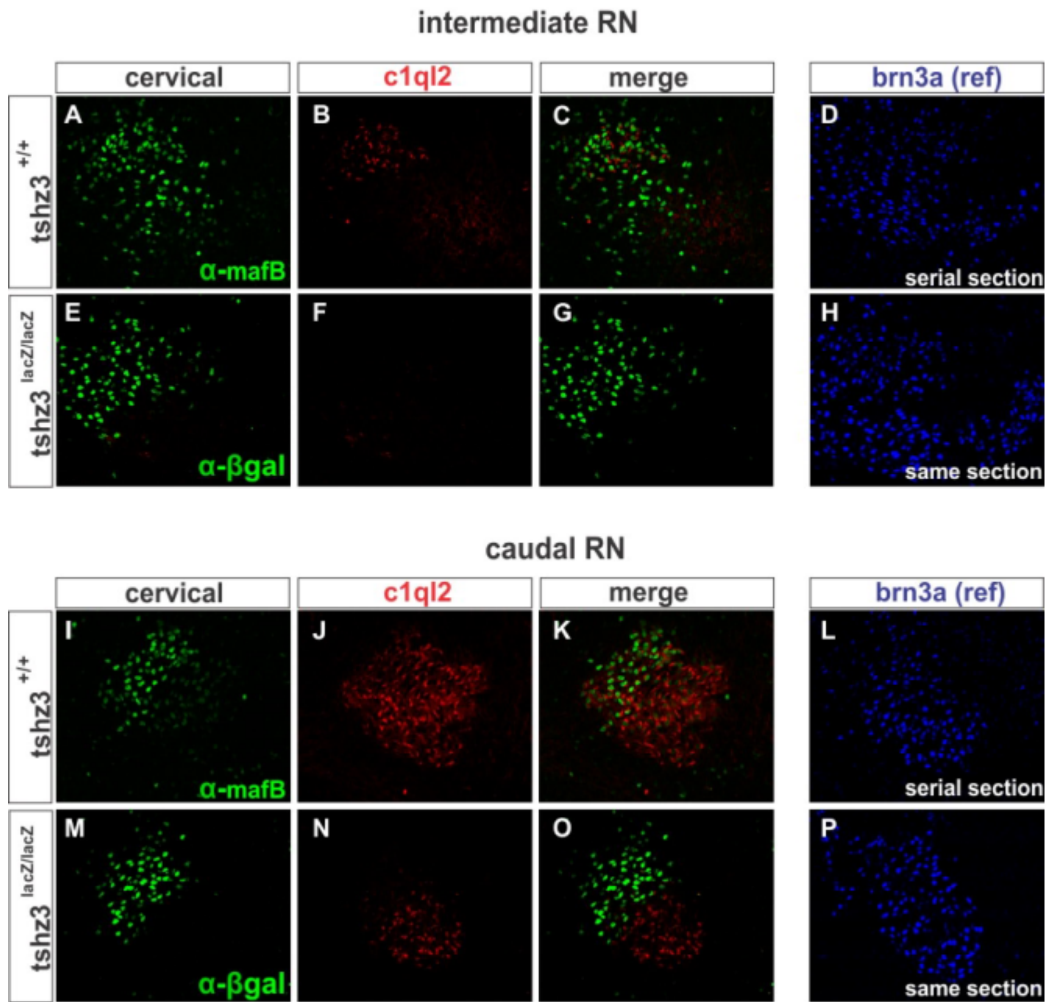


Figure 4.12 A loss of tshz3 does not affect early or late survival of RN neurons To determine whether tshz3 is required for the survival of cervical RN neurons, B-galactosidase expression was examined in Tshz3 lacZ/lacZ mutant e14.5 RN. B-gal expression demonstrates that dorsomedial neurons are still generated in the absence of tshz3 (D-F) in similar numbers as Tshz3 lacz/+ heterozygotes (A-C). B-gal expression was still present at e18.5 in Tshz3 lacZ/lacZ mutants (J-L), ruling out a role for tshz3 in the late survival of RN neurons.

Figure 4.13



Q

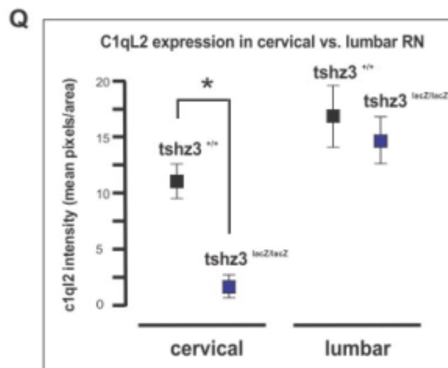


Figure 4.13 C1qL2 expression is selectively lost in the cervical domain of tshz3 mutants To determine whether tshz3 is responsible for the induction of downstream genetic programs in cervical RN neurons, gene expression was analyzed in tshz3 mutants. Brn3a was expressed in the absence of tshz3 (H, P), indicating that its expression is upstream of tshz3. However, C1qL2 expression was absentfrom the cervical RN (identified by B-gal or MafB immunostaining) in the absence of tshz3, although its expression was maintained in the lumbar domain. At intermediate RN levels, C1qL2 is first observed in the dorsomedial RN (A-C). This expression is completely lost in Tshz3 mutants (E-G). At caudal levels, C1qL2 is normally expressed throughout the entirety of the red nucleus (I-K), although levels appear to be slightly higher in the ventrolateral half (quantified in Q) even under normal conditions (I-K). In tshz3 mutants, c1ql2 is completely absent from the dorsomedial RN, but maintained within the ventrolateral RN (M-O). Quantification of C1qL2 mean pixel levels indicates a significant decrease of C1qL2 expression in the cervical domain Tshz3 LacZ/LacZ mutants as compared to WT (11.55 vs. 2.47), whereas lumbar levels between WTs and mutants are not significantly different (Q).

Discussion

There are two distinct spinal projection groups within the red nucleus

Although generally considered as one structure, the red nucleus has been subdivided into two functionally, anatomically, and histologically distinct regions: the rostral 'parvocellular' red nucleus, projecting to the inferior olive, and the caudal 'magnocellular' red nucleus which projects to the spinal cord. However, recent work in the cat (Pong et al., 2002) demonstrating that the parvocellular red nucleus projects to upper cervical spinal levels indicates that the parvocellular vs. magnocellular distinction may not be completely accurate.

I have previously identified two molecularly distinct populations within the red nucleus: A rostral Brn3a^{+} C1qL2^{-} populations, and a caudal Brn3a^{+} C1qL2^{+} population (figure 2.1a). My retrograde labeling experiments confirm that both the rostral and caudal red nucleus in the mouse contain spinal neurons that project the entire length of the spinal cord. The rostral rubrospinal neurons map to the Brn3a+ domain, whereas the caudal spinal group is contained within the Brn3a^{+} C1qL2^{+} domain, establishing a molecular distinction between these two populations of rubrospinal neurons. Interestingly, although both the rostral and caudal RN contain cervical and lumbar projection neurons, the segregation of these two populations within each domain is quite different. Whereas the caudal RN contained the expected somatotopic arrangement of cervical and lumbar neurons along the dorso-ventral and rostro-caudal axis, the rostral cervical and lumbar projection neurons were intermingled. Thus, although both rostral and caudal RN contain a significant spinal

projection, the anatomical and molecular differences that I have demonstrated supports a hypothesis that these two groups have different functional purposes in the control of motor output.

One potential reason for the clustering of neurons projecting to conserved target regions is that it also facilitates the topographic organization of afferent inputs onto these neurons. The parvocellular and magnocellular red nuclei both receive afferent input from the deep cerebellar nuclei. However, rostral RN inputs are from the dentate nucleus whereas caudal RN inputs are from the interpositus nucleus. A similar topographic organization has been demonstrated in projections from the interpositus nucleus to RNm (Daniel et al., 1988), but has not been investigated in the projections to RNp from the dentate. Given the lack of somatotopic organization within the spinal neurons of RNp, one would predict that the inputs from the dentate are equally mixed.

Finally, I have demonstrated that RNp contains a population of 'dual' neurons that send projections to both cervical and lumbar levels. One can imagine the purpose of these projections to be the coordination of forelimb and hindlimb activity, perhaps during movement or locomotion. However, an understanding of the post-synaptic targets of these neurons is necessary to further elucidate their unique contribution to motor output.

Somatotopic organization within the 'magnocellular' RN

I have confirmed that the caudal C1qL2$^+$ red nucleus contains distinct subdivisions correlating to cervical, thoracic, and lumbar projection neurons. Although the dorsomedial and ventrolateral divisions within the red nucleus corresponding to cervical and lumbar projection neurons, respectively, has previously been established, the rostro-caudal distribution of these two subdivisions within the red nucleus has not been well characterized. Retrograde labeling experiments demonstrate that the cervical subdivision of the magnocellular red nucleus is situated rostrally, whereas the lumbar subdivision occupies the caudal-most red nucleus (data not shown). This anatomical information proved crucial for LCM experiments isolating cervical and lumbar RN subdivisions for microarray analysis.

Interestingly, the rostral-most population of C1qL2$^+$ neurons is consistently unlabeled by spinal injections. The 'rubrospinal' designation has historically included projections from the magnocellular red nucleus to brainstem sensory and motor nuclei, which are thought to serve a similar function within hindlimb motor nuclei as those to the spinal cord. The distribution of brainstem-projecting neurons within the caudal red nucleus is unknown. Given that the rostro-caudal distribution of rubrospinal cell bodies in the C1qL2$^+$ caudal domain mirrors the rostro-caudal axis of their target regions in the spinal cord, I would hypothesize that the rostral-most C1qL2$^+$ neurons represent projections to the hindbrain.

Molecular correlates of rubrospinal subpopulations

Identifying patterns of gene expression within a larger structure enables the identification, access, and manipulation of individual aspects of a circuit. It also provides much needed insight into the organizational strategy used by a particular system to accurately and efficiently achieve its functional purpose. Although post-synaptic spinal targets of the rubrospinal tract have been identified (Jankowska, 1988), the molecular correlates of rubrospinal subsets projecting to various interneuron and motor neuron classes remains unknown.

There are three anatomically distinct domains within the red nucleus: a rostral domain which contains an intermingling of spinal projection neurons, a dorsomedial caudal domain corresponding to cervical projection neurons, and a ventrolateral caudal domain containing lumbar projection neurons. To identify potential molecules involved in various aspects of this topography, including the determination of cellular identity, cell body sorting, and axon guidance cues, I used microarray analysis to compare gene expression in these three populations at e17.5, an age in which the rubrospinal circuit is being formed. My study of gene expression within the red nucleus was aimed at addressing the following questions: 1. Can I identify molecular markers of rubrospinal divisions that may provide insight into the genetic regulatory networks that establish topography within the red nucleus? 2. What are the molecular mechanisms by which rubrospinal neurons select a particular spinal termination level? 3. Are there genes that correlate to specific functional subpopulations of rubrospinal neurons?

Differential gene expression in cervical and lumbar domains of the caudal RN

Although there are still a number of candidate genes to screen, I have identified two transcription factors which are restricted to rubrospinal neurons projecting to the cervical and thoracic spinal cord: *tshz3* and *mafB.* Immunostaining indicates that their protein expression overlaps within the dorsomedial half of the caudal red nucleus, although the tshz3+ population might extend further ventrally. I have also confirmed that tshz3 is expressed in thoracic neurons located between the cervical and lumbar domains, but do not know whether thoracic neurons also co-express mafB. Thus, it is possible that the differential expression of mafB might distinguish cervical and thoracic neurons.

The expression of multiple transcription factors in a given population raises the possibility that these genes exist within a genetic regulatory pathway, or that their expression is independently controlled. In either scenario, they can be responsible for regulating distinct aspects of neuronal development and connectivity. The possibility that one gene may be upstream of the other in a genetic pathway would be supported by a demonstration of staggered timing of gene expression onset within the developing RN. Likewise, maintenance of gene expression in the absence of the other transcription factor would support the existence of parallel regulatory pathways. I have acquired tshz3 mutant animals and have begun to examine gene expression in these animals. Brn3a expression is maintained in the cervical domain of tshz3 mutants, which is expected considering its immediate post-mitotic expression in all

neurons within the red nucleus (Prakash et al., 2009). It remains to be seen whether mafB expression remains on in the absence of tshz3.

In addition, transcription factors can not only initiate genetic programs that promote cellular identity, but can also repress transcriptional profiles leading to alternate cell fates (Dasen et al., 2005). Whether repressive transcriptional activity is required for the establishment of somatotopic domains within the red nucleus is unclear. It will be interesting to examine the emergence of lumbar markers such as *fam19a4* in the dorsomedial red nucleus of tshz3 knockouts to determine whether lumbar fate must be actively repressed. The homologous *teashirt* gene in *drosophila* is known to act as a transcriptional repressor, thus it is possible that mammalian *tshz3* plays a similar role in the developing red nucleus (Waltzer et al., 2001).

Molecular mediators of rubrospinal circuitry

C1qL2

I have established that the differential expression of C1qL2 delineates the rostral and caudal rubrospinal populations. Although the functional distinction between these two populations is unclear, the topographic organization within each domain differs significantly. C1qL2 is a member of the C1q/TNF family of proteins, of which multiple family members have been implicated in aspects of synaptic development, maintenance, and refinement (Chu et al., 2010; Hirai et al., 2005; Stevens et al., 2007). It is possible that C1qL2 plays a role in the establishment and/or refinement of specificity within the rubrospinal circuit, or has a novel function,

such as the establishment of somatotopy within rubrospinal populations. I have acquired *c1ql2* mutant animals, and am in the process of analyzing rubrospinal projections, both anterogradely to investigate the specificity of spinal circuitry, and retrogradely, to investigate the organization of rubrospinal neurons within the red nucleus.

KitL

The red nucleus of the mouse contains two distinct spinal representations within its rostral and caudal halves. Although the somatotopic organization within each region differs, I have demonstrated that both contain projection neurons to all levels of the spinal cord. Nonetheless, the majority of somatotopically relevant genes that I have identified are restricted to the cervical or lumbar domains of the caudal RN (*tshz3*, *mafB*, *fam19a4*). However, the expression pattern of *kitL* (*steel factor, stem cell factor SCF*) is unique in that it includes both the cervical domain of the caudal RN and both cervical and lumbar rubrospinal neurons in the rostral red nucleus. Furthermore, within the dorsomedial red nucleus, its expression is limited at intermediate levels, as compared to *tshz3* and *fam19a4*. This rostral-most region of the c1ql2+ dorsomedial red nucleus is predicted to contain projection neurons to motor nuclei of the hindbrain. KitL and its receptor, kit, have been identified as key regulators of axon guidance in commissural neurons (Gore et al., 2008). The role of kitL in establishing rubrospinal connectivity is unclear, although given its apparent distinction between hindbrain and spinal projection neurons, it is possible that kitL plays a key role in mediating this decision point.

Tshz3

Unfortunately, it is not possible to study the functional consequences of a loss of *tshz3* in cervical RN projection neurons, as the tshz3$^{lacZ/lacZ}$ mutants die at P0 due to defects in the respiratory circuit (Caubit et al., 2010). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) tracing experiments in P0 mice indicate that although the corticospinal projections have reached the cervical spinal cord at this age, the rubrospinal tract has not (data not shown). Nonetheless, transcriptional networks control a wide-range of early and late developmental cellular processes, such as differentiation, maturation, and circuitry.

I first examined neuronal survival in tshz3 mutants to determine if tshz3 was necessary for cell survival at early (e14.5) and late (e18.5) stages, and found that dorsomedial neurons were present in the same approximate numbers as in the WT at all ages examined. Although I did not observe a survival phenotype in tshz3 mutants, it was apparent that even at e14.5, tshz3 expression is restricted to the dorsomedial half of the red nucleus, indicating that not only are molecular subpopulations established at an early age, but that the somatotopy within the red nucleus is established prior to axon outgrowth.

As transcription factors are ultimately responsible for controlling the expression of downstream ‘effector’ genes, I then analyzed the expression of C1qL2 in tshz3 mutants. Members of the C1q/TNF family have been shown to play a role in multiple aspects of synaptic function, including synapse both stabilization and

elimination (Chu et al., 2010; Hirai et al., 2005; Stevens et al., 2007). C1qL2 is a novel family member whose function is undefined (Iijima et al., 2010). The restricted loss of C1qL2 expression in the cervical domain of *tshz3* mutants is particularly striking given its uniform expression in both cervical and lumbar neurons in the caudal red nucleus. One would perhaps expect its expression to be controlled by a common mechanism instead of distinct regulatory pathways in cervical and lumbar neurons. There is still the possibility that RNA levels of C1qL2 remain uniform, and that instead there is cervical vs. lumbar regulation at the protein level.

Fam19a4

Finally, although I have not yet identified a transcription factor expressed in the ventrolateral lumbar domain of the red nucleus, I have confirmed that the chemokine, fam19a4, is expressed in retrogradely labeled lumbar-projecting rubrospinal neurons in the caudal RN. Chemokine ligand-receptor pairs are known to play a role in axon guidance in the developing CNS (Lieberam et al., 2005). Future studies will require the identification of a putative receptor for fam19a4, and the generation of molecular tools with which to study its functional role in the patterning of rubrospinal projections to the lumbar spinal cord.

Functional subpopulations within cervical and lumbar domains

Although many post-synaptic targets of descending motor tracts have been identified, the circuitry of individual populations of supraspinal neurons has never been investigated. In vivo recordings of rubrospinal activity during movement

indicate that individual neurons are engaged in a temporal and activity specific manner (Gibson et al., 1985a, b), supporting the hypothesis that the red nucleus contains functionally distinct subsets within a larger organization. Furthermore, the results from chapters 2 and 3 demonstrate that a population of rubrospinal neurons are capable of making direct contacts onto motor neurons, which would likely be responsible for a different behavioral outcome than projections onto interneurons. Therefore, a next major step towards understanding the individual circuits contributing to rubrospinal functional output would be to identify and assign molecular correlates of subpopulations within the larger somatotopic organization.

I have identified two genes whose expression is restricted to a subset of neurons across both the cervical and lumbar domains of the caudal RN. The first, *cxcl13,* is a secreted chemokine. Although this gene might be expressed in a subset of rubrospinal neurons with a conserved post-synaptic spinal target, its nature as a secreted factor suggests that its molecular role is more likely to be in regulating pre-synaptic afferent inputs onto these neurons. Although expression databases indicate that its receptor, CXCR5, is expressed in the deep cerebellar nuclei (Forster et al., 1994), which provide the majority of inputs to the magnocellular red nucleus, the timing and localization of CXCR5 expression requires further detailed investigation. The second gene identified as a rubrospinal subpopulation marker is *gpr88*, an orphan trans-membrane g-protein coupled receptor, whose CNS expression is mainly restricted to striatal dopaminergic regions. As with *cxcl13*, its expression is also restricted to a subset of neurons across both the cervical and lumbar domains.

The prevalence of molecular subpopulations across rubrospinal domains projecting to all spinal levels indicates that the molecular strategies employed to determine spinal termination level vs. post-synaptic target choice might be independent. Although it is possible that there are functional subpopulations contained within the cervical or lumbar domains, I have yet to provide molecular evidence in support of this. Rubro-motoneuronal cells are most likely located within the dorsomedial region of the caudal red nucleus, given the location of the ED motor pool within the cervical enlargement. As of now, there has been no evidence of direct rubrospinal projections onto lumbar motor neurons in rodents, but this needs to be explored in further detail. If so, there might be aspects of a common molecular program shared between cervical and lumbar rubro-motoneuronal cells. If direct projections are limited to cervical motor neurons, I would expect there to exist a molecularly distinct subpopulation of neurons within just the cervical domain. Future experiments to identify and profile gene expression in direct projecting rubrospinal neurons will be discussed in the following chapter.

Conclusions

One of the key motivations to pursue the experiments outlined in this chapter was to provide a foundation of anatomical and molecular organization within the red nucleus with which to correlate the functional circuitry that I have established, namely the specificity of projections onto a single cervical motor pool. I have identified molecular substrates of the topographic domains observed within the rodent RN, and suggested the existence of molecularly distinct subpopulations within these

subdivisions. Although future experiments are required to provide a definitive link between molecular populations and their post-synaptic targets, this molecular 'description' of the red nucleus already serves to provide us with insight into the molecular strategy employed by the rubrospinal system to ensure precision and specificity within the circuit. Furthermore, it will be an essential tool enabling the genetic dissection of individual circuits within the rubrospinal system.

Chapter 5: Future directions and general discussion

In this thesis, I have established that in the rodent, cervical level motor neurons receive monosynaptic inputs from supraspinal motor regions. Specifically, I have determined that the red nucleus, a midbrain motor center that sends a large descending projection to the spinal cord, forms direct connections onto a single pool of motor neurons innervating the forelimb extensor digitorum muscle. This level of input specificity onto a single MN pool does not appear to be replicated by any other supraspinal, sensory, or interneuron population that I have examined. Despite the wealth of information about the functional role of descending projections, and specifically the significance of direct inputs onto motor neurons in the execution of skilled movement, their functional consequences remain to be directly determined. The degree of specificity observed in the rodent rubrospinal circuit seems to be unique, and implies 1. A high degree of specialization within the red nucleus, whose molecular diversity I have demonstrated for the first time 2. A highly specific level of guidance/refinement of rubrospinal axons during development and 3. A critical conserved role for the extensor digitorum muscle in the execution of skilled movement.

Although the majority of the rubrospinal tract projects onto interneurons, a small number of rubrospinal axons are capable of separating from the majority of the rubrospinal tract, and charting a new trajectory into the ventral horn, where from amongst a number of MN pools, they eventually synapse upon a very specific target.

How is this specificity encoded during development? Does the circuit develop specifically from its onset, or are the projections onto motor neurons initially exuberant, and then refined during a so-called 'critical period'? In the first half of this chapter, I will explore the possible mechanisms and strategies by which this circuit might be established. Then in the second part of this chapter, I will discuss how knowing the genetic 'identity' of individual rubro-motoneuronal cells would provide us with an incredible tool with which to study and manipulate the direct circuit for the first time.

Molecular establishment of the rubro-motoneuronal circuit in rodents

Identification of rubro-motoneuronal cells

The rubrospinal projection onto motor neurons represents only a small fraction the total fibers in the tract. Therefore, I would predict that rubro-motoneuronal cells comprise only a small percentage of neurons within the red nucleus. Since the C1qL2 reporter line labels the dorsolateral ventral horn-projecting rubrospinal axons, I would expect these neurons to be located within the Brn3a+ C1qL2+ caudal magnocellular domain, and express the appropriate cervical RN markers (tshz3, mafB, kitL) that I have identified. Within the topographic organization of the red nucleus, it will be interesting to see whether there is also a functional organization based on post-synaptic target type, represented by a clustering or segregation of rubro-motoneuronal cells. There is certainly a precedent for this type of organization in the CNS, for example the motor pool organization of MNs sharing the same muscle target seen in the spinal cord.

To identify rubrospinal neurons that make monosynaptic connections onto the ED motor pool, we hope to take advantage of trans-synaptic tracing from the extensor digitorum muscle, utilizing a circuit tracing method developed by Ed Callaway (Wickersham et al., 2007) and modified by Silvia Arber (Stepien et al., 2010). The ability of certain neuronal tracers (e.g. wheat germ agglutinin) to cross multiple synapses has been utilized to study connectivity within a neural circuit (Fabian and Coulter, 1985). In recent years, this technique has been facilitated by the development of fluorescent protein-expressing neurotropic viruses that can be used as anterograde or retrograde trans-synaptic tracers. These viruses fall into two classes, the alpha-herpesviruses (including pseudorabies virus) and the rabies viruses, both of which infect cells along a neural circuit in the retrograde direction (Card et al., 1990; Ugolini, 1995). Although trans-synaptic viruses have been indispensable tools in furthering our understanding of connectivity within a neural network, the order of cellular linkage can be difficult to decipher. The distinction between neurons linked monosynaptically and those linked polysynaptically is particularly difficult to decipher as the timecourse of trans-synaptic crossing varies based on viral concentration and the density of pre-synaptic terminations (Card et al., 1999; Ugolini, 1995).

With this in mind, the Callaway lab modified the widely used rabies virus to enable the unambiguous identification of monosynaptically linked neurons in a circuit (Wickersham et al., 2007). The rabies virion requires a membrane glycoprotein (G protein) on its surface to infect a neuron across a synapse. In the modified system,

the G protein is deleted from the rabies viral genome (rabies deltaG), thus limiting the viral spread to the initially infected neuron. However, if this initial cell can independently produce the G-protein, complete viral particles can be produced, and all neurons monosynaptically connected to the initial cell will be infected. Viral spread will cease beyond this point. Although this approach represents a significant advancement in the field of neural circuitry, two major limitations of note are the difficulty of selectively infecting a cell population of interest, and the question of how to specifically express the g-protein in this infected subpopulation. While an in vitro culture system can be manipulated via DNA transfection (Wickersham et al., 2007), in vivo tracing experiments have required the generation of transgenic mouse lines, a time intensive approach.

The Arber lab has developed an elegant solution to address both of those issues, specifically designed to facilitate analysis of the pre-motor spinal circuitry upstream of individual motor pools (Stepien et al., 2010). Motor pool specificity can be accomplished via injection of the modified deltaG rabies virus into individual muscles (figure 5.1 a-c). Expression of the missing G protein in the same MNs is accomplished by co-injection of AAV-G Protein (figure 5.1 d-g) With this approach, the entire pre-motor network projecting onto a specific motor pool can be identified, including any direct supraspinal projections. Thus, we expect that a co-injection of Rabies-deltaG virus and AAV2::G-protein into the extensor digitorum muscle would infect the rubrospinal neurons that project directly onto the ED motor pool (figure

Figure 5.1

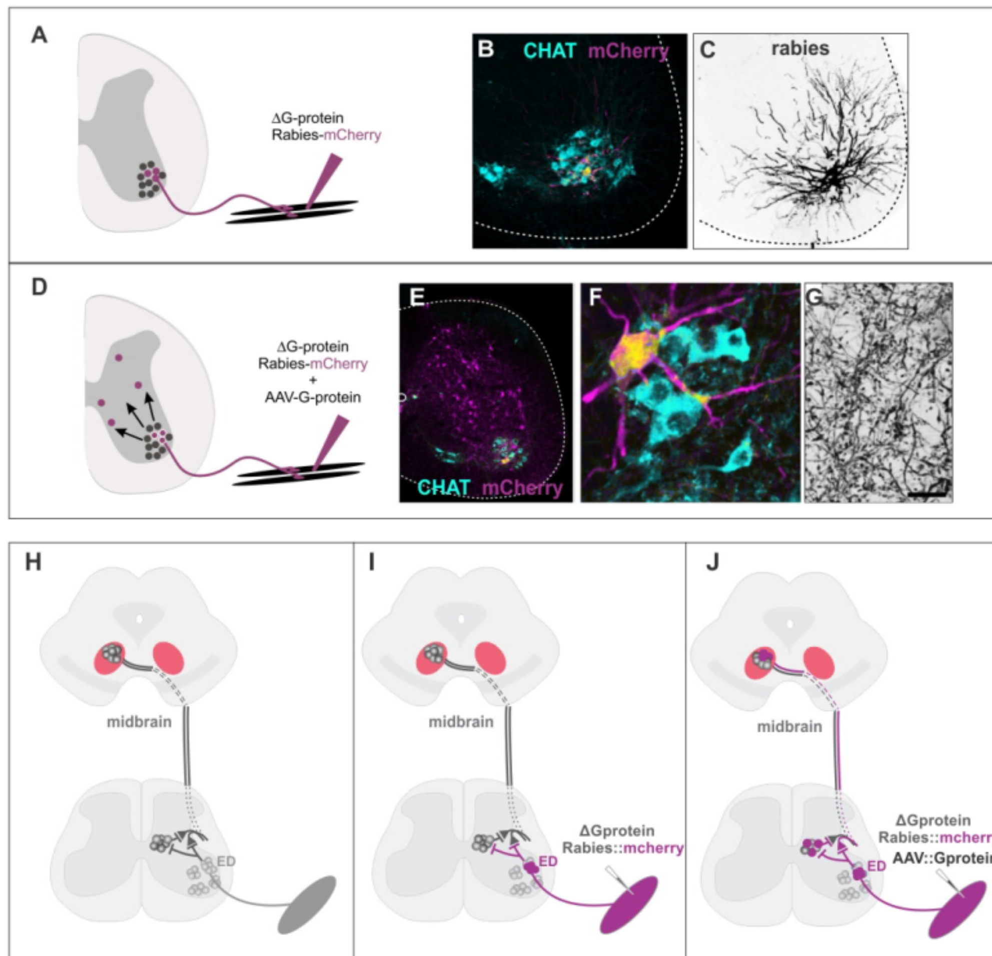


Figure 5.1 Monosynaptic tracing from the ED motor pool A-G: Modification of the Callaway monosynaptic tracing. Injection of a rabies virus lacking a critical protein required for trans-synaptic infection (ΔGprotein Rabies ::mcherry) into a specific muscle will label that motor pool (A-C). If the G protein is in introduced into the motor neurons by co-injection with AAV::Gprotein (D-G), the motor neurons and all monosynaptically connected neurons will then be infected. These first-order neurons lack the G-protein, and trans-synaptic infection will cease. H-I: A subset of rubrospinal neurons project directly onto ED motor neurons (H). If the ED muscle is injected with ΔGprotein Rabies::mcherry, the ED motor pool will be labeled (I). If the ED muscle is co-injected with ΔGprotein Rabies::mcherry and AAV::Gprotein, the ED motor pool and all monosynaptically connected neurons, including interneurons and the population of rubrospinal neurons that project directly onto ED motor neurons, will be labeled with mCherry (J).

From Stepian et al., 2010 (A-G)

5.1 j). Injections into a neighboring muscle, such as the flexor digitorum, would not be expected to label any rubrospinal neurons. We believe that this technique could be essential in answering the following outstanding questions raised by the work in this thesis: 1. Do the monosynaptic projections onto the extensor digitorum motor pool arise from the red nucleus? 2. Do neighboring motor pools lack direct rubrospinal input? and 3. Are distinct groups of rubrospinal neurons projecting directly to the ED motor pool segregated within the red nucleus?

Although many lines of evidence support our assertion that direct projections onto motor neurons from the midbrain originate within the red nucleus, such as the similarity of projections labeled in previous tracing experiments, the ventral projections seen in the c1ql2 reporter lines, and the co-localization of vglut2 in labeled descending terminals onto ED motor neurons, the fact remains that there are other descending tracts located in close proximity to the red nucleus, and inevitably labeled by my midbrain viral injections. Monosynaptic tracing from the extensor digitorum motor pool would provide the first definitive evidence that these inputs are in fact rubrospinal in origin.

Based on anterograde anatomical tracing experiments, I have also made the claim that of the motor nuclei studied, the extensor digitorum motor pool is the main recipient of monosynaptic rubrospinal inputs. The flexor digitorum, and other forearm muscle motor pools appear to be devoid of direct inputs from the red nucleus, although the intrinsic footpad muscles do appear to receive a small number of direct

inputs. Therefore, we can harness the inherent specificity of the monosynaptic tracing method to further strengthen our result suggesting that the direct circuit from the RN to the ED motor pool plays a specialized role in motor output in the rodent. Although viral co-injections into other accessible forelimb muscles should not result in labeling within the red nucleus, we will focus our initial comparison on injections into the extensor digitorum, flexor digitorum, and ventral footpad muscles.

Beyond the necessary confirmation that rubrospinal neurons project directly onto motor neurons, this line of experiments will reveal for the first time the location of rubro-motoneuronal cells in the rodent (or any other species). Whether these neurons reside in their own sub-nucleus within the larger population of the red nucleus will be important to determine. I have identified a single motor pool that receives dense rubrospinal input (the extensor digitorum) and an additional motor pool that receives limited input (the ventral footpad). Nonetheless, co-injection of the modified rabies virus and AAV::G protein into either muscle should result in the labeling of rubro-motoneurons within the red nucleus. It remains to be seen whether rubrospinal neurons projecting to these two distinct motor neuron targets will be segregated in an organizational scheme reminiscent of the motor pool distribution within the spinal cord. Finally, identification of rubro-motoneuronal cells is the first requirement in the molecular characterization of these neurons (discussed in the following section), which would allow unprecedented genetic access to a circuit that has intrigued scientists for many years.

Monosynaptic tracing from individual motor pools has begun to reveal intriguing levels of motor pool specificity in pre-motor spinal circuits (Stepien et al., 2010). We have high hopes that it will prove indispensable in our rubrospinal tracing studies as well. However, the main caveat of this modified monosynaptic tracing technique to-date is the inability of the rabies virus to efficiently infect MNs beyond the first 10 postnatal days (Stepien et al., 2010). Unfortunately, the success of the experiment is therefore dependent on the timing and nature of rubrospinal tract development, about which little is known. The rubrospinal tract in the rat has been shown to reach the cervical spinal cord by P0 (Shieh et al., 1983), thus would be expected to reach equivalent spinal levels in the mouse by the first couple postnatal days. Preliminary DiI tracing experiments that I have performed in newborn mice demonstrate that the rubrospinal tract does not reach the cervical enlargement by P0 (data not shown), and in fact, appears to lag behind the corticospinal tract in this regard (Bareyre et al., 2005). I would predict that the rubrospinal tract projects down the length of the spinal cord during the first postnatal week, and should have innervated its appropriate target region by P10.

The second concern is the manner in which the final rubrospinal circuit is established. Although we have established that there is a highly specific and reproducible rubrospinal projection onto only the ED motor pool in the adult mouse, the pattern of rubrospinal innervation during development is unknown. It is not unprecedented for a circuit to be promiscuous in its early days, and then undergo a process of refinement by pruning back the exuberant projections during a

developmental period until it reaches its final mature state (Penn et al., 1998). On the opposite side of the spectrum, some circuits come together with what is referred to as 'stringent specificity': the innate ability of two neuronal cells to recognize each other as their lifelong synaptic partner (Betley et al., 2009). We do not know which of these two developmental strategies the rubrospinal tract employs. If it is the latter, then as long as the direct circuit is in place by P10, the specificity of monosynaptic projections onto the ED motor pool should be as expected. However, if the rubrospinal circuit progresses through a period of developmental refinement, we might expect that the specificity of projections onto individual MN pools at p10 does not accurately reflect the specificity observed in the final circuit. It is also possible that the direct and indirect rubrospinal circuits onto motor neurons develop through different strategies.

Descending rubrospinal axons labeled at P12 by the C1qL2::ϕGFP reporter line indicate that the rubrospinal tract has indeed reached the cervical enlargement and entered the gray matter of the spinal cord by this age. I can visualize GFP+ rubrospinal axons projecting into the dorsolateral ventral horn, and forming synaptic connections onto motor neurons. However, given the paucity of GFP expression in this line, it is difficult to ascertain whether the exceptional specificity of the direct rubrospinal circuit is present at this age. Nonetheless, despite the potential experimental concerns, I remain hopeful that the monosynaptic tracing technique will enable the identification of rubro-motoneuronal cells within the red nucleus, and

further molecular and functional experiments expanding upon this technique will be explored in following sections of this chapter.

Molecular establishment of the direct rubro-motoneuronal circuit

The establishment of precise specificity within this circuit critically depends on two key elements. The first is the expression of a molecular profile that serves to distinguish rubro-motoneuronal cells from all other rubrospinal neurons, enabling them to de-fasciculate from the main tract at the appropriate spinal level, navigate their own distinct trajectory within the ventral horn, before synapsing onto a distinct post-synaptic neuronal class. The second element is the unique molecular identity conferred to the post-synaptic partner, the ED motor neuron, which separates it from other cervical motor neurons, making it uniquely receptive to receiving rubrospinal input. This might include the establishment of cell intrinsic properties, such as the determination or maintenance of a dorsolateral cell body location within the ventral horn, thus placing it in the direct path of incoming ventral projecting rubro-motoneuronal axons. Conversely, ED motor neurons might express a guidance signal or synaptic stabilizer that actively attracts and maintains rubrospinal inputs, or lack the expression of a repellant that prevents the ingrowth of rubrospinal axons. It is also possible that multiple guidance strategies are employed to direct rubrospinal neurons to their final ED motor neuron target. Regardless of the mechanism by which the specificity within this circuit is established, the first step is to identify and molecularly characterize the pre- and post-synaptic components.

We hope that the monosynaptic rabies viral tracing technique detailed above will allow us to identify and visualize the rubro-motoneuronal cells within the red nucleus. To genetically profile these neurons, we can employ the following two experimental approaches to examine gene expression in the fluorescent protein-labeled rubrospinal neurons. The first is to determine whether any of the subpopulation markers already identified in the microarray (chapter 4) co-localize with the rubro-motoneuronal population. Top candidate markers include *cxcl13* and *gpr88,* based on their expression in subpopulations of rubrospinal neurons within the cervical domain.

The second approach is to isolate virally infected neurons by fluorescent LCM and then perform a microarray analysis of gene expression. One potential concern is that there will likely be a predominance of cellular response genes related to the cells' viral infection state. However, we have already accumulated a list of genes that are expressed in the red nucleus under normal conditions, thus we will likely be able to rule out this subset of candidate genes. An additional drawback is that even if I am capable of specifically performing intramuscular viral injections at P0, as the virus takes a further 10 days to replicate and infect first-order neurons, we would only be able to identify rubro-motoneuronal cells at P10. A number of developmentally relevant genes will likely have downregulated their expression by this age. However, a significant number of genes that I do identify will likely have initiated and maintained expression from an early age.

Ideally, there are two 'categories' of genes I am interested in: The first includes transcription factors that may be involved in the establishment of a cellular 'identity,' important for both the initiation and repression of cell fate decisions, and the subsequent activation of genetic pathways. As noted, this first category may be underrepresented in our gene list given the age at which gene expression will be profiled. The second category of genes can be considered 'molecular effectors': the actual proteins responsible for establishing precision and specificity within a circuit. These include axon guidance molecules (ligands/receptors), cellular adhesion molecules (fasciculation/defasciculation, cell-cell recognition), and proteins involved in various aspects of synaptic development or maintenance.

Profiling of the Extensor Digitorum motor pool

On the post-synaptic side, the level of specificity in the direct rubrospinal circuit is equally astounding. Faced with a choice of hundreds of potential motor neuron targets, rubrospinal axons are capable of selecting a single motor neuron pool with which to synapse or maintain synaptic contact upon. How do the extensor digitorum MNs distinguish themselves from other MNs? Do the ED MNs actively attract incoming rubrospinal axons, or is it simply a case of being in the right place at the right time? There is also the additional possibility that initial motor neuron innervation lacks the precision of the adult circuit, and the extensor digitorum motor pool maintains rubrospinal innervation through activity dependent mechanisms.

I propose to genetically profile the extensor digitorum motor pool during the time period in which rubrospinal axons are projecting to the spinal cord and selecting a post-synaptic target. The extensor digitorum motor pool can be identified by injecting a fluorescent retrograde tracer (Alexa488-CTB) into the muscle, and the labeled motor neurons can then be isolated using fluorescent laser capture microdissection, and subjected to microarray analysis. This technique has been recently employed by the Henderson lab to genetically profile single motor neuron pools over a developmental timecourse, and the results have been validated by independent measures of gene expression (Tim Spencer, Chris Henderson). Gene expression in this pool will be compared with a neighboring pool that is devoid of rubrospinal input, the flexor digitorum motor pool. I have not identified another class of pre-synaptic input that differentiates between these two motor pools; both receive similar levels of sensory, corticospinal, and cholinergic C-bouton inputs, although given that these two muscles form an antagonistic pair, the pre-motor networks for each are likely quite separate. It is important to note that I have not yet completed a similar analysis of pre-synaptic inputs in synergistic muscle motor neurons such as the wrist extensors ECU and ECR.

Although the majority of differentially expressed genes will be related to other aspects of motor pool development, such as axon outgrowth and target muscle innervation, we hope to identify candidate genes involved in the specification of pre-synaptic inputs (Arber et al., 2000; Vrieseling and Arber, 2006). If I can evaluate this post-synaptic gene set in the context of the pre-synaptic genetic profiling of rubro-

motoneuronal cells, I might be able to identify putative ligand-receptor or cell adhesion pairs between the two. Possible candidates include classical axon guidance molecules such as semaphorins/plexins and ephrins/ephs (Cohen et al., 2005; Iwasato et al., 2007), chemoattractive pairs such as chemokines and cytokines/receptors (Lerner et al., 2010; Lieberam et al., 2005; Ma et al., 1998), or adhesion molecules such as cadherins (Price et al., 2002). Candidate molecules identified that fulfill the appropriate criteria, such as timing of onset of ligand/receptor expression and specificity within the red nucleus, can then be tested in loss of function scenarios, focusing on the specificity of rubrospinal projections to the ED motor pool. For example, the role of guidance molecules can be tested in loss of function mutant animals by anterograde tracing of rubrospinal projections.

An alternate possibility is that specific cell-cell guidance cues play a secondary role to motor neuron cell body location. Recent work from the Jessell lab, examining the establishment of specificity within the sensory-motor circuit in the developing spinal cord has revealed that incoming sensory neurons appear to have an autonomous pre-determination of their final target region within the spinal cord. If the ventral horn is divided into 'columels,' consisting of the complement of motor neuron pools that innervate synergistic muscles at the same proximo-distal limb position, a sensory neuron has a pre-determined columel target, independent of cues from motor neurons (Gulsen Surmeli, Tom Jessell).

The aforementioned clustering of motor neuron pools into 'columels' that dictate sensory fiber termination zones is one example of an organizational scheme in which motor neuron subdivisions are present within the columnar organization, but that are broader than the level of individual motor pools. Interestingly, vestibulo-motoneuronal projections originating from the Nucleus of Deiters appear to distinguish between extensor and flexor motor neurons in the cat lumbar spinal cord (Lund and Pompeiano, 1968). It is possible that there is an underlying general molecular distinction between extensor and flexor motor neurons that provides the initial specification for descending innervation. Thus, incoming rubro-motoneuronal axons might have an inherent knowledge of their termination zone within the dorsolateral ventral horn; once within that area, a motor neuron target might be selected through other mechanisms.

A motor neuron-dependent vs. -independent guidance mechanism could be tested in mutant animals that lack motor neuron differentiation. One such example of this is the Foxp1 mutant animal (Dasen et al., 2008), in which motor neurons are present but have failed to acquire a further subtype identity. Analysis of rubrospinal projections in these animals would provide valuable insight into the developmental strategy employed by the motor system to establish connections between the red nucleus and the extensor digitorum motor pool. If extensor digitorum motor neurons possess a specific characteristic or identity that renders them "receptive" to receiving rubrospinal input, one might expect that all rubro-motoneuronal connections are abolished in the FoxP1 mutant. Alternatively, rubrospinal neurons might actively

seek out motor neurons innervating the extensor digitorum muscle, as innervation persists but loses its motor pool organization in these animals. Finally, if locational guidance signals play a role in patterning rubrospinal connectivity one might expect to see MNs from the vicinity of the original ED motor pool receiving direct rubrospinal input regardless of their muscle target.

Role of c1ql2 in the establishment of the magnocellular rubrospinal circuit

Although we would like to undertake screening efforts to exhaustively characterize pre- and post-synaptic gene expression, I have already identified a candidate molecule, c1ql2, that I hypothesize will play a role in the establishment of a mature rubrospinal circuit. C1ql2 is part of the C1q/TNF protein superfamily, of which multiple members have been identified as regulators of synaptic development, maintenance, and elimination in the CNS (Chu et al., 2010; Hirai et al., 2005; Stevens et al., 2007). The restricted expression of *c1ql2* to the caudal rubrospinal population suggests that it might play a particular role in establishing the intraspinal circuit that functionally distinguishes this population from the rostral rubrospinal population. I have acquired C1qL2 transgenic knockout mice, and will begin to analyze the accuracy of rubrospinal projections through anterograde viral tracing, based on what I have learned about the specificity of the monosynaptic rubrospinal circuit onto motor neurons.

We predict that these experiments will provide insight into the maturation of specificity within the rubro-motoneuronal circuit. It is well established that in other

systems, the juvenile circuit is initially quite expansive and is then refined through a combination of molecular and activity-dependent mechanisms. On the opposite side of the spectrum, it is possible that the formation of rubro-motoneuronal connections might in fact replicate the specificity seen in the adult circuit. As proteins in the C1q/TNF family have been implicated in circuit refinement through elimination of synapses, it is plausible that C1qL2 might play a similar role in the establishment of the mature rubrospinal circuit. An overabundance of synapses in C1qL2 knockout mice could reflect one of two possibilities: 1. An increase in rubrospinal synaptic density on extensor digitorum motor neurons or 2. An increase in the number of motor pools which receive direct rubrospinal input. Both possibilities would reflect an inherent defect in the refinement of the rubro-motoneuronal circuit, but would also provide insight into the mechanism by which specificity is established. Any aberrations in the direct circuit (i.e. loss of monosynaptic connections, exuberant projections, etc) can be confirmed using the retrograde monosynaptic viral tracing introduced earlier.

Plasticity and compensation amongst descending pathways

One of the dominant theories explaining the role of the corticospinal and rubrospinal tracts is that they function in a complementary manner to each other in the execution of a skilled movement. Specifically, the rubrospinal tract appears to provide tonic support to the hand and digits, whereas the corticospinal tract is thought to layer individual digit movements upon this basal position. I have established that the anatomical substrate of rubrospinal control appears to be a direct circuit onto the

extensor digitorum motor pool. In the rodent, I found no evidence for similarly dense corticospinal projections onto motor neurons, and the prevailing thought is that there is a more general level of diffuse cortico-motorneuronal projections. It is possible that corticospinal control of digit movement may be encoded through a more extensive pre-motor pathway controlling digit motor neurons.

What would be the effect on skilled movement if the inputs from one pathway were to be removed? Would the remaining pathway compensate for the functional loss of the first? Experiments in the rat have demonstrated that if the corticospinal tract is lesioned in the mature animal, the rubrospinal tract is capable of re-wiring its spinal connectivity, albeit in the presence of a myelin neutralizing antibody (Raineteau et al., 2002). One interesting experiment in the light of my data would be to study the extent of rubrospinal tract re-wiring following an acute removal of the corticospinal tract, focusing in particular on the potential expansion of the direct circuit. In parallel, it could be informative to study the effects of a complete developmental loss of the corticospinal tract. In fact, such a tool has already been developed: the Fez1 (Fezf2) knockout animal, in which the corticospinal tract fails to develop (Figure 6.3) (Chen et al., 2005). These animals are viable until adulthood, thus we could trace the circuit of the mature rubrospinal tract in a situation where it has provided the sole descending control over voluntary motor output. These experiments would also shed light on the distribution of rubrospinal and corticospinal pre-motor inputs. There is a great deal of overlap in the spinal territory occupied by each pathway, especially in the intermediate zone. However, the CST expands

dorsally whereas the RST projects further ventral. Do interactions between the two pathways play a role in shaping the final intraspinal circuit? Tracing the rubrospinal circuit in adult Fez1 mutant mice will provide important insight into the amount of inherent plasticity within the rubrospinal tract, and whether compensation of corticospinal function would arise from the rubrospinal tract, or through a re-wiring of other descending and intraspinal circuits. In addition, any resulting defects in forelimb movements during a skilled reach-to-grasp task could be compared with those seen following an acute lesion of the corticospinal tract (Whishaw et al., 1998).

Functional relevance of direct vs. indirect descending projections

At this point, it is widely accepted that direct descending projections onto motor neurons evolved to enable precise and skilled individual digit movements. However, the evidence for this is correlative: an expansion of the cortico-motorneuronal projection is seen in species with increased digit dexterity. Furthermore, when the corticospinal or rubrospinal tracts are lesioned in monkeys, whereas general arm movement is eventually regained, individual digit movements are permanently lost (Lawrence and Kuypers, 1968a, b). However, it has been difficult to separate the indirect and direct contributions of supraspinal pathways. In this section, I will detail the behavioral and genetic experiments that will allow us to definitively characterize and study the direct rubrospinal circuit.

Is activation of the ED muscle responsible for hand/digit extension?

I postulate that the direct activation of the extensor digitorum motor pool by rubrospinal projections is responsible for the maximal hand and digit extension and separation seen during the arpeggio movement. This assertion is supported by three arguments: the functional role of the extensor digitorum as a digit and hand extensor, the loss of this particular component of movement seen after a rubrospinal lesion, and the demonstrated direct circuit from the red nucleus to the ED motor pool. If instead of removing rubrospinal inputs, we inactivated the downstream component of the circuit, the extensor digitorum muscle itself, we would expect to see a similar impairment in forelimb movement during a skilled reach-to-grasp movement.

We can prevent nerve conduction from activating the extensor digitorum muscle by injecting it with a short-term muscle paralyzing agent such as botulinum toxin, which prevents pre-synaptic acetylcholine release at the neuromuscular junction (Kao et al., 1976). High-resolution video analysis of the rat's performance in a reach-to-grasp task will then reveal whether paralysis of just the extensor digitorum muscle will mimic the defects seen after a rubrospinal lesion. Will the loss of ED muscle function result in a removal of the 'tonic support' necessary for fractionated digit movements, and if so, what will be the subsequent effect on digit flexion and grasp? Given that complete rubrospinal lesions affect multiple motor pools through the indirect and direct circuits, the extensor digitorum loss of function experiment would not be expected to replicate all the effects seen after a rubrospinal lesion. Instead, I imagine that any overlap in rubrospinal lesion deficits and ED paralysis

deficits would reveal the relevant aspects of the movement related to indirect and direct rubrospinal projections to the ED motor pool.

Separation of the direct vs. indirect rubrospinal circuits

I have made the assertion that the direct rubrospinal projection to the extensor digitorum motor pool is responsible for the 'whole hand' extension movement seen just prior to and during the arpeggio and grasp components of a skilled forelimb reach-to-grasp movement. This declaration is supported by an overwhelming amount of anatomical and functional evidence. However, I cannot definitively rule out the possibility that the pre-motor rubrospinal circuit is in fact responsible for this movement, and the direct projections onto the ED motor pool serve a separate purpose.

One of the main goals of this thesis was to be able to correlate circuitry with functional output. Our anatomical tracing results have provided insight into the indirect and direct rubrospinal circuits. What we would now like to do is be able to combine these two approaches within the same experiment, allowing us to directly probe the functional significance of the underlying circuit. The single most important step is to be able to distinguish between directly- and indirectly-projecting rubrospinal neurons, and our experimental approach for doing so is detailed in the first part of this chapter. By utilizing what I have discovered about the specificity of the direct rubrospinal circuit, I have developed a tracing assay that I hope allows us to identify rubro-motoneuronal cells in the red nucleus. The most exciting result from

this project would be the ability to selectively manipulate direct rubrospinal neurons. The identification of a genetic driver selectively expressed in rubromotoneuronal cells would allow us to take advantage of the many genetic and optical tools available to study and manipulate the activity of this elusive population of neurons.

Selective lesioning of the direct rubrospinal circuit: effects on digit extension

The first key experiment will be to address the effects of selective lesioning of the direct rubrospinal circuit during the execution of a skilled movement. Given the specificity of this direct circuit, we expect to observe a deficit in digit extension and separation. However, understanding the timing and amplitude of the deficit will be most revealing. Are the direct rubrospinal projections onto the extensor digitorum motor pool responsible for ED muscle activation prior to and during the arpeggio movement? Will a selective lesion of the direct rubrospinal tract completely mimic the effects of ED muscle paralysis during a reach-to-grasp task, or are there other supraspinal and intraspinal pathways that modulate the activity of this muscle during a skilled movement?

Selective lesioning of the direct rubro-motoneuronal cells can be accomplished by the genetic expression of a neurotoxin, such as the diphtheria toxin alpha subunit (DTA), specifically in these neurons (Ivanova et al., 2005; Maxwell et al., 1987). However, an acute or adult lesion would likely be much more informative than a developmental lesion, given the potential for plasticity, compensation, and re-wiring of other spinal pathways. Therefore, we would need to use an ablation

approach that includes a temporal level of control. For example, instead of using a rubro-motoneuronal specific cre driver to induce gene expression in a conditional DTA line, we could instead utilize cre-ER, in which cre recombinase activity is blocked by fusion to the estrogen receptor (Kellendonk et al., 1996). Rather than being constitutively active, Cre activity is initiated by injection of the estrogen receptor agonist, tamoxifen. An alternative approach would be to use a viral delivery approach, in which toxin expression is driven by a rubro-motoneuronal specific promoter.

The behavioral assays that can be used to probe the functional consequences of a loss of direct rubro-motoneuronal connections have already been well characterized (Sacrey et al., 2009; Whishaw et al., 1998). High definition frame-by-frame video analysis of arm and hand position during a reach-to-grasp task in the rodent has proved an incredible resource with which to identify the subtle yet significantly unique contributions of each descending motor pathway. This method also allows for the analysis of digit flexion/extension and separation, providing a quantifiable measure of impairment that will be essential for comparing the effects of complete vs. direct circuit rubrospinal lesions.

Selective lesioning of the direct rubrospinal circuit: effects on overground locomotion

Although the stereotypic forelimb movements utilized by the rodent to execute a skilled reach have been well defined, one would imagine that other normal

behaviors in the rat might also utilize a similar pre-determined set of basic hand movements (or muscle synergies) in different combinations to accomplish the task at hand. Despite the continuing emphasis placed on supraspinal control through muscle synergies, the rubrospinal tract appears to specifically control the activation of a single muscle, the extensor digitorum, through direct projections onto its corresponding motor pool, and the potential role of this direct circuit during skilled reaching has been discussed. It would be interesting to determine whether the direct rubrospinal circuit exerts a similar focused influence over digit extension during other motor behaviors, or whether this circuit has emerged with the specific purpose of modulating hand movements required for finer control of dexterity.

Rodent movements commonly used to evaluate lesions and movement disorders include tests of overground locomotion, ladder rung walking, and cylinder exploration. To evaluate the 'lexicon' of movements that comprise these various tasks, and the overlap of this set with those observed during the skilled reaching task, the Whishaw lab has undertaken a similarly extensive analysis of limb, hand, and digit movement during each of these behaviors (Whishaw et al., 2010). Interestingly, the same three 'components' of movement can be recognized in each behavioral task: release (initial hand lift), collection (digits flexed, hand advancing forward), and manipulation (extension of digits and grasp of object/target).

Of particular interest are the hand movements observed in overground locomotion, as red nucleus lesions lead to clear deficits in this behavior (Muir and

Whishaw, 2000). Furthermore, a rubrospinal tract is observed in species that lack skilled limb movements, suggesting an initial role in more primitive behaviors such as locomotion (ten Donkelaar, 1988). The extended positioning of the hand and digits when the paw is lifted off the ground is similar to the initial phase of skilled reach. Likewise during the swing phase of locomotion, the wrist and digits are fully flexed as the arm moves forward. Finally, in the 'manipulation' step, the digits are first fully extended, and then lowered onto the ground in a pronated manner, reminiscent of the 'arpeggio' movement. Particularly relevant is the observation that during overground locomotion, the digits become fully extended at a precise temporal timepoint during the movement. However, in contrast to skilled reach, although the hand is extended and lowered in a pronated manner, once the hand contacts the ground, there is no subsequent flexion or fractionated digit movements. Analysis of the deficits in overground locomotion following lesioning of just the direct rubrospinal circuit might provide insight into the behavioral context dependency of the rubrospinal projection onto the extensor digitorum motor pool, and its role in the evolutionary development of motor behavior.

The proposed line of experiments evaluating the role of direct rubrospinal projections in the context of functional motor output will not be undertaken in the immediate future, and will require initial development of the experimental tools required. However, I believe that this is the most fundamental direction that we can progress in, and would represent the most significant contribution of this thesis project to the advancement of our understanding of motor control. The role of direct descending projections onto motor neurons in the emergence of skilled movement has

proved an intriguing and controversial subject in neuroscience for the past 50 years. We hope to be at a point where we can, for the first time, distinguish direct from indirect descending projections, and begin to study each circuit individually. While our immediate interest lies in the functional contribution of monosynaptic connections to motor control within the rubrospinal system, we also anticipate the extension of this study to more general unresolved questions regarding the direct modulation of motor neuron activity by supraspinal motor regions.

Physiological relevance of direct descending projections onto motor neurons

Although the existence of direct cortico-motorneuronal and rubro-motoneuronal connections is well established in monkeys, one of the outstanding questions in the field is the physiological relevance of these projections. Are the supraspinal inputs capable of causing a motor neuron to fire independently, or is there instead a method of summation with other pre-synaptic inputs, or another form of modulation that is occurring at the motor neuron level? In addressing this issue for the corticospinal system, Roger Lemon states that the answer to this question can be addressed by the following: 1. How extensive is the cortico-motoneuronal (CM) projection? 2. How many CM cells project to a given motor neuron or motor pool? And 3. How large are the post-synaptic CM effects in a given motor neuron? (Lemon, 2008)

I hope to provide insight into the first two questions from my retrograde labeling experiments. As my anatomical tracing results indicate that the majority of

direct rubrospinal projections are onto a single motor pool, providing the efficiency of labeling is high, monosynaptic tracing should identify a significant proportion of the rubromotoneuronal population. As I have molecular markers to identify the total number of rubrospinal neurons, I will be able to estimate the percentage of these that are rubromotoneuronal neurons. Based on the small number of axons that are seen deviating from the main tract and projecting to the ventral spinal cord, and their restriction to a single spinal level, we predict that this number will be quite low. However, the majority of the direct projection appears to be concentrated on the activation of a single, albeit larger muscle, suggesting that the overall effect on ED motor neuron activity might be fairly significant. In addition, I have found that individual ED motor neurons appear to receive multiple inputs from a single rubrospinal axon.

In the monkey, two methods have been used to estimate the amplitude of CM connections. The first is to measure the monosynaptic EPSP evoked in forelimb motor neurons in response to pyramidal tract or motor cortex stimulation in the anaesthetized monkey. The amplitude of motor neuron response varies from microvolts up to ~5 mVs, with the larger effects seen in motor neurons of the intrinsic hand muscles (Porter and Lemon, 1993). However, this method is not an accurate representation of CM influence; instead of replicating the normal level of activity within the system, large numbers of PT fibers are synchronously stimulated. Furthermore, as both the direct and indirect pathways are active, disynaptic inhibition can decrease the maximal monosynaptic EPSP observed (Maier et al., 1997). An

alternative approach is to use spike-triggered averages (STA) from single identified CM cells to estimate the CM effect. The discharge of a single motor unit or a gross EMG in hand and forelimb muscles can be altered by the activity of a single CM cell (Cheney et al., 1991a; Lemon et al., 1986). While these experiments certainly provide support for, and an estimate of, a strong cortico-motorneuronal effect, we still lack a more direct measurement of the EPSP evoked by the direct supraspinal inputs onto motor neurons, which would be provided by the intracellular recording of MN response following stimulation of the cortico- or rubro-motoneuronal pathway.

Thus, we turn to the emerging field of 'optogenetics', the optical stimulation of populations of genetically specified neurons, which provides us with the ability to not only visualize connections between nerve cells, but to stimulate and record activity within a neural circuit and even correlate it with a behavioral outcome. The earliest examples of such 'optically' driven neural activators relied on the genetic expression of ion channels gated by 'caged' molecules. With a brief flash of light, the molecule would undergo rearrangement, allowing ion flow through the channel (Lima and Miesenbock, 2005). A striking example of this system was the expression of an ATP gated channel in just the two *Drosophila* giant fiber neurons that control the escape reflex. After injection of the caged ATP substrate, a brief flash of light shone on the entire fly resulted in a dramatic 'escape' response (Lima and Miesenbock, 2005). Although these preliminary light-sensitive methods represented the most direct way to date to probe activity within a specific circuit, one of the major

drawbacks was the inherent complication built in to the system, such as the additional need of caged ATP or glutamate.

The Deisseroth lab simplified and strengthened the optical system by identifying a directly light-activated channel in green algae that bore a strong resemblance to those found in the mammalian retina, naming it channel rhodopsin 2 (ChR2) (Boyden et al., 2005). When genetically expressed in neurons, this channel allows cation influx in response to light, causing the neuron to fire. The neuronal response is immediate, thus allowing a precise temporal control within the system that can mimic the neural activity seen in a normal circuit. We anticipate being able to specifically express this light-sensitive channel in rubro-motoneuronal cells, and then stimulating the circuit to probe both the effect on motor neuron activity, and to link the circuit with the behavioral response generated.

Motor neuron activity can be recorded intracellularly in spinal cord slice preparations. Given the distance between the red nucleus and the spinal cord, keeping the rubrospinal cell body attached in an in vitro spinal preparation is unfeasible. However, it is possible to optically stimulate the nerve terminals of virally infected ChR2-expressing corticospinal neurons, and evoke EPSPs in post-synaptic spinocerebellar neurons (A. Hantman, personal communication). In my experimental proposal, extensor digitorum motor neurons can be identified by intramuscular injection of a retrograde fluorescent tracer. Although the difficulty level of this experiment appears considerable, pilot experiments have demonstrated

that it is feasible (A. Hantman, personal communication), and will allow us to directly monitor the MN response generated by direct rubrospinal inputs in a manner that closely replicates the in vivo circuit.

One of the key questions which has intrigued and motivated neuroscientists for many years is an understanding of exactly how activity within neural circuits is capable of eliciting a behavioral output. The development of optogenetic tools with which to precisely stimulate and monitor activity in targeted circuits of awake, behaving animals has already demonstrated its potential to provide unprecedented insight into the connection between neural activity and behavioral output. Unfortunately, the limitations of optical access have so far dictated that behavioral studies be restricted to superficial brain regions, or more accessible genetic model systems like *drosophila* or *c. elegans*. As the red nucleus of the mouse is located deep in the ventral midbrain, in vivo stimulation of the rubrospinal tract would have proved impossible, unlike the more superficial location of the motor cortex. Recently however, flexible optical fibers have been developed that can be secured in vivo to successfully reach deep brain structures (Kravitz et al., 2010), and we hope that this technology will enable activation of direct rubro-motoneuronal cells in the red nucleus. From the anatomical circuit, we predict that the behavioral outcome will involve extension of the digits as a result of extensor digitorum muscle activation, but this remains to be seen.

General discussion and conclusions

In this thesis, I have characterized the motor pool specificity of direct projections from one of the major descending pathways involved in motor control in the rodent, the rubrospinal tract,. This work has the potential to alter the way we think about the role of supraspinal motor control in two key areas: 1. Despite a growing body of evidence, both anatomical and functional, suggesting similarities in motor control between rodents and higher species, the existence of direct projections from supraspinal motor centers onto motor neurons in the rodent has not been widely accepted. My data establish that forelimb muscle motor neurons in the mouse receive monosynaptic inputs from motor regions in the midbrain, prompting a re-evaluation of the role of direct descending inputs in the regulation of motor output. And 2. Even in species in which direct supraspinal control of motor neuron activity is well established, direct projections are considered to be a general neural specialization for the control of all individual distal forelimb musculature. My results instead suggest that direct projections from one of the major descending pathways involved in voluntary motor control, the rubrospinal tract, are instead focused on the activation of a single muscle, the extensor digitorum.

Although projections from supraspinal motor control systems involved in the involuntary control of movement, such as interstitiospinal projections onto neck motor neurons to coordinate eye and head rotatory movements (Fukushima et al., 1979a), can be of a monosynaptic nature in lower species, supraspinal control of movement through the direct activation of forelimb motor neurons has long been considered a primate and human specialization. I have demonstrated that descending

projections from the midbrain, likely originating from the red nucleus, project directly onto motor neurons involved in the control of digit movements. That these direct projections in the rodent originate from the rubrospinal tract presents an interesting insight into the functional distribution of motor control. Although rubro-motoneuronal connections have been demonstrated in the monkey, in addition to the well-known cortico-motoneuronal pathway, whether the contributions of these two pathways are distinct or overlapping has not been clearly determined.

I now present an organization within the rodent motor system in which direct projections from the rubrospinal tract onto motor neurons appear to control a very specific aspect of skilled movement: the stereotypic extension and separation of the digits in preparation for a task requiring digit manipulation. How the corticospinal tract fits in to this organization is currently unclear, both in rodents and in higher species. I have proposed a model in which cortico-motoneuronal projections might have evolved to provide the additional control necessary for fractionated digit movements seen in higher species. A detailed investigation of the motor pool specificity of corticospinal projections in primates is required to support this hypothesis. It will be interesting to see whether the specialized rubrospinal control over ‘whole hand’ extension is maintained following the emergence of increased cortical control of skilled movement in higher species.

The identification of a small number of rubrospinal fibers that project onto motor neurons has raised the question as to how this functional distinction between

populations of rubrospinal neurons is represented within the red nucleus. The diversity of post-synaptic spinal targets contacted by the rubrospinal tract has long suggested the existence of rubrospinal subpopulations, and I have established a level of molecular heterogeneity within the red nucleus that supports this concept. Identifying molecular correlates of functional rubrospinal populations is the logical next step in further understanding the specific circuitry that encodes descending motor commands. This work will facilitate the dissection of the rubro-motoneuronal circuit, enabling the establishment of a direct link between neural connectivity and individual muscle control during a skilled movement.

Chapter 6: Experimental Methods

Mouse strains

All animal work was performed in compliance with Columbia University IACUC protocols.

The following transgenic mouse lines were used in this thesis (ϕ=lox-stop-lox): C1qL2::ϕmGFP (described below), Nestin::Cre (Tronche et al., 1999), ChAT::Cre (Jackson Labs stock number 006410, Rosa:: ϕtdtomato (Madisen et al., 2010), Rosa::ϕYFP (Srinivas et al., 2001), Emx1::Cre (Gorski et al., 2002), Tshz3::LacZ (Caubit et al., 2010)

Generation of C1qL2::mGFP BAC transgenic

The *C1ql2* conditional reporter mice was generated using a modified BAC, clone RP23-184E2, purchased from the CHORI bacpac resource center. Homology arms (HA) for homologous recombination into the BAC were generated by PCR amplification using the original BAC construct as a template. The 5'-HA was generated from a sequence 600bp upstream of the endogenous start codon and the 3'-HA was generated from a 600bp sequence immediately downstream of the first 3 codons. Concurrently, the expression cassette was generated by ligation of a 5' splice acceptor with a floxed selection cassette (PGK-Neo-pA-STOPx4) and with the MARCKS-GFP (a kind gift from S. Arber). The HAs were cloned into the TOPO-

pCRII vector (Invitrogen) and the expression cassette ligated into restriction sites between the HAs to create the minitargeting vector. The minitargeting vector will be electroporated into BAC-containing SW102 cells and recombinase activity induced by heat shock. Positive clones were screened for Neo(Kan)-resistance. In preparation for pronuclear injection, the BAC vector backbone was removed by a not1 restriction digest and the modified BAC fragment gel purified and dialyzed against BAC injection buffer (10mM Tris, 0.1mM EDTA, 100mM NaCl). The BAC construct was injected into BalbC/C57Bl6 hybrid fertilized ova and implanted into recipient mothers. Seven positive founder lines were identified by polymerase chain reaction for GFP, and screened by mating with a ubiquitous neuronal cre driver. GFP expression was compared with that of endogenous C1qL2 in the red nucleus, and for the presence of a labeled tract in the dorsolateral funiculus of the spinal cord. Founder line 14 has been used for the experiments in chapter 2.

Immunohistochemistry

Antibody staining of 12-20 uM cryosections was performed as previously described (Price et al., 2002; Tsuchida et al., 1994). Animals were first perfused intracardially with PBS followed by 4% paraformaldehyde in .1M PB, the tissue removed, and then post-fixed for two hours at 4° followed by multiple washes with 1X PBS. Tissue was then cryoprotected overnight in 30% sucrose in .1M PB, and frozen in OCT at -80°. Following cryosectioning, slides were first blocked for twenty minutes in a solution of PBS with 1% BSA and .1% Triton X-100. Slides were then incubated overnight in primary antibody, diluted in PBS with .1% BSA and .1% Triton X-100. For detection

of synaptic proteins, the concentration of Triton X-100 was increased to .3%.

The following primary antibodies were used: rat anti-c1ql2 1:200 (R&D systems), rabbit anti-brn3a 1:20,000 (generous gift from E. Turner), guinea pig anti-tshz3 1:1000 (generous gift from L. Fasano) goat anti-ß-gal 1:1000 (Biogenesis) chick anti-ß-gal1:500 (Millipore), goat anti-ChAT 1:100 (Millipore), rabbit anti-mafB 1:2000 (Millipore), rabbit anti-GFP 1:1000 (Molecular Probes, Invitrogen), sheep anti-GFP 1:2000 (Biogenesis), rabbit anti-RFP 1:500 (Millipore), goat anti-vAChT 1:2000 (Chemicon), guinea pig anti-vGlut1 1:20000 (Millipore), guinea pig antivGlut2 1:3000 (Millipore).

Secondary antibodies used were generated in Donkey (Jackson Immunoresearch Laboratories, West Grove, PA). Those conjugated to FITC and Cy5 were used at a 1:200 dilution while those conjugated to Cy3 were used at a 1:800 dilution. The DyLight secondary antibodies were used at 1:1000 (Jackson) and the Alexa fluorophor-conjugated secondaries also at 1:1000 (Molecular Probes, Invitrogen).

Secondary antibody incubation was performed in PBS with .1% Triton X-100 for 2 hours at room temperature. Sections were then washed repeatedly with PBS and mounted in VectaShield and images acquired on a Zeiss LSM510 confocal microscope.

In situ hybridization

In situ hybridization was performed on 12-20µm cryostat sections as previously described (Price et al., 2002). Briefly, postnatal animals were anaesthetized and underwent intracardial perfusion with PBS followed by 4% paraformaldehyde in .1M PB. Spinal cords and midbrain were removed and then postfixed in PFA for an additional 2 hours at room temperature. Tissue was washed in PBS and then cryopreserved in 30% sucrose overnight. Tissue was then embedded in OCT and frozen.

In situ hybridization probes with a T7 promoter were generated by PCR from mouse e17.5 midbrain cDNA. Primers were designed to be 600-1000 bps in length using the primer3 program, targeted to the 3' UTR. The antisense mRNA probes were labeled with digoxigenin-UTP.

The detailed in-situ hybridization protocol can be found here:
http://sklad.cumc.columbia.edu/jessell//pdf/DIG_Labelled_In_Situ.pdf

Double fluorescent in situ hybridization

Double fluorescent in situ hybridization (FISH) histochemistry was performed as previously described (Price et al., 2002) using one FITC-labeled and on DIG-labeled probe. The TSA Plus Cyanine 3/Fluorescein kit was used to detect each probe.

Retrograde labeling of adult rubrospinal neurons

Adult animals (p30-p50) were deeply anaesthetized with a mix of ketamine and xylazine, and the head secured in a small animal stereotaxic frame. An incision was made in the skin overlying the spinal cord (cervical or lumbar region), and the underlying muscle and tissue bluntly dissected until the vertebral column was visualized. Using spinal clamps, the vertebrae caudal to the injection site was stabilized, and an opening in the laminae created using rangiers scissors. The dura was removed with fine forceps, and a glass pulled micropipette attached to a microinjector inserted into the spinal cord. Multiple injections of tracer (100-300nl, 10-12 % rhodamine-dextran or fluorescein-dextran) were made at appropriate spinal levels. The wound was filled using gelfoam, and the skin closed with sutures. The animal was allowed to recover on a heating pad. Ten days post-surgery, animals were sacrificed and subjected to intracardial perfusion of 4% paraformaldehyde fixative, before removal of the spinal column and midbrain. The spinal cord and midbrain were post-fixed in 4% paraformaldehyde for 2 hours before being rinsed in 1X PBS. If the tissue was to be cryosectioned, it was then incubated overnight in a solution of 30% sucrose. Following cryoprotection, tissue was mounted in OCT and stored at -80. Midbrains were sectioned at 20uM and immunohistochemistry performed as above. For vibrotome sectioning, midbrains were mounted in 4% low melting point agarose, and sectioned at 250 uM. Immunohistochemisry was performed as above.

Retrograde labeling of p7 rubrospinal neurons

P7 mice were deeply anaesthetized using a modified isofluorane mouthpiece. An incision was made in the skin overlying the spinal cord (cervical or lumbar region),

and the underlying muscle and tissue bluntly dissected until the vertebral column was visualized. An opening in the laminae was created using fine forceps. A glass pulled micropipette attached to an aspirator tube was inserted into the spinal cord. A single injection of tracer (100nl, 5% alexa488-CTB, alexa555-CTB, alexa647-CTB) was made at the appropriate spinal level. The wound was filled using gelfoam, and the skin closed with sutures and vetbond. The animal was allowed to recover on a heating pad before being returned to its mother. Three days post-surgery, animals were sacrificed and subjected to intracardial perfusion of 4% paraformaldehyde fixative, before removal of the spinal column and midbrain. The spinal cord and midbrain were post-fixed in 4% paraformaldehyde for 1 hours before being rinsed in 1X PBS, and prepared for cryosectioning as described above.

Stereotaxic injection of the red nucleus

Adult animals were deeply anaesthetized with avertin, and secured in a small animal stereotaxic frame. An incision was made in the skin overlying the midbrain, and small burr holes were made with a dental drill at a location of -3.5 mm from lambda, and .5 mm from the midline bilaterally. A glass pulled micropipette attached to a microinjector was inserted into the brain at the following coordinates for the red nucleus (-3.5-4.2 AP, .5-.75 ML, 3.6 depth) (Paxinos and Franklin, 2004). Three injections of 100-200 uL of virus each were made at -3.6, -3.9, and -4.2 from lambda. The needle was withdrawn after 1 minute. The wound was filled with gelfoam, and the scalp closed with sutures.

AAV2::Cre (from vector biolabs) 10E6 pful/mL

AAV2::Synapsin-GFP (generous gift of K. Franks)

Intramuscular backfills

Adult (p30-p50) animals were deeply anaesthetized with isofluorane. *A* small incision was made along various locations of the skin to expose the underlying muscle. Approximately 5 uL of retrograde tracer (Alexa488-CTB, Alexa 555-CTB, Alexa647-CTB, all 1% in PBS) was injected into the identified muscle with a pulled glass micropipette connected to an aspirator tube. One or several injections was made into the same muscle. Skin was then closed with sutures, and the animal allowed to recover on a heating pad. Four to five days post-surgery, animals were sacrificed and subjected to intracardial perfusion of 4% paraformaldehyde fixative, before removal of the spinal column and midbrain. The forelimb was then dissected, and muscle injection specificity determined.

Laser capture microdissection

E17.5 midbrains were dissected in 1X PBS and then immediately frozen in OCT solution in dry ice. 10uM sections were collected on special coated PALM Membrane Slides 1.0 PEN for laser capture microdissection. Every third section was collected on a separate slide for fixation and immunostaining with Brn3a and C1qL2. Once the tissue was sectioned, it was stored at -80 for a maximum of overnight. The slides were removed from the freezer, and cresyl violet staining performed as follows: The slides were fixed in cold 100% ethanol for 2 minutes and then incubated in cold

50% ethanol for 30 seconds (with slight agitation) to remove remaining OCT. The slides were then laid flat and 800 uL of 1% cresyl violet added to cover the slide. The slides were incubated at room temperature with occasional agitation for up to 3 minutes. The slides were then rinsed in cold 50% ethanol for 30 seconds, followed by incubation in cold 75%, 90%, and 100% ethanol for 30 seconds each, and then air dried for 5-10 minutes.

Tissue was then isolated from rostral, caudal cervical, and caudal lumbar regions of the red nucleus using laser capture microdissection. Tissue was collected in lysis buffer from the Absolutely RNA nanoprep kit (stratagene). Tissue from two midbrains was collected during each LCM session, and tissue from three LCM sessions was pooled to generate enough RNA for one sample. RNA was then purified with the absolutely RNA nanoprep kit, and samples were tested for integrity and quality on a bioanalyzer. Amplified cDNA was then prepared using the Ovation Pico RNA Amplification system (NuGen). Two replicates from each condition were then analyzed on Affymetrix Mouse Genome 430 2.0 gene arrays, by Vladan Miljkovic in the Herbert Irving Cancer Institute at Columbia University. Microarray statistical analysis was performed by Paolo Guarnieri in the Hervert Irving Cancer Center at Columbia University.

Chapter 7: References

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