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Cortical circuits, learning, and behavior: Local reorganization of synaptic partners and the expansion of the motor repertoire

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The Dissertation of Jeremy Stanford Biane is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

For mom

Georgia Leann Biane

1944 - 2005

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ABSTRACT OF THE DISSERTATION

Cortical circuits, learning, and behavior: Local reorganization of synaptic partners and the expansion of the motor repertoire

by

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Appropriate patterning of synaptic circuitry is vital for proper central nervous system function, and neurons retain a significant capacity for synaptic reorganization throughout life. To better understand how synaptic alterations mediate the development and refinement of complex behavior, this dissertation investigates the neurophysiological and circuit-level changes accompanying 1) the emergence of fine motor behavior during development, and 2) motor skill learning in adulthood.

We developed methods for identifying individual neurons of the motor cortex that are associated with specific motor domains to enable study of synaptic modifications among neural subpopulations associated with discrete behaviors. This was accomplished by labeling individual corticospinal motor neurons of layer V motor cortex that are associated with either proximal or distal forelimb control, in the same animal.

By way of thousands of paired whole-cell recordings, we find that the emergence of fine motor behavior is associated with a developmental switch in connection strategy and intrinsic cell properties, which fundamentally alter the manner by which excitation is spread within the corticospinal system in rats during development. These changes parallel the emergence of fine motor behavior, and may indeed be necessary for its expression. Motor skill learning in the adult rat is next discussed, where we find that task-related corticospinal neurons specifically increase excitatory interconnectivity, inhibitory input, and intrinsic excitability following skilled motor training. Neighboring corticospinal neurons not associated with the motor task, on the other hand, exhibit no changes in connectivity or neurophysiology. Such population-specific changes may enable local encoding of motor behavior, thus automating skilled motor execution and freeing up higher-order cognitive processes, such as attention, for other tasks. Furthermore, such learning-related changes are likely a ubiquitous feature of the neocortex and underlie numerous forms of cortical learning. In total, these findings identify, for the first time, neuronal properties

of connectivity and synapse function that characterize the cortical underpinnings of complex behavior and the learning engram.

Chapter 1

INTRODUCTION

The brain exhibits a remarkable capacity for reorganization throughout its lifespan, as an ongoing ability to acquire new skills and adapt to an ever-changing environment is crucial for survival. While even phyolgenetically ancient animals are capable of simple associative learning, mammals demonstrate a level of sophistication that is unmatched throughout the animal kingdom. The neocortex, present only in mammals, plays a vital role in such advanced behavior. Plasticity within the cortex allows us to develop brains optimized for our sensory environment, execute precise movements, and learn to speak new languages (while simultaneously comprehending their subtle pronunciations). Cortical plasticity also enables functional recovery following traumatic insults to the brain. Thus, understanding the mechanisms that underlie cortical plasticity could enhance recovery following brain injury, reduce cognitive decline associated with aging, and even enhance learning in healthy adults.

Previous studies identifying experience-related changes in cortical function have provided great insight into the mechanisms associated with learning. However, an important feature absent these studies is the ability to classify the role of individual neurons with respect to the learned skill/behavior. For example, synaptic transmission, task-related firing, and neural synchrony are

all known to increase in the motor cortex following skilled motor learning. But are these changes specific to neurons that control the motor behavior, or are they a general property of learning applied throughout the motor cortex? Addressing this issue and others like it will greatly inform how the cortex processes and stores information, and how specific neuronal interactions generate learning in adults.

In comparison to adult learning, where changes in synaptic circuitry are relatively modest, connectivity is extensively refined throughout development. During this time, axons traverse tortuous paths across the brain to innervate postsynaptic targets, while dendrites infiltrate their surroundings. Most mammals are born devoid of fine motor control, possessing only a limited repertoire of motor behaviors necessary for survival. But this situation can drastically change over a period of weeks, as in the rat where fine motor control emerges within one month of birth. What are the physiological and synaptic changes that support the emergence of such fine motor behavior? How might these changes differ from adult modifications associated with skill learning?

This thesis investigates the cortical processes underlying the initial development of complex behavior, and the further refinement and long-term storage of such behavior in adulthood. It specifically examines these issues in the context of motor behavior, investigating discrete populations of corticospinal neurons of the motor cortex with known behavioral function. This ability to identify individual neurons associated with select motor outputs represents a significant methodological advancement, allowing investigation of specific neurons

preferentially engaged during learning. This technique also enables the examination of developmental changes in cortical circuitry with exceptional resolution. In addition to the above technical advantages, there is extensive documentation on motor development and skill learning in the motor cortex, providing a rich context for the interpretation and application of experimental results.

It should be noted that, in addition to the motor cortex, several subcortical regions contribute significantly to motor output, including the cerebellum, basal ganglia, red nucleus, and intrinsic circuits of the spinal cord [1]. Proper motor control requires the interaction of all motor systems, as is readily apparent in subjects with compromised function to any of the above regions. However, the motor cortex occupies a unique position in the control of fine motor behavior and will therefore be the focus of this thesis.

With a concentration on the motor cortex in general, and the corticospinal system in particular, this introduction will discuss the structure and function of the motor system, as well as its development. This is followed by an examination of motor learning and the various forms of plasticity associated with the acquisition of motor skills. Finally, a qualitative model of motor learning is presented, with testable predictions on how behaviorally relevant movements are stored within M1 to form a motor engram.

A very brief history of early motor cortex inquiry

The motor cortex has long been the province of neuroscientists interested not only in motor behavior, but cortical function in general. Initially considered by the scientific community to be no more than a nutrient-rich protective coating, the cerebral cortex was commonly accepted to be “inexcitable” and thus could not possibly give rise to motor behavior, forming the basis for Paul Broca’s assertion in 1861 that, “Everybody knows that the cerebral convolutions are not motor organs” [2, 3]. This misconception was soon rectified when, in 1870, Gustav Fritsch and Eduard Hitzig were able to consistently evoke muscle twitches after stimulating the cortices of both anesthetized and unanesthetized dogs [2]. These seminal experiments showed that movement could only be evoked from the anterior portion of the cortex, and that particular regions of the cortex were associated with activation of specific muscles, providing the first evidence of a somatotopically organized motor cortex.

Elaboration of Fritsch and Hitzig’s findings were quick to follow. By the early 1900s, notable contributions by David Ferrier, John Jackson, Charles Beevor, Victory Horsley, and Charles Sherrington refined our understanding of the somatotopic organization in M1, showing that the motor cortex was generalizable to numerous different species, and even uncovering evidence of plasticity within this structure [4-7]. These early experiments provided a solid foundation from which our understanding of the structure and function of the motor system has developed.

With its advantageous positioning for experimental inquiry, and an output that is readily observable (and quantifiable), the motor cortex continues to be the focus of intense scientific scrutiny. Not only has this led to increased understanding of the processes controlling motor behavior, but because the organizational principles of the motor cortex may generally be shared across the cortex, many of the operations governing motor cortex function are likely repeated throughout the neocortex.

Structural organization of the primary motor cortex

As a whole, the neocortex has access to an unparalleled array of sensory inputs and behavioral outputs, and extensive interconnectivity enables communication between distinct cortical modalities. Repeated structural motifs across these modalities suggests similarity in how information is extracted and processed, thus providing a “universal language” for communication.

The canonical organization of the neocortex consists of six layers, each differentiated by cellular density and morphology. While there exists a multitude of neuronal phenotypes within each layer, at a basic level neurons can be classified as excitatory or inhibitory, with the former outnumbering the latter roughly 5:1 [8]. Similarly, the ratio of excitatory to inhibitory synapses within the rat cortex is approximately 8:1 [9]. This balance of excitation and inhibition is important for regulating information processing, and preventing seizure or coma.

Traditionally, motor areas of the cortex have been classified based on their ability to evoke movement when stimulated, with the primary motor cortex (M1) exhibiting the lowest threshold for stimulation [10]. An exception to the standard six-layered organization of the neocortex, the primary motor cortex lacks the granular layer IV [11], (but see [12]). Nevertheless, the remaining layers of M1 display analogous structure, and presumably function, to surrounding cortical areas. Layer I contains the apical dendritic tufts of pyramidal neurons positioned in lower laminae, and is the target of many local inhibitory axons as well as diffuse thalamocortical projections, corticocortical input, and afferent modulatory input [13-15]. Pyramidal neurons with long horizontal projections (up to 3 mm in rat) occupy much of layer II/III [16], and may act to unify neuronal ensembles controlling distinct aspects of complex movement [16, 17] and/or laterally inhibit neighboring domains [18]. Layer II/III is also the predominant source of transcallosal projections [8]. Pyramidal neurons of layer V send long-range axonal projections to various subcortical targets, including the striatum, brainstem, and spinal cord[19], while layer VI is the primary origin of corticothalamic projections[20]. In sensory cortex, layer IV contains spiny stellate neurons that are the primary recipients of thalamic input [21]. The absence of layer IV in primary motor cortex does not mean this region lacks thalamic input, however, as neurons in all layers receive some level of monosynaptic input from the thalamus [22].

The corticospinal system

The corticospinal neurons of the primary motor cortex – also known as upper motor neurons, pyramidal tract neurons, or Betz cells – are among the largest neurons of the CNS. As the name suggests, corticospinal neurons send a long axonal projection to the spinal cord, where they terminate in the dorsal horn and intermediate zone [23-25]. In rodents, the majority of these projections terminate on intermediate neurons of the spinal cord (i.e., propriospinal neurons). In primates, however, a considerable number of corticospinal neurons terminate directly onto motor neurons that project to and control skeletal muscles [26]. These direct projections are traditionally believed to mediate fine motor behavior, such as skilled grasping, primarily for two reasons: 1) direct projections are more prominent in species with advanced manual dexterity, and 2) direct connectivity with motor neurons bypasses propriospinal circuits that innervate motor neurons in a manner that is considered too diffuse to control fine movement [27]. Recent work has challenged this assumption, however, as targeted disruption of propriospinal pathways in the macaque monkey impairs hand dexterity and skilled grasping behavior [28]. Furthermore, animals lacking a direct corticomotoneuron pathway are still capable of dexterous motor control, such as skilled grasping behavior in rodents [27]. Whether rats possess a direct corticomotoneuron pathway remains controversial, and evidence supports both its presence [29, 30] and absence [31-33]. Regardless, rats exhibit remarkable dexterity of the distal forelimb, even showing evidence of individual digit control

[34, 35]. Importantly, this fine motor behavior is dependent on input from corticospinal neurons [34].

Of note, corticospinal projection neurons are not confined to the motor cortex proper. In fact, retrograde labeling studies in adult animals have localized corticospinal somata to the somatosensory cortex, posterior parietal cortex, and frontal cortex [36, 37]. These projections are somewhat sparse compared to outputs arising from the motor cortex and may, in part, play a non-motor role, such as modulating incoming sensory information or influencing spinal neurons indirectly [8].

Corticospinal organization within M1

Within the motor cortex, corticospinal somata are confined to layer Vb [38, 39]. Despite their significance for spinal cord activation, and therefore movement, it is estimated that corticospinal neurons constitute less than 0.1% of cells in the motor cortex [16], and only approximately 10% of all layer V neurons (although personal observations estimate a figure closer to 30%). Morphologically, corticospinal cells possess a single apical dendrite, with sparse branching in layer II/III and a thick apical tuft in layer I (Fig. 1.1) [40]. The basal dendrites of corticospinal neurons branch much more extensively, extending hundreds of microns across multiple laminae [40, 41]. Local horizontal collaterals of descending axons extend even farther, with horizontal projections up to 2 mm in distance [16], creating substantial axo-dendritic overlap between neighboring corticospinal neurons. Within the spinal cord, a single corticospinal neuron can

terminate across multiple spinal segments [26, 42], although a slight majority of neurons innervate only a single segment [43]. Interestingly, corticospinal cells that control distal muscles show a more restricted branching pattern in the spinal cord than those controlling proximal musculature, which may contribute to the finer movements generated by the distal forelimb [44].

At a gross level, the motor cortex is somatotopically organized, with spatial segregation of corticospinal neurons that project to divergent levels of the spinal cord and control biomechanically independent muscles [45-47]. For example, there is virtually no intermingling of neurons controlling the foot and those controlling the arm. At a finer level, however, there is substantial overlap of corticospinal neurons that project to neighboring segments of the spinal cord, such as neurons controlling the shoulder and those controlling the digits [41, 46, 48]. Similarly, motor movements induced via intracortical microstimulation (ICMS) show intermingling of stimulation sites that evoke proximal or distal forelimb activity [49]. This intermingling may serve at least two purposes: 1) facilitate communication between neurons controlling diverse, yet functionally related muscles, and/or 2) provide resistance to injury such that focal damage (e.g., stroke) does not completely abolish control of a particular muscle group [50].

Despite abundant axo-dendritic overlap, recurrent connectivity among corticospinal cells – that is, direct synaptic coupling between corticospinal neurons – is relatively low, with an overall connectivity rate among neighboring cell pairs of approximately 4 % in cortical slices containing M1 [51, 52], indicating

a high degree of functional specificity within corticospinal circuits. Although there appear to be no studies systematically examining the dendritic location of these recurrent corticospinal synapses, Cho et al report that “axon varicosities in close apposition to dendrites” are restricted to basilar dendritic branches [53] (Fig. 1.1b). Thick tufted layer V pyramidal neurons of unknown long-range axonal target, but within the sensorimotor cortex, form recurrent synaptic contacts primarily on basilar dendrites, with the remaining one-quarter of contacts located on apical oblique branches [54]. Thus, it seems the vast majority of recurrent corticospinal synaptic contacts are positioned along the basilar dendrite.

Locally, layer Vb neurons of the mouse cortex receive their strongest excitatory input from other layer Vb neurons, followed closely by layer II/III and layer Va inputs [55-57]. Local output of layer Vb, however, is largely restricted to other layer Vb neurons, with little excitatory signaling to layer II/III, layer Va, or layer VI [55]. Furthermore, layer Vb corticospinal neurons show little to no connectivity with neighboring corticocortical and corticostriatal neurons [52]. Thus, although corticospinal neurons express a low rate of interconnectivity, this within population signaling represents a strong pathway for local excitation. Moreover, the fact that corticospinal neurons minimally influence local neuronal populations further underscores the selective nature of corticospinal signaling, and suggests a hierarchical organization of M1 where corticospinal neurons occupy a downstream position [52]. Exactly how this high level of synaptic specificity is established and maintained is unknown.

Long-range inputs and outputs

Although there are numerous reports detailing regions with projections to the motor cortex (Fig. 1.2), these studies were not conducted with regard for the specific identity of recipient neurons. However, a recent study by Hooks *et al* found that corticopontine neurons in layer Vb of mouse motor cortex receive direct input from the frontal cortex and motor (ventrolateral) thalamus [22]; , the latter "relays" signals from the basal ganglia and cerebellum [22, 58]. These findings are consistent with personal observations of direct synaptic input from the thalamus and frontal cortex in the rat to corticospinal neurons (data not shown). Aside from these known corticospinal inputs, the motor cortex in general receives afferents from the somatosensory cortex, posterior parietal cortex, and transcallosal input from contralateral motor cortex [22, 57, 58]. In addition, subcortical neuromodulatory systems terminate throughout all layers of the motor cortex [58], and unpublished studies from our lab reveal that M1 is also the target of projections arising from the amygdala, and the claustrum/endopyriform cortex (James Conner, personal communication).

In addition to axonal projections to the spinal cord, corticospinal neurons also send axon collaterals to the reticular formation and red nucleus of the brainstem [25], and ipsilateral (but not contralateral) striatum [38]. As such, these systems receive an efferent copy of the motor signal destined for the spinal cord. These "auxiliary" projections may be important for predicting sensory and behavioral consequences of an intended movement, and comparing these

predictions to actual feedback to generate an error signal and guide corrective movements [59].

Electrophysiological characteristics

The electrophysiological properties of a neuron dictate how signals are processed and thus contribute greatly to how information is transmitted. Several characteristics of corticospinal neurons highlight the importance of coincident, highly active inputs and bursting output for corticospinal communication. For example, excitatory postsynaptic signals decay at a faster rate in corticospinal cells compared to surrounding neuronal populations [52], due in part to elevated hyperpolarization-activated current (I_h) [60]. This fast decay lowers the integration window for synaptic signals, placing a premium on coincident input. Furthermore, in general, synapses between corticospinal neurons are facilitatory, as are corticospinal inputs to the spinal cord [52, 61, 62]. This facilitation suggests that sustained bursting can increase activity within the motor network. Indeed, corticospinal neurons display sustained, or even accelerating firing rates to depolarizing current injections [52, 63, 64], and layer V of M1 is capable of sustaining 10 Hz oscillations in vivo, whereas S1 is not [65]. Collectively, these attributes help minimize the effect of background noise, such that aberrant input or activation should minimally influence downstream spinal neurons and therefore motor output.

The role of M1 and the corticospinal system in movement control

Patients with damage to the motor cortex or corticospinal tract exhibit substantial deficits in fine motor control, with force generation and manual dexterity of the hand particularly affected [66]. Similarly, nonhuman primates with lesions to the corticospinal tract display impairments in digit control, precision grip, and the ability to retrieve food from small wells [67-70]. In rats, damage to the pyramidal tract disrupts various features of forelimb function, including skilled grasping ability [71-74]. Thus, an intact corticospinal system is crucial for complex forelimb behavior.

How might the corticospinal system, and the motor cortex in general, control such fine motor behavior? This question has occupied experts for decades, and remains an active topic of research in many labs. At the heart of this debate is the uncertainty over what neurons of the motor cortex encode. Muscles? Movements? Direction? Synergies? Despite a lack of consensus within the field, numerous studies have shed light on various characteristics of motor cortex function.

The idea that corticospinal neurons control individual muscles has strong intuitive appeal, and was widely adopted in early theories. By way of meticulous Intracortical microstimulation (ICMS) experiments showing brief current pulses could evoke movement about a single joint, Hiroshi Asanuma was a strong proponent of the idea that the motor cortex controls individual muscles, and further argued that M1 was organized in a columnar fashion [75, 76]. Subsequent experiments challenged this view, however. Direct measurement of

EMG muscle activity showed that intracortical stimulation routinely activated multiple muscles [77]. Spike-triggered averaging experiments corroborated these findings by demonstrating the spiking of a single neuron is correlated with activity patterns across various muscles [78, 79].

Around the same time, experiments from Georgopoulos and colleagues showed that neurons controlling arm movements could display directional tuning. That is, a neuron encoded a preferred direction of movement and, when active, would incrementally bias movement in this favored direction [80]. A major caveat of these experiments was that movement was restricted to a center-out reaching task along a 2-dimensional plane. As such, it is possible that these neurons were “tuned” to aspects of movement other than direction, such as final hand position or forelimb posture [79]. Indeed, later experiments showed that the directional tuning of a neuron could be altered by the initial position of the hand or posture of the arm [81, 82].

An important consideration in any discussion of motor control is that of muscle synergies, which can be defined as a stored pattern of stable activation levels across multiple muscles [7]. Many complex movements can be broken down into a limited number of synergies, suggesting the CNS uses these activation patterns as building blocks of movement [83]. These muscle synergies appear to be stored, at least in part, within internal circuits of the spinal cord [84] and can be combined in a linear fashion [7]. Like a puppeteer pulling marionette strings with varying force and sequence to produce fluid movement, the motor

cortex differentially activates combinations of motor primitives to generate a vast array of motor output. Importantly, the use of muscle synergies can decrease the degrees of freedom associated with movement compared to individually controlling muscles. However, one trade off of this simplification is a loss of motor resolution, as muscle synergies restrict the range of muscle activation patterns [85]. Therefore, it is possible that muscle synergies may not have dominion over the entirety of motor behavior [86], especially fine motor movements.

In addition to the features above, M1 activation has also been associated with force, velocity, joint angle, and final posture [79, 87-89]. How can one reconcile these seemingly disparate observations of M1 function? The answer is likely that the motor cortex can be “tuned” to a variety of parameters, depending on the task. For example, reaching actions may preferentially control spatial variables such as direction, while dexterous motor movements may require control of individual muscles. This idea is captured by the theory of optimal feedback control [90], whereby the motor system learns to control the movement parameters essential for a particular task, while allowing for variability in task irrelevant parameters [85]. Thus, there may not be a specific parameter, such as endpoint or direction, preferentially controlled by the motor system, but a multitude of available parameters employed in accordance with the task being performed [79].

Finally, it must be remembered that neurons do not act in isolation, but maintain a vast array of inputs and outputs; activation of even a single cortical

neuron will impact thousands of cells. In an attempt to limit activation of such networks, ICMS experiments apply the minimum stimulation necessary to surpass movement threshold. However, when the applied stimulation is increased in duration and amplitude, Graziano *et al* [91] found that a variety of complex, coordinated movements are evoked. Interestingly, these movements closely resembled common behaviors of the animal, leading Graziano to hypothesize that ethologically relevant movements are stored and mapped across the motor cortex [92]. First observed in monkey, these results have been generalized across species [93-95], including reach-to-grasp movements in the rat [96]. Thus, while understanding the contribution of individual neurons to movement is of great scientific interest, meaningful behavior arises only through the coordinated activity of carefully structured networks. How these networks are initially assembled is explored next.

Development the corticospinal system

Having established that fine motor behavior is contingent on the motor cortex and corticospinal neurons, it is not surprising that the emergence of skillful movement parallels development of these systems [61]. Development of corticospinal input to the spinal cord can be roughly broken up into three progressive phases: axonal migration, gray matter innervation, and synapse refinement.

In the rat, pioneering axons of the corticospinal tract reach the upper cervical spinal cord at birth, and reach the caudal extent of the spinal cord by postnatal day 9 (P9) [97]. Myelination begins soon after (P10), and increases steadily through the fourth postnatal week before gradually tapering off into the third postnatal month [98]. The entirety of the corticospinal tract is not myelinated, however, as a substantial number of unmyelinated corticospinal axons are present in mature rats [98].

Descending axons are guided by a variety of elements during outgrowth into the corticospinal tract [99]. Glial cells appear to form channels in the extracellular space that enable outgrowing corticospinal axons to traverse the brain with greater ease [100], while numerous adhesion molecules direct axons along the proper pathway. These include L1, which directs decussation at the medulla [101, 102], and neural cell adhesion molecule (N-CAM), important for path finding and fasciculation (i.e., neurite growth along existing neurites) [103, 104]. Growth factors such as insulin-like growth factor-1 (IGF-1) [105] and ciliary neurotrophic factor (CNTF) [106, 107] promote axon elongation. Additionally, inhibitory factors, such as Wnt proteins and myelin-associated molecules, may direct axons along the appropriate pathway by repelling them from improper locales, although the role of myelin-associated molecules during development is uncertain [99, 108, 109].

Interestingly, during early development, layer V neurons throughout all regions of the neocortex project axons through the pyramidal tract and into the

spinal cord. This phenomenon is not restricted to spinal targets, as it appears that layer V neurons throughout the cortex initially send axons to multiple subcortical targets, only to have improperly targeted axons withdrawn. In the case of the spinal cord, axons originating from inappropriate areas are selectively retracted during the second postnatal week, and the mature distribution of corticospinal axons is in place by postnatal day 17 [110].

Corticospinal axon entry into the spinal grey matter shows an approximate delay of two days following arrival of the growth cone at the target [99, 111]. At lower cervical segments (i.e., C7 and C8), innervation begins at P5 and increases until approximately P10 [112]. Axonal arborizations within a particular segment display an initial exuberance throughout the spinal laminae that is pared with development [112] (but see [113]). Despite this within-segment exuberance, corticospinal neurons do not display exuberance across segments. That is, they do not innervate segments in which they are not present in adulthood [98].

During the third phase of corticospinal development, the initial broad termination pattern of axons is refined over a period of weeks. During this time, dense clusters of synapses develop within spinal cord laminae III – VII [1, 30]. At the same time, connections are formed and degraded, and the overall percentage of terminals containing synaptic vesicles increases [114, 115]. This refinement of connectional specificity is activity dependent, as projections compete for space onto local dendrites much like that seen in the development of ocular dominance columns in the visual cortex [61, 116, 117].

The increase of targeted, functional synapses in the spinal cord results in increased spinal potential amplitudes in response to stimulation of the pyramidal tract [115]. Additionally, corticospinal input begins to show temporal facilitation [61, 115]. These developments strengthen cortically mediated activation of spinal neurons, and as such are accompanied by the emergence of motor maps in response to intracortical stimulation of M1 [118, 119].

At this point (~P15-P20), the motor cortex is primed for an increasing role in motor control. Over the next several weeks motor abilities increase, with concurrent changes in motor maps, which exhibit substantial reorganization and growth [61, 118-120]. Furthermore, ICMS current threshold for muscle activation is reduced, indicating more effective signal transduction between corticospinal neurons and their spinal targets [61, 118-120]. In rats, motor maps assume mature topography by P60 and are accompanied by a capacity for fine motor behavior, as animals as young as P45 learn a skilled grasping task at performance levels equal to adults [118].

In addition to expansion and lowered activation threshold, representational motor maps in M1 undergo two other notable changes with development. First, a predominance of proximal forelimb representations in early development gradually gives way to expanding distal representations [119, 120]. This progression corresponds with the development of motor behavior in human infants, where control of the hand lags behind that of the shoulder and elbow

[121, 122]. Therefore, this increased representation of distal musculature may, at least in part, signal the emergence of skilled distal movements.

Second, the number of stimulation sites that evoke movement about multiple joints increases with maturation [120]. This is intriguing, as it shows that muscle representations exhibit greater overlap at a time when the animal is learning complex movements that require coordination across multiple joints. Furthermore, if the animal is prevented from engaging in such complex movements, these overlapping representations are reduced [123]. Reinforcing the conclusions from Graziano's long stimulation experiments [7], these findings suggest that motor maps reflect the behavioral experience of the animal.

Development of the motor cortex

Altered motor representations during development reflect not only changes in the pattern and efficacy of terminations in the spinal cord, but also local changes within the motor cortex itself. If behavioral experience influences motor maps – and by extension local connectivity – in adulthood, what initially determines connectivity within a developing motor cortex that is dissociated from behavior? Little is known of how corticospinal properties within M1 are modified during development, although such knowledge would illuminate how local interactions support the emergence of fine motor output. There is, however, substantial documentation on the development of local microcircuits within the cortex in general.

Despite the considerable elongation of subcortically projecting axons at birth, axonal and dendritic branching of layer V neurons within the motor cortex itself at this time is extremely sparse, with little to no presence of dendritic spines [124, 125]. But by day 14, rats exhibit complex dendritic trees and axon collaterals that fundamentally mimic their adult form [124, 125]. Dendritic spine density sharply increases from birth to P30, at which point there is slow reduction in spine number until stabilizing at 2-3 months of age [126, 127]. Interestingly, unlike most long-range subcortical inputs, which show an initial axonal exuberance that is subsequently pruned [116, 128-130], local axonal branching develops with considerable specificity and largely avoids targeting inappropriate laminae during development [131, 132].

By day 15, when representational motor maps are first identifiable [119], there is considerable axo-dendritic overlap of neighboring neurons, and local connectivity likely plays a vital role in the expression of these maps. It is tempting to partially attribute the progressive increase in motor map size and decrease in stimulation threshold over time to an upsurge in local interconnectivity. Yet, the rate of connectivity between neighboring cortical neurons appears to remain static [133] or even decrease [134] from P15 onward. There is, however, significant reorganization of recurrent synapses. For example, in mouse visual cortex at P30 and beyond, layer II/III neurons with similar receptive field characteristics show preferential connectivity [133, 135]. At eye opening (~P15), however, such functional connectivity does not exist, and presumably develops over time via experience-dependent processes [133].

What determines initial connectivity among neighboring neurons? Although it was long assumed that much of synaptic formation was somewhat random and scaled with the extent of axo-dendritic overlap (e.g., Peters' rule) [136], it has been shown that even in young animals (~P18) axo-dendritic overlap does not consistently explain differences in connectivity among neighboring pyramidal neurons [137]. Instead, molecular cues likely guide the initial path of neuronal processes [132], while properties such as gene expression [138], long-range axonal targeting [137, 139] and clonal lineage [140] may dominate initial connection specificity. Furthermore, early connectivity may be activity-dependent, as transient electrical coupling is correlated with subsequent chemical synapse formation among sister neurons [141].

Finally, synaptic and electrophysiological properties undergo substantial changes with development, which affects how information is distributed throughout cortical circuits. Between the second and fourth postnatal weeks, excitatory signals between layer V pyramidal neurons exhibit a decrease in synaptic efficacy, as exemplified by decreases in excitatory postsynaptic potential (EPSP) amplitude, decay rate, and presynaptic release probability [133, 134, 142, 143]. Moreover, synapses switch from depressing to mostly facilitating over this same time period [134, 135, 142-144]. This amplification of early synaptic signals may be important for initial synaptogenesis or stabilization of nascent synapses [142]. On the other hand, the more "selective" signaling properties of mature animals allow for greater temporal resolution of synaptic

inputs, placing greater importance on features typical of finely tuned circuits, such as coincidence detection and an ability to follow high frequency inputs.

Overall, the developmental trajectory of motor control appears to encompass several features, including: 1) pruning and refinement of corticospinal inputs to the spinal cord, as well as an increase in synaptic efficacy; 2) reorganization of recurrent connectivity within M1; and 3) decreased synaptic efficacy for intracortical connections. Together, these modifications could give rise to a progressive facility for spinal activation while still increasing the resolution of signaling in the motor system. However, whether features 2) and 3) actually apply to corticospinal neurons is unknown.

Properties of adult cortical circuits

In the adult cortex, synaptic circuitry is meticulously organized and exceptionally complex, and has thus proven difficult to decipher. Common characteristics observed across cortical regions and species support the existence of canonical properties that are at the heart of cortical function. Prominent among these is the columnar organization of the cortex, first proposed by Lorente de Nó [145], and expanded upon by Mountcastle [146] and later Hubel and Wiesel in their Nobel-prize winning work on visual receptive fields [147]. To summarize, a column consists of a collection of neurons expressing similar functional properties, such as receptive field characteristics. These neurons are spaced throughout the cortical laminae and are more or less

vertically aligned. Although they have been documented throughout the neocortex, there is no strict or consistent anatomical correlate underlying these functional columns [148].

Another canonical property of the cortex is the interlaminar signaling pathway. Thalamic afferents enter the cortex and preferentially synapse on spiny stellate cells of layer IV. This information is then shuttled to layer II/III, and subsequently passed onto layer V neurons, which constitute a final cortical level of neural processing before projecting to other cortical and subcortical regions [149]. However, this is an oversimplified caricature of local signaling, as information is passed across all layers bidirectionally and simultaneously [150]. Recurrent connectivity plays a prominent role in local information processing, as regional excitatory and inhibitory signals can silence certain input streams and amplify others [151]. For example, although spiny stellate cells of layer IV are the primary target of thalamocortical inputs, the vast majority of synaptic input to these cells come from neighboring spiny stellate cells and interlaminar projections from layer VI pyramidal cells [152]. These local interactions may enable pattern completion or winner-take-all type computations, important features of many network models [152].

Among neighboring pyramidal neurons displaying extensive axo-dendritic overlap, axons do not bias projections towards the dendrites of particular neurons, but contact all neighboring dendrites without preference [153]. Despite this anatomic potential to form functional synapses with virtually all nearby cells,

connectivity is selective and deliberate [154, 155]. For example, as discussed earlier, neighboring corticospinal neurons connect to only 3-4% of adjacent corticospinal cells, and avoid synapsing on nearby corticostriatal cells altogether [52]. The determinants of local connectivity are not completely understood, but appear to be preferential among neurons with similar function, such as receptive field characteristics [135] or long-range axonal targets [137, 139], and can also depend on the function of downstream neurons [17]. In stark contrast, inhibitory connectivity is promiscuous and largely nonspecific [156, 157].

An additional organization principle of local networks is the tendency of recurrent connections to cluster together [154, 158]. That is, if neuron A and neuron B are both connected to neuron C, there is an increased probability that neurons A and B will also be connected. Furthermore, synaptic strength is disproportionately large within these clustered networks. It is hypothesized that these circuits are not shaped by experience, but rather are innate building blocks forged during early development, and encode perceptions (and perhaps muscle synergies) that can be strung together to form memories [159].

To reiterate, cortical circuitry is highly specific, but retains the potential to communicate with diverse synaptic partners as evidenced by the close proximity of axons and dendrites of neighboring cells. How synaptic connectivity evolves with experience in adulthood is of great interest and is the main focus of the remainder of the introduction.

Plasticity in the adult vs. newborn brain

Throughout the CNS, the developmental changes previously described prepare the brain to effectively process and interact with environmental stimuli. But environments change, challenges arise, and many systems require extended training to optimize their function. Therefore, an ongoing capacity for synaptic reorganization is essential.

In adulthood, flexibility is traded for stability. To remain in a state of ubiquitous change, such as encountered during development, would inflict chaos on the brain. While an enhanced ability to adapt and learn may seem beneficial, it would seriously challenge the capacity to retain information and therefore predict outcomes: this is arguably the primary function of the brain. The upshot is the existence of developmental critical periods that, once closed, effectively lock in certain brain properties, and limit the modification of others [160].

In the case of the motor system, developmental events establish the building blocks of motor function: spinal innervation patterns, muscle synergies, and central pattern generators, among others. Once these features are in place the adult brain must learn to control and choreograph these building blocks to express flexible, purposeful movement. On a systems level, there are many strategies by which the brain could accomplish this task [161-163]. But a universal requirement of these strategies is modification of intrinsic and network properties of neurons. Such changes are a prominent feature of the motor cortex

during skilled motor learning, implicating M1 as a primary site for the acquisition of fine motor behavior.

Motor learning and M1

Motor learning can take many forms. The most generous definition might include the formation of instincts and reflexes that become genetically encoded over many generations. This thesis, however, is concerned with the acquisition of motor skills within an animal's lifetime. Simply put, motor skill learning involves extending the motor system's performance beyond its prior limits [164]. A form of implicit learning, motor skills require a longer period of time for acquisition than the learning of facts or events (declarative learning), but also are more enduring and resistant to degradation [165]. Further, a distinction between motor skill learning and motor adaptation should be made. Whereas motor skill learning increases the capabilities of the motor system, motor adaptation allows the motor system to regain these capabilities in altered circumstances, such as changes in muscle or limb growth, or in the presence of abnormal external forces [164]. While there is likely a rich interaction between these two processes throughout an animal's life, this thesis is primarily concerned with the acquisition and consolidation of fine motor skills.

Among the many neural regions that contribute to motor performance, the primary motor cortex is essential for fine motor behaviors, especially those involving the digits and distal forelimb [66, 166]. For example, the acquisition of a

skilled reach-to-grasp task, which requires the grasping a small food pellet with a single arm/forepaw, is severely compromised following damage to the primary motor cortex [167, 168]. Even in well-trained animals displaying a high level of proficiency, subsequent damage to M1 greatly impairs performance [74, 169, 170]. These findings point to the vital role of M1 for skilled motor output, but they do not necessarily implicate it in motor learning. After all, the motor cortex could be just a simple readout of a system where changes in upstream inputs (or downstream outputs) regulate the acquisition of new motor skills. However, there are numerous changes within M1 during motor training that strongly implicate the motor cortex as a locus for motor learning.

Learning-related plasticity in the motor cortex

Learning-related changes associated with motor skill acquisition have been documented at virtually every physiological level of the motor cortex, from genes to systems. Animals undergoing reach-to-grasp training exhibit increases in such M1 parameters as spine density, synaptic transmission, neural synchrony, forelimb map representation, movement-related firing rates, and expression of immediate early genes. Of note, many of these findings are explicit to the learning condition, and cannot be ascribed to simple motor activity [171-173]. Collectively, these changes indicate that reorganization of local circuitry is closely tied to the acquisition and storage of motor skills.

Inhibition of protein synthesis by means of focal anisomycin injection during the early stages of grasp training disrupts learning when injected into the motor cortex, but not the cerebellum or parietal cortex [174, 175]. As this study used a global protein inhibitor, the particular genes/proteins involved in learning were not addressed. However, a recent study identified specific genes with altered expression levels during early grasp training [176]. Many of these genes are affiliated with synaptic transmission, neurite remodeling, and synaptogenesis (or, more broadly, circuit reorganization) [176]. Correspondingly, repeated grasp training increases dendritic spine density within layer V of the forelimb region of M1 [177]. In rats this increase is detected during late (7 – 10 days), but not early (3 days), stages of grasp training [178]. However, in vivo studies in mice detect a net increase in spine formation among the apical tufts of layer V neurons just hours following an initial training session [179]. This temporal discrepancy in spine formation may highlight differential roles for local input, which predominately synapses on basilar dendrites in layer V, versus long-range thalamocortical, corticocortical, and neuromodulatory inputs that synapse on apical tufts.

In a follow-up study, Zuo and colleagues found that spines formed in response to grasp-training exhibit a greater tendency to grow in clusters, whereas “spontaneously” generated spines or those formed in response to an alternate motor task do not cluster with “grasp-related” spines [180]. This synaptic clustering may reflect input from the same presynaptic axon, or perhaps nearby axons of functionally related cells. Co-activation of neighboring spines

can cause nonlinear summation of their inputs, in some cases drastically increasing the postsynaptic response [181, 182] and facilitating the formation of long-term memory [183]. Both clustered and single spines formed during training were preferentially stabilized and endured for months after training ended, suggesting long-term storage of skilled movements may partially reside in the inputs to layer V neurons [179, 180].

Reorganization of recurrent synaptic patterns should be reflected in alterations in synaptic transmission and representational motor maps within M1. Indeed, synaptic transmission is potentiated following skilled grasp training selectively in the hemisphere contralateral to trained forepaw [184-186] (but see [187]). These changes appear to be mediated by LTP-like mechanisms, and are present following three days of training [188-190]. Changes in synaptic efficacy are followed by alterations of ICMS-evoked motor maps, where training induces an expansion of the distal forelimb representation that is first detected following 10 days of training [49, 178, 191]. Blocking this cortical reorganization through depletion of cholinergic inputs to M1 impairs learning (while previously learned motor skills remain intact) [192, 193]. Likewise, ablation of dopaminergic terminals in M1 impairs induction of LTP among horizontal connections of layer II/III neurons, as well as impairing motor learning [194]. Curiously, although blocking the cholinergic- or dopaminergic-mediated modifications of M1 impairs learning, it does not completely abolish performance gains, suggesting other neural mechanisms support more limited forms of learning.

Extracellular recordings have provided further evidence that changes within M1 contribute to motor skill learning. For example, M1 neurons from monkeys trained over a period of several years show increased synchrony of firing while performing the trained movement [195]. Rat unit recordings by Kargo and Nitz [196] showed that layer V neurons in the forelimb region of M1 demonstrate decreased background firing, increased burst-related firing rates, and enhanced muscle recruitment following forelimb reach training. These results suggest that, with practice, neurons become more precise and reliable in their activation of muscles, an important provision for skilled motor control. In the same study, EMG recordings from various forelimb muscles identified three temporally distinct patterns of motor adjustments associated with skilled grasp learning. Specifically, during the first day of training, muscle patterns of successful and unsuccessful trials were substantially different, suggesting the animal was auditioning different motor strategies for pellet retrieval. After a particular strategy had been chosen, movement patterns were adjusted over the course of single training sessions during days 2-5, as if slowly converging on a more successful combination of muscle activations through trial and error. Finally, at later stages of training (days 8-10), muscle activation became stereotyped and movement patterns showed little to no change within a session. Interestingly, this decrease in trial-to-trial variability was accompanied by increased signal-to-noise ratio and reliability of muscle recruitment in layer V neurons of M1 [196, 197].

Finally, systems level inquiry of motor learning in humans has clarified the chronological involvement of different brain regions during motor learning. These studies show a number of areas are active during the acquisition of new skills, including the motor cortex, prefrontal cortex, cerebellum, basal ganglia, and parietal association cortex [198, 199]. With repeated practice, most higher-order areas, such as the prefrontal and premotor cortices, show declining participation during task performance, while activity in the motor cortex remains high and can even increase [198, 200, 201].

A qualitative model of motor skill learning and accompanying plasticity

Collectively, the above findings strongly support the interpretation that motor skill acquisition and retention are mediated by local circuit reorganization. They also suggest that motor skill learning is a multilevel process that may involve several potentially independent yet interdependent stages of learning. Furthermore, as evidenced by cholinergic and dopaminergic depletion studies, local reorganization of M1 may not contribute to all stages of learning.

To a first approximation, learning can be broken up into three stages roughly corresponding to the stages of motor pattern adjustments observed by Kargo and Nitz above [196]. **Stage 1** is straightforward and involves selection of a general motor strategy to optimize success. For example, faced with the challenge of acquiring a sugar pellet as in the skilled grasping task, the animal may attempt to scoop the pellet into his cage or retrieve it with his tongue. When

these strategies prove unsuccessful, a grasping strategy may be adopted. This decision is rapid, likely made within the first training session, and almost certainly utilizes a top-down mechanism (i.e., does not require modification of local M1 circuitry).

Stage 2 might be considered the conventional “learning” phase of motor skill acquisition. After selection of a general movement strategy, the motor system must learn the spatiotemporal pattern of muscle activations that leads to an optimal result. This includes which muscles/synergies need to be activated, when, for how long, and with what ratio of activity. Much effort is required as varying patterns are tested and evaluated. During this time, the motor system may also be learning which parameters, or degrees of freedom, are important for success and which need not be constrained (i.e., optimal feedback control). Sensory input is paramount at this stage [202], as the system learns to associate motor commands with expected sensory feedback. Finally, some form of supervised and/or reinforcement feedback is necessary to strengthen neural patterns leading to successful trials and to weaken those that fail [161]. refine

Like stage one, regions outside M1 are expected to be highly involved during this phase, as is seen in human studies during initial learning. Dopaminergic signaling may provide reinforcement signals, and the basal ganglia and cerebellum may play a role in motor sequencing and supervised feedback, respectively. The elevated activity of prefrontal areas (mediating attention) during this time may be important for balancing excitation and inhibition within M1 and

coordinating the relative activation of neuronal ensembles. The pattern of inputs from all of these regions is expected to be considerably restructured with training, and may correspond to the early-onset changes seen in spine dynamics across the apical tufts of layer V dendrites.

In **Stage 3**, simultaneous with the changes above, unsupervised learning in the form of Hebbian plasticity occurs within the motor cortex. Repetitive training produces repetitive coactivation of corticospinal neurons that control the skilled grasp behavior. This repeated coactivation leads to the formation and/or potentiation of synapses, generating a local “grasping network” of corticospinal neurons within M1. Acetylcholine (ACh) release, which is primarily confined to behaviorally relevant events [203, 204], can facilitate plasticity within the cortex [205-209]. As such, depletion of cholinergic input to M1 or routine motor activity that does not evoke ACh release does not induce local reorganization [192, 193, 203].

Increased interconnectivity among grasp-related corticospinal neurons could account for numerous effects associated with skilled reach training, including increases in neural synchrony, movement-related firing rate, and reliability of muscle recruitment, as well as the increased spine density in layer V, where the vast majority of recurrent corticospinal connections are made. This boost in interconnectivity could also strongly contribute to the expansion of the distal forelimb representation detected with grasp training. Of note, the above alterations emerge only after extended training. This delay might be attributable

to the considerable variation that occurs in task-related movement patterns during early training, which should correspond to unstable and fluctuating corticospinal activation patterns. Once movement patterns are relatively stable and there is consistent coactivation of particular corticospinal ensembles, the influence of Hebbian-like plasticity is amplified.

After repeated training, the newly learned motor skill is consolidated and solidified within M1 and the movement pattern has become, to some extent, locally encoded. Such local encoding should increase trial-to-trial movement consistency, and augmented interconnectivity should enable pattern completion within the network and facilitate activation of the motor program. That is, movement becomes stereotyped and requires less attention to execute [210, 211]. At this point, the need for supervised feedback and higher-order oversight are diminished, as evidenced by the decreased activation of regions such as the prefrontal cortex. Thus, high level cognitive functions can be harnessed for other demands.

The evolution of inhibitory circuitry with learning must also be addressed in this model. Absent a shift in inhibition, the observed increase in excitatory transmission could simply lead to elevated background noise or even runaway excitation. Thus, increased inhibition is vital and likely plays an important role in the depressed background firing observed with training [196]. Furthermore, Inhibition is known to constrain the time window for synaptic integration [212-214], and likely plays an important role in the temporal precision of corticospinal,

and thus muscle, activation. Finally, an important part of automating movement may be the inhibition of neurons controlling interfering movements. For example, a single neuron in the motor cortex can contribute to a variety of behaviors [215-218], suggesting it is a constituent of numerous, independent movement networks (Fig. 1.3). Accordingly, activation of a single neuron would incrementally excite other potentially irrelevant or even conflicting networks. Although this minimal excitation is likely incapable of activating undesirable networks with much consistency, inhibiting competing circuits would promote a winner-take-all scenario that would actively suppress interfering movements, ensuring their silence. Intriguingly, computational models with winner-take-all dynamics can learn to autonomously encode temporal movement sequences [219], suggesting that such dynamics may be important for locally encoding behavior within M1.

To summarize, this model describes three major stages of motor skill learning (Fig 1.4): 1) **selection** of an appropriate motor strategy; 2) **refinement** of muscle activation patterns; and 3) **encryption** of the movement within M1. A major prediction of this last stage is that repetitive motor training will increase the interconnectivity of neurons controlling the learned motor behavior. This increase could take many forms, but may manifest by way of *de novo* synaptogenesis and/or strengthening of preexisting contacts between neurons actively participating in the learned behavior.

Testing such a prediction has been problematic in the past, largely due to difficulties with identifying individual neurons that: 1) control particular motor behaviors, and 2) are specifically engaged during learning. For example, although there is good evidence that layer V neurons controlling forelimb movements are altered with reach training, targeting of these cells is complicated by dispersed and intermingled muscle representations in the motor cortex [48]. Thus, although previous studies have identified numerous learning-related changes in cortical properties, these features have never been investigated amongst neurons known to actively participate in the learned behavior. Such information is vital for understanding how network interactions support learning and the specificity with which they operate. As such, modifications particular to learning neurons may be obscured by current, relatively broad sampling methods, potentially leading to an inaccurate understanding of the synaptic mechanisms that specifically support learning

However, we recently developed methods for identifying a subpopulation of corticospinal neurons that is functionally associated with skilled grasping behavior. Following skilled grasp training, animals exhibited increases in spine density and dendritic complexity selectively in this grasp-related subpopulation, while neighboring corticospinal neurons controlling separate forelimb behaviors unrelated to grasping were unchanged [41]. In addition to supporting the learning model proposed above, this study provides a method for identifying individual neurons that actively participate in learning a new motor skill, as well as

neighboring neurons that are not functionally engaged during learning, permitting unprecedented resolution for investigating cortical mechanisms of learning.

Experimental proposal and hypotheses

This thesis explores the role of cortical microcircuits in the initial development of fine motor behavior, and their subsequent refinement in adulthood. Specifically, modifications in the intrinsic and network properties of corticospinal neurons are examined in the context of normal development and adult motor skill learning with the general hypothesis that motor behavior is highly dependent on the pattern and strength of recurrent corticospinal connections.

Question 1: *How do developmental changes in corticospinal properties support the emergence of fine motor output?*

The corticospinal system plays a primary role in the development of fine motor behavior. Although the evolution of spinal cord projections has been well documented, cortical properties have been largely ignored and likely play a vital role in the emergence of dexterous movement. Thus, synaptic circuitry and electrophysiological properties were investigated throughout development in functional subpopulations of corticospinal neurons.

Hypothesis: **The emergence of fine motor behavior is accompanied by increasingly selective interactions within the corticospinal system.**

Question 2: *How do network and synaptic properties of corticospinal neurons change following motor skill learning? Are these changes confined to learning-related neurons?*

A number of plastic changes within M1 suggest reorganization of local circuitry is a fundamental property of motor learning. Furthermore, the encoding of ethologically relevant behaviors within the motor cortex suggests M1 may contain the motor engram of skilled motor behavior, prospectively due to synaptic coupling of neurons controlling the movement. In order to test these ideas, learning-associated modifications in cortical circuitry were examined among corticospinal neurons either related or unrelated to the learned motor skill.

Hypothesis: **Repetitive motor training increases the strength and rate of recurrent connectivity specifically between learning-related corticospinal neurons. Such occurrences may reflect the local encoding and long-term storage of motor skills.**

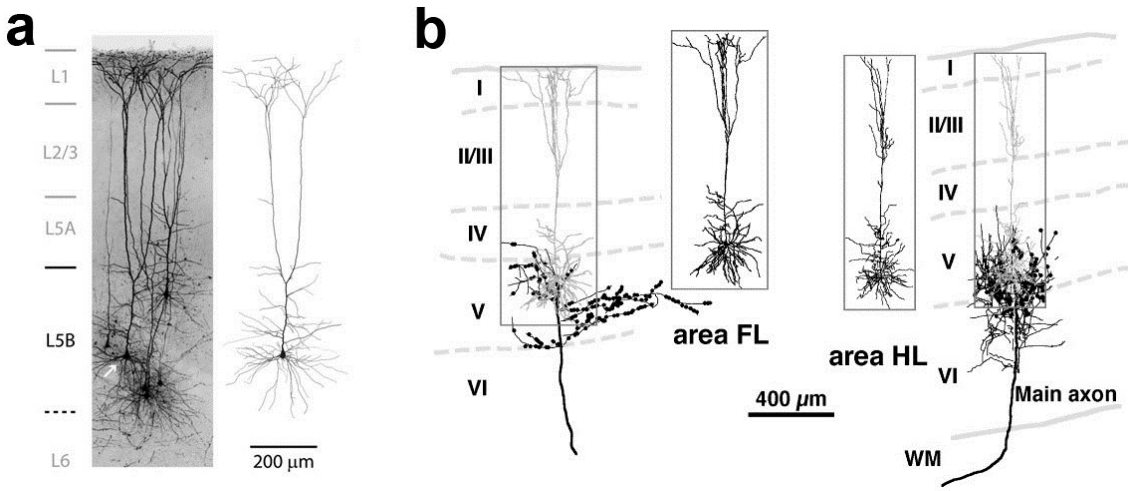

Figure 1.1. Morphology and recurrent synapses of corticospinal neurons. a) Somatodendritic morphology of corticospinal cells. Cell bodies are confined to layer Vb. Arrow indicates identify of reconstructed neuron at right. Taken from Suter et al., 2013. **b**) Two corticospinal neurons located in sensorimotor areas of the cortex (FL = forelimb, HL = hindlimb; note the presence of layer IV). Dendrites are shown in insets (and in light gray in main trace). Filled circles indicate axon varicosities in close apposition to dendrites or cell bodies of other corticospinal neurons. Thus, recurrent corticospinal connections are mainly along the basilar dendrites of layer V. The lateral extension of axon collaterals, which can span up to 2mm, produces substantial axo-dendritic overlap among neighboring corticospinal neurons. Taken from Cho et al., 2004.

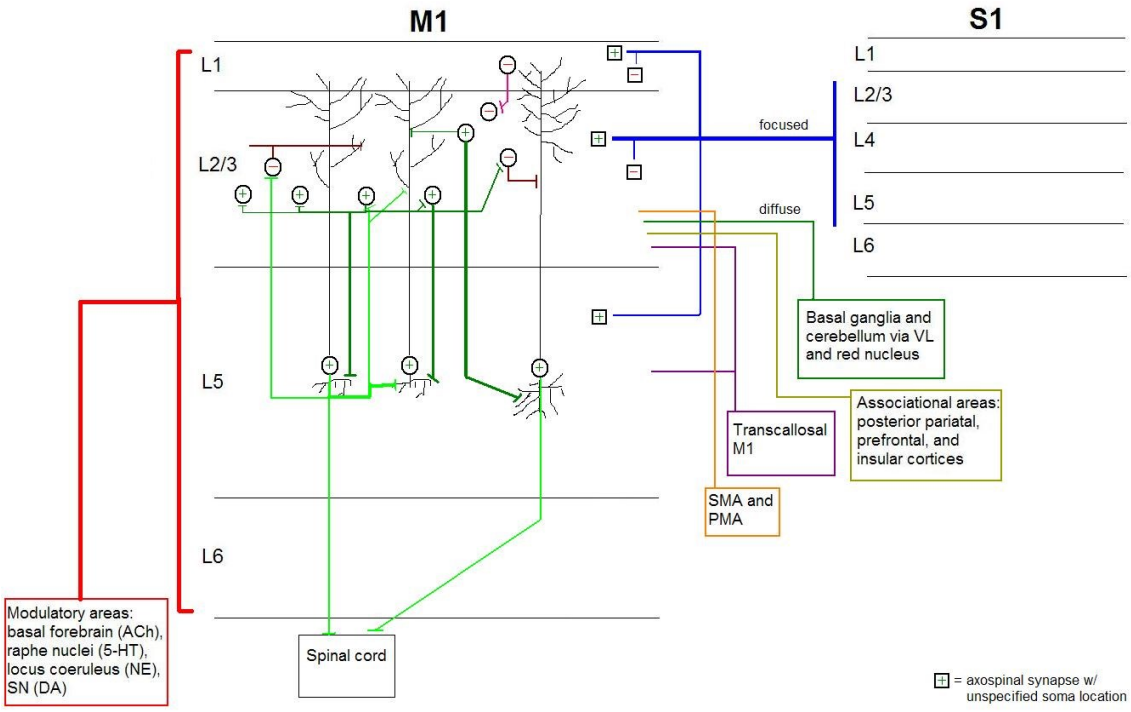

Figure 1.2. Local and afferent projections of the primary motor cortex. Plus sign enclosed by a circle denotes pyramidal cell. Minus sing enclosed by a circle denotes inhibitory neuron. Colored lines indicate the laminar region(s) of afferent termination for the specified projection area. For example, transcallosal projections (purple) terminate in L2/3 and L5, while neuromodulatory inputs (red) terminate across all layers.

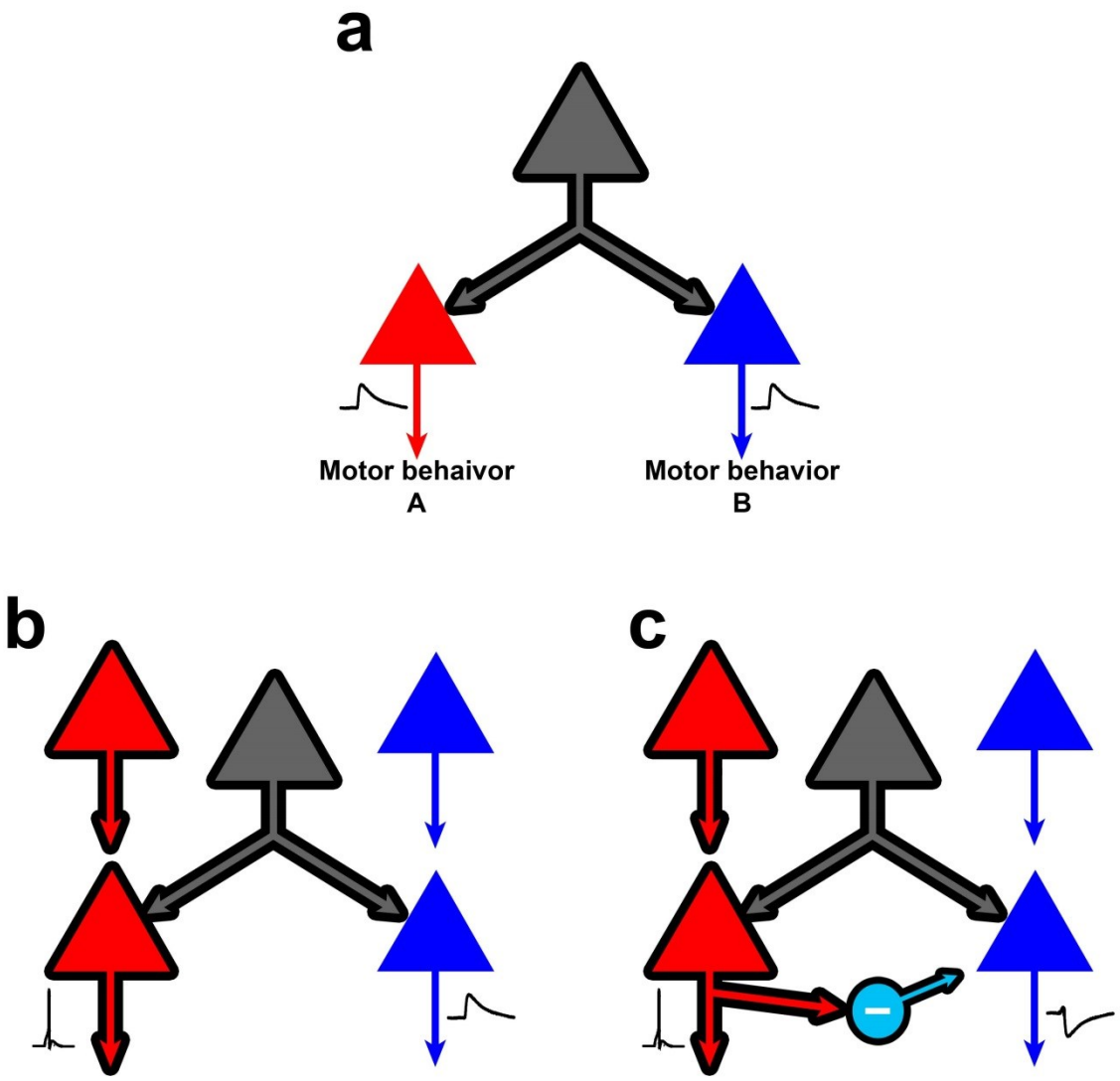

Figure 1.3. A single neuron participating in multiple functional networks. Two sample networks (**red** and **blue**) controlling distinct motor behaviors are shown. The **grey** neuron represents a corticospinal neuron(s) controlling an individual movement that is a component of both motor behaviors. Black outline denotes activation. **a**) Isolated activation of the grey neuron induces subthreshold excitation across both networks. **b**) However, when coactive with other neurons, activation will propagate through the circuit, triggering the encoded movement. In this way, single corticospinal neurons can participate in multiple autonomous motor networks. **c**) To safeguard against undesirable activation of parallel networks, lateral inhibition can be applied.

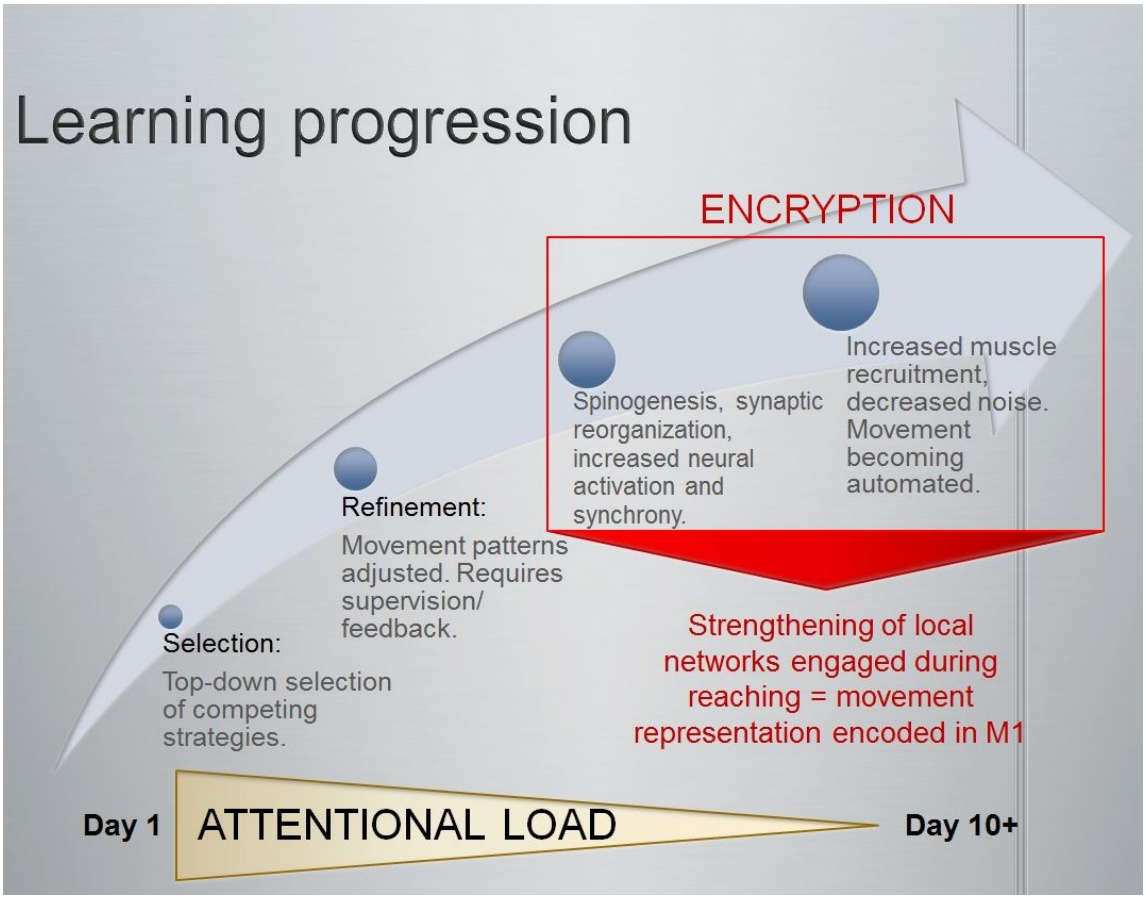

Figure 1.4. Stages of skilled grasp learning. Learning progresses through 3 overlapping stages: 1) Selection; 2) Refinement; 3) Encryption. As learning evolves and the movement sequence is encoded within M1, less attention is required for skilled performance.

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CHAPTER 2

A DEVELOPMENTAL SHIFT IN SYNAPTIC ORGANIZATION AND SIGNAL TRANSMISSION AMONG CORTICOSPINAL NETWORKS DURING THE EMERGENCE OF FINE MOTOR BEHAVIOR

ABSTRACT

Appropriate patterning of synaptic circuitry is vital for central nervous system function, and connectivity is extensively refined during development. At birth, the ability to express fine motor behaviors is absent in most mammals, and gradually emerges in parallel with the developing corticospinal system. While developmental changes within the spinal cord have been well documented, modifications in cortical motor circuits have been largely unexplored despite the fact that local corticospinal interactions exert a critical role in fine motor behavior in adulthood. We investigated connectivity and synaptic signaling among distinct corticospinal populations at time points ranging from postnatal day 18 through 75, using methods of multiple whole-cell recordings. Individual layer V corticospinal neurons associated with distinct motor outputs were identified by injecting retrograde tracers into specific spinal cord segments. Several days following tracer injection, acute slices containing M1 were collected, and labeled corticospinal cells were targeted for simultaneous patch clamp of up to four neurons. In total, 3,489 potential connections were tested, from which 130

excitatory connections were identified. We find that, during development, local connectivity is biased toward corticospinal neurons projecting to the same spinal cord segment, suggesting intralaminar connections are initially established according to similarity of long-range axonal targets. This within-population connectivity diminishes through development until adulthood, when connection frequency is similar between neurons projecting to the same or different (but neighboring) spinal segments. This reduced interconnectivity may reflect the evolution of smaller, more independent networks which enable fine motor movements to emerge, while the change in network composition may reflect a shift toward functionally related cells controlling complex movements. Accompanying these developmental changes in connection specificity is an overall decrease in synaptic efficacy and an increase in intrinsic neuronal excitability, indicating a fundamental change in how excitation is spread across corticospinal circuits of young and adult animals. Collectively, these changes in synaptic patterning and physiological function may provide a basis for the increased fine motor capabilities of the mature versus developing brain.

INTRODUCTION

At birth, synaptic circuitry of the cortex is diffuse, nonspecific, and overlapping [1-6], with axonal/dendritic patterning established by such factors as gene expression, clonal lineage and environmental guidance cues [3, 7-9]. This initial configuration can differ greatly from the mature pattern of synaptic

connectivity [8]. Consequently, most animals are born with limited abilities that progress only as synaptic circuitry is refined over time [10]. In the motor system, for example, brainstem regions for motor control are well developed at birth and mediate survival-related behaviors such as respiration and feeding [10, 11]. Fine motor movements, however, do not emerge for several weeks, and closely parallel development of the corticospinal system of the motor cortex (M1) [10, 12].

As the name suggests, corticospinal somata reside in layer V of the cortex and send a long-range axonal projection to the spinal cord. In the rat, pioneering axons of the corticospinal tract reach the upper cervical spinal cord at birth, and project throughout the entire spinal cord by postnatal day 9 (P9) [13]. Initially, gray matter innervation in the spinal cord is broad and synapses are weak [14, 15], although axons do not innervate spinal segments in which they are not present in adulthood [16]. Within the first postnatal weeks, this within-segment exuberance is refined [10], the proportion of synaptic terminals containing vesicles increases [17], and synaptic transmission begins to show temporal facilitation to repetitive activation [10, 15]. These developments strengthen cortically mediated activation of spinal neurons. Consequently, muscle activation can be elicited by intracortical microstimulation (ICMS) of L5 motor cortex beginning at P15 [18, 19].

In adulthood, corticospinal neurons are essential for fine motor control [20-25], and the pattern of CS interconnectivity likely plays a crucial role in the

execution of motor behavior. For example, despite substantial axo-dendritic overlap between neighboring CS neurons [26, 27], recurrent connectivity is highly specific in adults, with CS neurons making synaptic contacts with only 4% of neighboring CS neurons [28, 29]. Furthermore, representational motor maps, which are an expression of CS output and are influenced by local signaling [30, 31], are altered with changes to the motor repertoire [32-34]. Corticospinal neurons also exhibit changes in dendritic morphology and spine dynamics during acquisition of new motor skills [35-39], further implicating CS circuits as a foundation for skilled motor behavior.

Although the development of corticospinal projections within the spinal cord are well documented, relatively little is known with regard to how local network properties (within M1) evolve during maturation of the motor system. Given its importance in adulthood, the initial establishment and subsequent alteration of recurrent CS connectivity are likely key developments in the emergence of fine motor behavior. Indeed, progressive changes in ICMS-evoked motor maps during early life [18, 19, 40] suggests that network and cell-intrinsic properties of CS neurons undergo considerable change during development. Broad changes in dendritic morphology and spine dynamics among L5 neurons of the motor cortex during development [5, 41, 42] further indicate that reorganization of CS circuitry may enable fine motor performance.

In the current study, we examined changes in CS circuitry during the timeframe of fine motor development in order to elucidate neural modifications

associated with the emergence of fine motor behavior. We utilized two subpopulations of corticospinal neurons associated with distinct forelimb behaviors: CS neurons that project to segment C4 of the spinal cord and control musculature of the upper forelimb (C4-projecting), and CS neurons that project to segment C8 of the spinal cord and control musculature of the distal forelimb (C8-projecting). This use of independent yet interrelated CS populations allowed us to probe the evolving nature of recurrent CS interactions within and across populations during development, with the hypothesis that the emergence of fine motor behavior is accompanied by increasingly selective interactions within the corticospinal system of M1.

By way of *in vitro* paired recordings, we find that, in young animals, excitatory connectivity is more frequent between cells targeting the same spinal cord segment (within-population connectivity) versus different spinal segments (across-population connectivity). Within-population connectivity specifically decreases with maturation, until within- and across-population connectivity are of uniform probability in adulthood. Furthermore, synaptic efficacy decreases for all cell pairs during development, while intrinsic excitability increases. We postulate that these findings signify a developmental switch in corticospinal communication, whereby innate synaptic networks are slowly refined with experience to both enable and support functional motor behavior.

METHODS

Peripheral motor control is largely modulated by motor neuron pools within distinct spinal segments, and these segments are primarily innervated by discrete subsets of corticospinal neurons [35]. In rats, the C8 spinal cord segment contains lower motor neurons that activate muscles controlling distal forelimb movements of the wrist and digits [43, 44]. Lower motor neuron pools located in the C4 spinal segment are associated with control of proximal forelimb, shoulder, and neck musculature [45, 46]. Injection of separate retrograde tracers into the C4 and C8 spinal cord segments allows identification of layer 5 corticospinal neurons controlling distinct motor domains (proximal vs. distal forelimb), a property we exploited to investigate synaptic networks among neural subpopulations associated with discrete behaviors (Fig. 2.1). Because corticospinal innervation patterns are established soon after birth [14, 16, 47], these dissociable populations can be stably examined over the course of motor system development.

At the earliest time point investigated (P18), the efficacy of M1 to stimulate muscle activation is low [15, 18, 19], and as such the cortical contribution to movement is little to none. Correspondingly, fine motor behaviors have yet to emerge, although basic motor behaviors associated with the brainstem motor system (e.g., righting, climbing, and rearing with support) are present [10, 48].

Neuronal labeling: Male F344 rats, between postnatal ages 15 – 75 days, were anesthetized with a cocktail (2 ml/kg) containing ketamine (25 mg/mL), xylazine (1.3 mg/mL), and acepromazine (0.25 mg/mL). To label

corticospinal neurons projecting to the C8 cervical spinal cord, the overlying dura between C7 and T1 was resected and a glass micropipette (tip < 40 μm) containing red or green fluorescent latex microspheres (Lumafluor, Durham, NC) was inserted into the dorsal horn of spinal cord (depth 0.75 mm, 0.55 mm lateral to midline). Using a Picospritzer II (General Valve), ~350 nL of fluorescent latex microspheres was injected into each side of the spinal cord (Figure 2.1). To label corticospinal neurons projecting to the upper cervical spinal cord, the same procedure was repeated between C3 and C4 spinal vertebra, using a different colored dye (green or red) than that used for C8 injections. In all cases, tracer diffusion was assessed postmortem in 50 μm coronal slices of the spinal cord (see Figure 2.1c). Animals with tracer diffusion into the dorsal columns were excluded from further study. In total, 142 male F344 rats were included for analysis. Because the exact tracer (red or green) injected into C4 or C8 was counterbalanced and varied from animal to animal, the experimenter was blind to the exact projection target of labeled cortical cells during recording. All procedures and animal care adhered to American Association for the Accreditation of Laboratory Animal Care, Society for Neuroscience, and institutional guidelines for experimental animal health, safety, and comfort.

Slice preparation: Three to twelve days following tracer injection, rats were anesthetized and perfused for 3 minutes with ice-cold, oxygenated, modified sucrose ACSF containing (in mM) 75 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 0.5 $CaCl_2$, 1NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 52.6 sucrose, 10 HEPES, 10 choline chloride, 1 pyruvate, 1 L-ascorbic acid (~300 mOsml, pH 7.4). The brain

was rapidly dissected and 330 μm-thick slices spanning the motor cortex were cut at 15° anterior to the mid-coronal plane to match the projection pattern of layer 5 corticospinal neurons [35]. Cortical slices were cut and collected in ice-cold, oxygenated, modified sucrose ACSF. Slices were transferred to an interface chamber containing the same modified sucrose ACSF solution and incubated at 34° C for 30 min. Slices were then held at room temperature (23° C) in the interface chamber for at least 45 min before initiating recordings. Recordings were made in a submersion-type recording chamber and perfused with oxygenated ACSF containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 26.0 $NaHCO_3$, 20 glucose (~300 mOsml) at 23° C at a rate of 2-3 ml / minute.

Electrophysiology: All recordings were performed within the primary motor cortex. Neurons were selected based on emission spectra (red or green, reflecting tracers injected at either the C8 or C4 spinal segment) and then visualized under infrared differential interference contrast videomicroscopy (Olympus BX-51 scope and Rolera XR digital camera). Whole-cell voltage and current clamp recordings were made at room temperature using pulled patch pipettes (4-7 MΩ) filled with internal solution containing (in mM) 150 K-Gluconate, 1.5 $MgCl_2$, 5.0 HEPES, 1 EGTA, 10 phosphocreatine, 2.0 ATP, and 0.3 GTP. Post-synaptic data were analyzed exclusively from cells with a resting membrane potential ≤ -55mV, with drift less than 6 mV over the entire recording period, with access resistance ≤ 35 MΩ, with the ability to evoke multiple spikes with >60 mV

peak amplitude from threshold. Series resistance was not compensated, but was continuously monitored via negative voltage steps. In a minority of cell pairs (~15%), the “presynaptic” neuron was fired in cell-attached mode and was not reciprocally tested for synaptic input.

Data acquisition and analysis: Whole-cell patch clamp recordings were obtained using Multiclamp 700A patch amplifiers (Molecular Devices) and data analyzed using pClamp 10 software (Molecular Devices). To characterize basic membrane properties, a series of hyper- and depolarizing current steps were applied for 500 ms in 10-45 pA increments at 5 sec intervals. Action potential threshold was determined for the first spike at the lowest level of depolarizing current required to evoke at least one spike. Action potential spike measurements were taken from the first action potential on the first sweep to reach threshold. Spike height was measured as the peak membrane voltage relative to threshold and half-width was measured at the half amplitude of the action potential. Input resistance (R_{in}) was determined from the slope of the linear regression taken through the voltage-current relationship in the hyperpolarizing range.

To determine connectivity among C4- and C8-projecting cell populations, simultaneous whole-cell recordings were made in groups of 2-4 retrogradely labeled cells. In many cases, a recorded cell in one pipet was replaced with a new cell while keeping the other patched cells intact. Repeating this procedure allowed us to test many (up to 11) synaptic partners for a particular cell, although

the mean (± SD) number of tested synaptic partners per cell was 3.7 ± 2.1. The distribution of C4- and C8-projecting cells, which are almost entirely intermingled across the primary motor cortex [35], enabled all types of paired recordings to be obtained within a single field (C4→C4, C8→C8, C4→C8, and C8→C4). Within-population cell pairs consisted of neurons both terminating in the same spinal cord segment (i.e., C4→C4 and C8→C8, as well as pairs of double-labeled neurons (< 5% of total cell pairs)). Across-population consisted of cell pairs where neurons did not project to the same segment (C4→C8 and C8→C4, as well as pairs of unlabeled corticospinal neurons (also < 5% of total cell pairs)).

Data were collected from cells greater than 25 μm below the slice surface (mean ± SD = 66 ± 25 μm). Connectivity was determined by evoking paired action potentials spaced 50ms apart in the “presynaptic” cell while monitoring responses in postsynaptic cells held at -65 mV in voltage clamp. Presynaptic action potentials were evoked by a 7 ms depolarizing current injection of 2 nA. Individual sweeps were separated by 5 s. Postsynaptic response properties were measured in response to the first pulse.

The responses to 30-100+ evoked action potentials were measured for each paired recording. In connected cell pairs, failure rate was calculated as the percentage of single trials in which the postsynaptic peak current was < 2 SD below baseline current noise. All traces were manually inspected for signal consistency, including monotonic rise and decay and reliable onset latency. Postsynaptic response amplitude was calculated as the averaged current over a

1.5 ms time window of peak response current compared to baseline, which was defined as the average current in a 17 ms window prior to presynaptic firing. Response potency, latency, rise time, decay time and half-width were calculated using only traces where a postsynaptic response was detected (failures omitted). Response latency was measured from the peak of the presynaptic spike to the onset of the EPSC. Rise time was calculated as the time between 20 and 80% peak EPSC amplitude. Decay time was calculated as the time between 80 and 20% of peak EPSC amplitude. For paired-pulse analysis, the peak response to each pulse was averaged over all trials (that is, failures were not omitted), and the average response of the second response was divided by that to the first pulse. To preserve the relative differences in magnitude for ratios above and below a value of 1, the logarithm of each ratio was used for statistical comparisons.

Current-spike relationship and maximum afterhyperpolarization (AHP) were measured in cells requiring less than -150 pA to hold at a membrane potential of -65mV. AHP was calculated in cells for which a 300 pA depolarizing current delivered for 500 ms evoked at least 4 spikes. Reported p-values reflect the main effect of training for a two-way ANOVA (IV1 = training; IV2 = number of spikes elicited by current injection).

Statistical comparisons were performed using JMP software, version 10.0 (SAS Institute Inc., Cary, NC). Comparisons of connectivity were made using Fisher's exact test. Pairwise comparisons utilized Student's t-test unless

otherwise noted (e.g., Wilcoxon test for synaptic potency comparisons). Linear regression analysis was used for all comparisons where both variables were continuous (e.g., synaptic failure rate by weight). Ten thousand iterations were simulated for Monte Carlo analyses. Significance level was set at 0.05. In text data values are presented as the mean ± standard deviation.

RESULTS

As rats were recorded across a continuum of ages, there was no way of grouping animals by individual postnatal ages (sample sizes would be too low for meaningful comparison). Alternatively, we categorized animals as developing or mature, with 125 g (approximately postnatal day 45) as a cutoff. At this time point (p45), cortical dendrites and spinal innervation patterns have attained their adult form [14, 17, 47], and the dynamics of dendritic spine growth and elimination has begun to level [17, 42]. Representational motor maps have largely stabilized [18, 19] and rats are capable of performing and learning fine motor behaviors [18, 42].

Excitatory connectivity is preferential between neurons projecting to the same spinal cord segment during development, but not adulthood

Overall, corticospinal neurons in developing animals (bodyweight < 125 g) demonstrated a greater level of interconnectivity compared to more mature animals (bodyweight > 125g; Fisher's exact test; $p = 0.02$). More detailed analysis showed this increase was attributable to increased connectivity between

neurons projecting to the same spinal cord segment, as within-population connectivity was over 2x greater in developing (45 connections detected out of 681 potential connections tested, 6.6%) versus mature animals (40/1265, 3.2%; $p < 0.001$; Fig.2.2). On the other hand, across-population connectivity – that is, connectivity between neurons projecting to different spinal cord segments and controlling distinct motor outputs – did not differ between developing (16/575, 2.8%) and mature animals (29/948, 3.1%; $p = 0.88$). A logistical regression analysis comparing bodyweight with connection probability further confirmed that connectivity within-populations ($p < 0.01$), but not across-populations ($p = 0.73$), dropped as animals aged (Fig. 2.2 inset).

These changes were not attributable to differences in intersomatic distance or depth of recorded cells beyond the slice surface. For example, although the average distance between recorded cell pairs increased with age (as would be expected as the brain expands in size), this increase was similar for both within- and across-population cell pairs (Fig. 2.3). The depth of recorded cells below the slice surface slightly decreased with age for within-population cells ($p = 0.04$; Fig. 2.4). However, the mean difference of cell depth between developing (< 125 g) and adult (> 125 g) animals was 3 μm (68 μm vs. 65 μm). Neuronal depth did not differ with age for across-population cell pairs ($p = 0.83$).

Examination of connectivity according to individual cell pair groups showed that interconnectivity within the C4-projecting population and interconnectivity within the C8-projecting population both significantly decreased

with maturity (C4-C4: p = 0.02; C8-C8: p = 0.02). Connectivity between projection populations, on the other hand, did not change (C4-C8: p = 1.0; C8-C4: p = 0.64; Fig. 2.5). Thus, the decrease in within-population connectivity during maturation was not due to changes within a specific projecting population, but appears to be a general property of development.

uEPSC response properties are similar for within- or across-population cell pairs, but differ overall with age

We analyzed several properties of unitary excitatory postsynaptic currents (uEPSCs) to see whether the initial elevation of within-population connectivity might correspond to a cohort of synapses with unique properties. However, we found no significant differences between within- and across-population groups in immature animals (under 125 g) for any of the uEPSC responses measured, including failure rate (p = 0.15), synaptic potency (p = 0.18; Wilcoxon test), paired-pulse ratio (p = 0.15), onset latency (p = 0.98), rise time (p = 0.34), and decay time (p = 0.15; Fig. 2.6). In addition, uEPSC response properties also did not differ when comparing within- and across-population cell pairs for mature animals (over 125 g; Fig. 2.6). Together, these results indicate that synaptic signaling does not differ based on the projection targets of synaptic partners. As such, we combined within- and across-population responses to see how synaptic transmission changed overall with age. Analyzing uEPSC properties over weight values (i.e., age) revealed that failure rate significantly increased with age (p <

0.01, linear regression), as did the paired-pulse ratio ($p < 0.01$) and uEPSC coefficient of variation ($p = 0.03$), collectively indicating a decrease in presynaptic release probability as animals mature. Notably, the variance in all cases was relatively large, leading to weak coefficient of determination (R^2) values (Fig. 2.7). Such large variance suggests that corticospinal synapses are highly variable and remain capable of substantial modification throughout development and into adulthood. Synaptic potency trended toward decreasing with age ($p = 0.08$), however this was driven by a minority of responses with exceptionally high amplitudes (6-fold greater than the mean) in young animals (Fig.2.7). Other measured response properties did not vary with age (Fig. 2.7), including onset latency ($p = 0.83$), rise time ($p = 0.17$), and decay time ($p = 0.22$), indicating that the temporal probability of presynaptic release and average electrotonic distance of the synapse from the soma do not change with age.

Bidirectional connectivity is greater among cells projecting to the same spinal cord segment

Both within- and across-population cell pairs exhibit greater levels of bidirectional connectivity than expected from the overall connection probability (within: $p < 0.001$; between: $p = 0.01$; Monte Carlo simulation testing for overrepresentation; Fig. 2.8). Although both cell pair groups exhibited reciprocal connectivity that was greater than chance, the rate of bidirectional connectivity for cell pairs projecting to the same spinal cord segment (within population) was

greater than for cell pairs terminating in different segments (across population) ($p < 0.01$; logistic model with cell-pair category and weight as factors). The rate of reciprocal connectivity did not change with age ($p = 0.75$).

Although others have found the postsynaptic response amplitude is increased in reciprocally connected compared to unidirectionally connected cell pairs [49, 50], EPSC potency among bidirectionally connected cell pairs only showed a weak trend toward increasing in the current study ($p = 0.16$, Wilcoxon test; Table 1). Interestingly, EPSC rise time was significantly lower in reciprocally connected cell pairs ($p < 0.01$), while decay time trended toward decreasing ($p = 0.07$). These results suggest “reciprocal” synapses may be preferentially located closer to the soma, and present a shorter window for synaptic integration. However, there was appreciable overlap of both rise and decay time values for unidirectionally and bidirectionally connected cell pairs.

Intrinsic excitability increases with age

Although changes to neuronal intrinsic properties have been well documented during early development (from birth to early adolescence) [51-55], less is known about how properties are altered during late development and into adulthood. With respect to the corticospinal system, these late changes may be of particular importance, as movement is not under cortical control in neonates, and skilled motor behavior does not emerge until relatively late in development

[10]. Therefore, alterations beyond the juvenile period are important for the evolution of fine motor control.

We find that several properties related to excitability are altered during late development (Fig. 2.9). For example, linear regression analysis by weight shows that action potential threshold ($p < 0.01$) decreases with age, as does medium afterhyperpolarization amplitude ($p < 0.001$). Additionally, spike frequency in response to depolarizing current injections increases with age ($p < 0.01$). Thus, intrinsic excitability of corticospinal neurons increases as the motor system matures.

We did not find any progressive differences with age for resting membrane potential ($p = 0.37$), input resistance ($p = 0.37$), or for the spiking parameters of action potential half width ($p = 0.21$) and spike height ($p = 0.22$; data not shown). Although such measures are known to change during development, previous studies show that these modifications largely occur prior to the developmental period examined in the current study [52, 53, 55]. Therefore, it appears that developmental changes in ion conductance are not specific to a particular time point, as some changes in electrophysiological properties can lag behind others.

Connectivity rate and intersomatic distance

The average intersomatic distance between *synaptically connected* cell pairs did not significantly change with age, nor was there any difference on this measure when comparing within-population cell pairs to across-population cell

pairs. Thus, we combined data from all animals to better characterize the anatomical properties of connectivity.

Overall, connectivity was tested between neurons separated by up to 425 μm (mean ± SD = 133 ± 80). At distances greater than 250 μm only one connection was found out of 363 potential connections. Unsurprisingly, the probability of finding a connection over 0 – 425 μm decreased with increasing distance between cell pairs ($p < 0.0001$). When the intersomatic range was restricted to 0 – 150 μm (approximately 70% of all cell pairs), this relationship disappeared ($p = 0.41$). Overall connection probability within this range of extensive axo-dendritic overlap was still low at 4.9% (112/2267), demonstrating the high level of selectivity for synaptic coupling among corticospinal neurons. In fact, further restricting the range of intersomatic distance from 0-50 μm still yielded a connectivity rate of 4.9% (25/513). We found no significant relationship between intersomatic distance and any uEPSC response properties (data not shown).

Figure 2.10 shows the overall breakdown of intersomatic distance of tested cell pairs separated into medial-lateral (x-axis) and dorsal-ventral (y-axis) dimensions. We tested connections from cell pairs with a medial-lateral separation of up to 400 μm, over which connectivity was inversely correlated with distance ($p < 0.001$). However, for cell pairs within 100 μm of each other, which comprised over 1000 tested connections, there was no significant relationship between connection probability and intersomatic distance ($p = 0.57$). Intersomatic

distance along the dorsal-ventral axis (y-axis) was not correlated with connection probability, even over the entire sample space of 0 – 375 μm ($p = 0.16$). The rostral-caudal axis (z-axis), which corresponds to the plane of tissue slicing, was sampled over a more limited range (0-70 μm). Distance between cell pairs along this axis was also not related to connection probability ($p = 0.54$).

Connectivity rate, synaptic signaling, and intrinsic electrophysiological properties do not differ between C4- and C8-projecting corticospinal neurons in adulthood

Previous findings demonstrate that baseline structural morphology differs between C4-projecting and C8-projecting corticospinal populations in adulthood [35]. Furthermore, structural variability was remarkably low within each population, suggesting that the mechanisms shaping neuronal structure may be unique for each projection subpopulation. To test whether these structural discrepancies generalize to other attributes, we examined the network and electrophysiological properties between C4- and C8-projecting neurons in adult animals (125+ g).

We found no differences for connectivity rate within the corticospinal population regardless of the identities of potential synaptic partners (Fig. 2.5). That is, connection probabilities between C4-projecting neurons (C4-C4: 18/480), C8-projecting neurons (C8-C8: 17/683), or between a C4- and a C8-projecting neuron (C4-C8: 12/428; C8-C4: 14/428) did not significantly differ ($p = 0.63$; Pearson's Chi Square). Moreover, uEPSC response properties did not differ

across cell-pair groups (Table 2). These results indicate that fundamental network properties are similar across functional subpopulations of corticospinal neurons.

Assessment of intrinsic electrophysiological properties, including multiple measures of excitability, also showed no difference between C4- and C8-projecting subpopulations (Table 3). Thus, despite differences in dendritic structure and axonal targeting in the spinal cord, corticospinal neurons controlling different aspects of forelimb behavior express similar properties for network connectivity, synaptic signaling, and intrinsic membrane ion conductance, suggesting information is processed homogenously across the corticospinal system.

DISCUSSION

The motor cortex (M1) provides a model system for examining developmental changes in local circuitry that support the emergence of adult-like behavior. As opposed to other cortical systems, such as the visual cortex, where sensory networks show significant functionality at birth [8, 56, 57], cortical control of motor behavior is absent in neonates of most species, including rodents. We examined network and cell intrinsic properties of corticospinal subpopulations associated with control of different forelimb behaviors (upper vs. lower forelimb) during the timeframe for the development of fine motor control. During “early” motor system development (P18 - 45), we find that connectivity is elevated

between corticospinal neurons projecting to the same spinal cord segment. This increased interconnectivity gradually declines with age, until connection probability is similar between neurons projecting to the same or different spinal cord targets. Accompanying this rearrangement of synaptic configuration is a general reduction in synaptic efficacy, and an increase in intrinsic excitability. Finally, we find that, despite known structural differences between C4- and C8-projecting corticospinal neurons, synaptic and electrophysiological properties do not differ between these subpopulations.

The elevation of within-population connectivity in developing animals indicates that synaptic coupling is initially favored between neurons projecting to the same long-range axonal target. We believe this biased connectivity in young animals may be partially driven by shared clonal lineage. Corticospinal neurons that project to distinct spinal cord segments are anatomically intermingled in M1 [35]. This intermingling makes it improbable that local cues within M1 specify spinal cord innervation patterns. Instead, cell intrinsic factors, such as gene expression profile, in conjunction with environmental cues within the spinal cord, likely guide specific neurons to innervate particular spinal cord segments [58-60]. Such similarity of gene expression may be a manifestation of shared clonally lineage. Indeed, in the visual cortex, sister neurons derived from the same parent cell show a high frequency of interconnectivity and similarity of functional response properties in early life [7, 9, 61], consistent with our finding that functionally related neurons show higher connectivity in young animals. Furthermore, clonally related neurons exhibit anatomical dispersion and are

spatially intermingled with neurons derived from other parent neurons [62], consistent with the intermingling of C4- and C8-projecting populations within M1.

Altogether, this presents a scenario where C4-projecting corticospinal neurons have inherited similar molecular expression profiles which guide their mutual innervation of spinal segment C4. This clonal relationship leads to an increased probability of connectivity, perhaps due to early electrical coupling [7], or co-expression of complimentary adhesion molecules [63]. C8-projecting neurons are derived from a different progenitor cell than the C4-projecting population, but are subject to comparable forces for axon guidance and synapse formation.

Whether the initial levels of across-population connectivity reflect random synaptic sampling or directed connectivity cannot be answered at present. Moreover, it cannot be ruled out that some fraction of across-population cell pairs may in fact be “within-population” due to axon collaterals that terminate in overlapping spinal segments, e.g., C4- and C8-projecting neurons with mutual collaterals in segment C6. Finally, our earliest recordings were from animals just under postnatal day 20. Thus, local circuitry may have experienced substantially reorganization prior to our examined timeframe.

Unitary EPSC properties did not differ for within- or across-population cell pairs, suggesting synaptic function is similar regardless of projection target similarity between individual neurons. We did, however, find a global trend for decreased presynaptic release probability as the motor system developed,

consistent with findings for other neural populations and cortical regions [51, 64-66]. Furthermore, intrinsic excitability increased with age. These results are indicative of a developmental switch in how information is transferred between neurons; whereas single action potentials can effectively transmit excitation in young animals, mature circuits rely on sustained activation for effective transmission. These different modes of signaling may promote different functions. For example, enhanced synaptic efficacy early on may be important for establishing and stabilizing nascent synapses [67]. Over time, however, this relatively large synaptic strength could hinder fine motor control by allowing even "counterproductive" synaptic connections to influence postsynaptic activity. Globally reducing synaptic strength would decrease the ability of functionally unrelated neurons to induce activity in recurrent targets, thereby decreasing noise in motor networks. This decreased synaptic influence could also facilitate synapse destabilization between incongruous cell pairs via Hebbian or spike-timing-dependent plasticity. Therefore, the observed loss of corticospinal interconnectivity may be necessary for the emergence of fine motor control.

That this decrease in connectivity was restricted to cell pairs projecting to the same spinal cord segment is intriguing, and suggests the role of within-population networks decreases over the timeframe examined. Indeed, many connections among neighboring cortical neurons present during development are unlikely to be functionally relevant in adulthood and are lost with increasing experience [2]. For example, in the visual cortex, local excitatory connectivity is biased toward functionally related neurons in adulthood [68]. Such functional

connectivity is not detected at eye opening, however, but instead emerges after weeks of visual experience [65]. Similarly, motor experience may drive reorganization of preliminary corticospinal networks when animals begin to engage in complex motor behaviors that require coordination across multiple body parts.

This view is supported by studies that indirectly assess network connectivity via intracortical microstimulation (ICMS) [69]. Interestingly, as the motor system develops and animals engage in complex movements, ICMS increasingly evokes movement about multiple joints [40]. Because ICMS output is highly dependent on local signaling within M1[30, 31], increased multi-joint representations likely reflect a greater influence of across-population connections with age. Indeed, If complex movements are restricted during development, thereby limiting coactivation (and strengthening) of across-population networks, multi-joint ICMS representations are reduced [70]. Therefore, it is feasible that the observed synaptic reorganization during development is due to experience-dependent mechanisms that selectively stabilize (or refrain from destabilizing) across-population connectivity required for increasingly complex behavior.

Consistent with previous reports [49, 50], bidirectional connectivity was more frequent than predicted by chance. This was true for both within- and across-population cell pairs. However, we found bidirectional connectivity was significantly higher between cells projecting to the same spinal segment versus different segments, indicating that the rate of reciprocal connectivity is

heterogeneous even within a specific subclass of neurons, and may be dependent on the functional relationship of cells. Why bidirectional connectivity would be more prevalent between neurons targeting the same spinal segment is unknown, although it is possible that reciprocal connectivity is elevated between neurons controlling the same muscle(s). Reciprocal connections may thus enhance activation within a “muscle network”, thereby increasing stimulation of downstream spinal neurons and increasing the reliability of muscle recruitment.

In total, the results of the current study suggest the emergence of fine motor behavior is associated with several alterations within the corticospinal network. Decreased interconnectivity may increase the quantity of independent networks, thereby enabling greater fractionation of motor behavior, and may allow neurons with similar output to segregate into separate functional networks encoding distinct motor behaviors. This reorganization is likely experience dependent, and may be augmented by decreased synaptic efficacy that ensures only functionally related cell pairs remain connected. Additionally, increased excitability promotes sustained activation of corticospinal neurons, amplifying descending signals and facilitating cortical control of movement.

Finally, in adult animals C4- and C8-projecting corticospinal neurons exhibit unique structural characteristics, with greater dendritic complexity and spine density present in C4-projecting neurons [35]. However, we found no differences in electrophysiological properties, interconnectivity, or uEPSC response properties between these two projection populations in adult animals.

Thus, synaptic and membrane properties appear not to differ across projection subclasses of corticospinal neurons. Further, the increased synaptic input onto C4-projecting neurons seemingly originates from outside the corticospinal network.

FIGURES

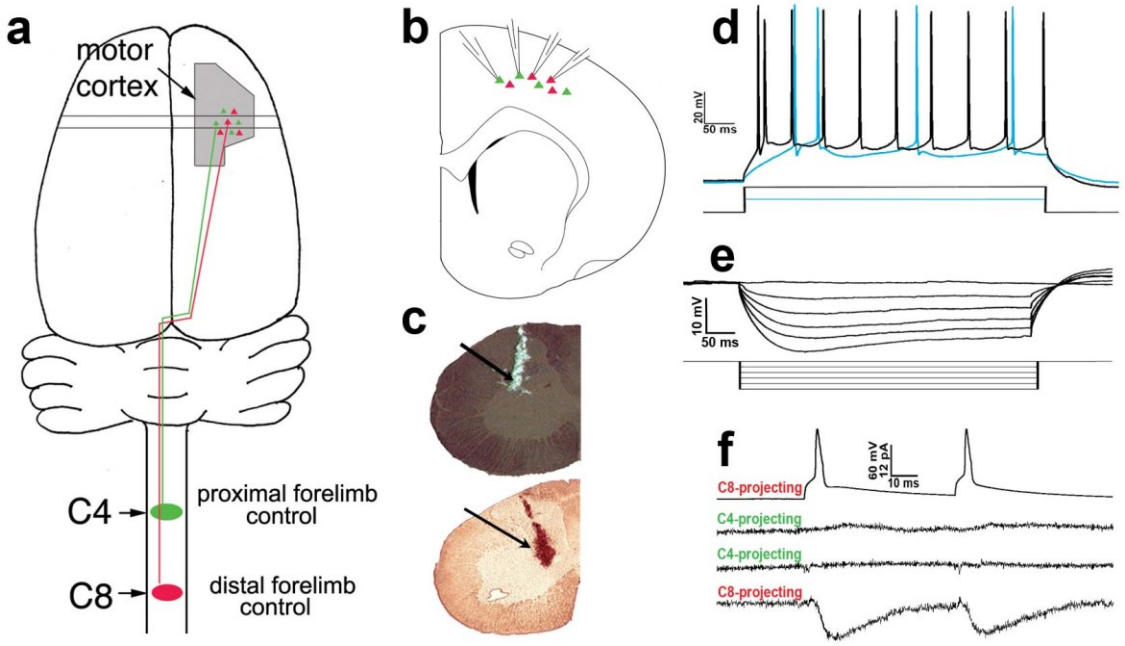

Figure 2.1. Experimental overview. a) Retrograde tracer injections at levels C4 and C8 of the spinal cord enabled identification of distinct corticospinal projection populations originating in layer V motor cortex. **b**) Labeled cells were targeted for *in vitro* whole-cell patch clamp of up to four neurons simultaneously. **c**) Tracer injections targeted the dorsal horn and intermediate zone (indicated by arrow), and did not inadvertently spread into the corticospinal tract. **d**) All recorded cells displayed a regular spiking pattern to suprathreshold current injections, often exhibiting a doublet at the onset of spiking (blue trace = 200 pA current injection; black trace = 400 pA). **e**) hyperpolarizing current injection (20 pA steps) produced a noticeable sag in the membrane potential. **f**) Quadruple recordings exhibiting an excitatory response in a single postsynaptic neuron.

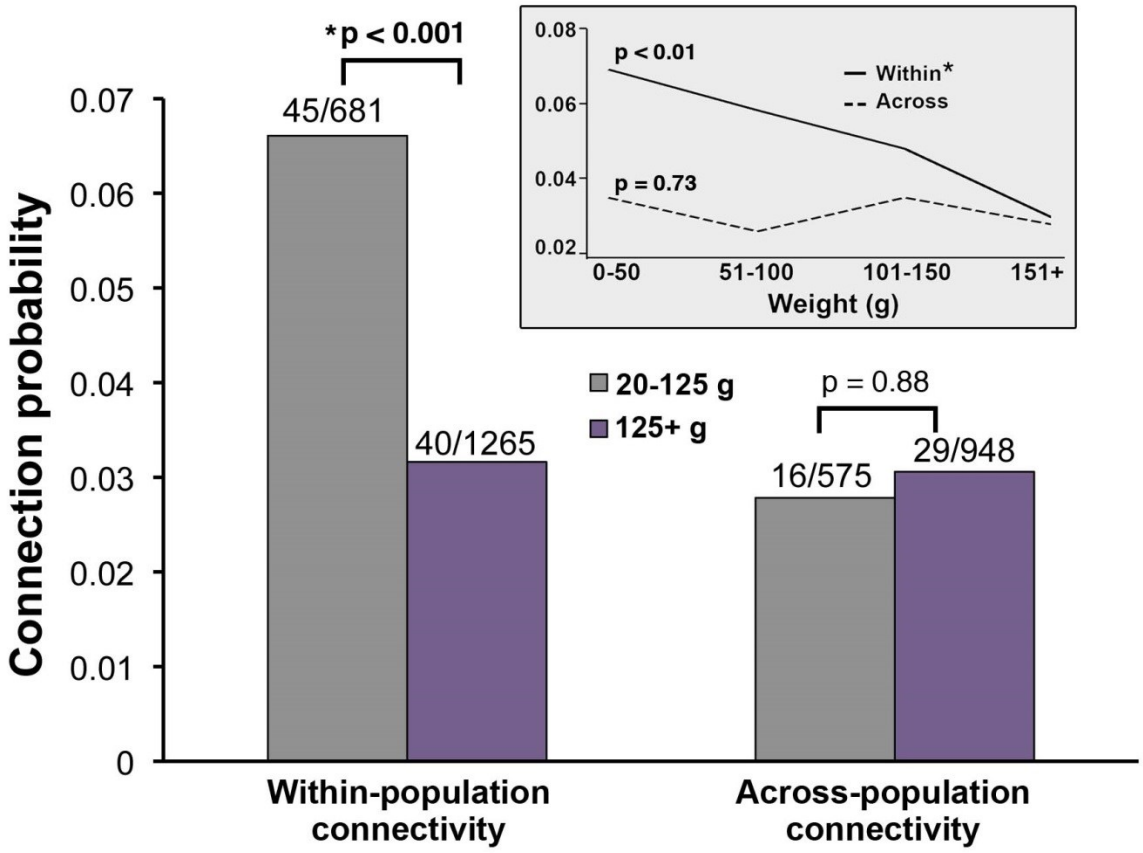

Figure 2.2. Connectivity is higher during development among corticospinal neurons projecting to the same spinal cord segment. Within-population connection probability was significantly higher in developing (20–125 g) versus adult (125+ g) animals. Conversely, interconnectivity between cell pairs targeting different segments of the spinal cord (across-population) was not different between developing and adult animals. Inset shows connection probability binned in 50 g animal weight increments, with p-values based on linear regression of connection probability over the total (unbinned) weight range.

Although we did not measure connectivity during the earliest period of synaptogenesis, these data suggest initial synapse formation is preferential between corticospinal neurons innervating the same spinal segment, and indicate an innate mechanism whereby neurons directed to a particular spinal segment are also directed to interconnect locally within M1. Many of these initial connections may not be functionally relevant, and are consequently lost with experience (see discussion).

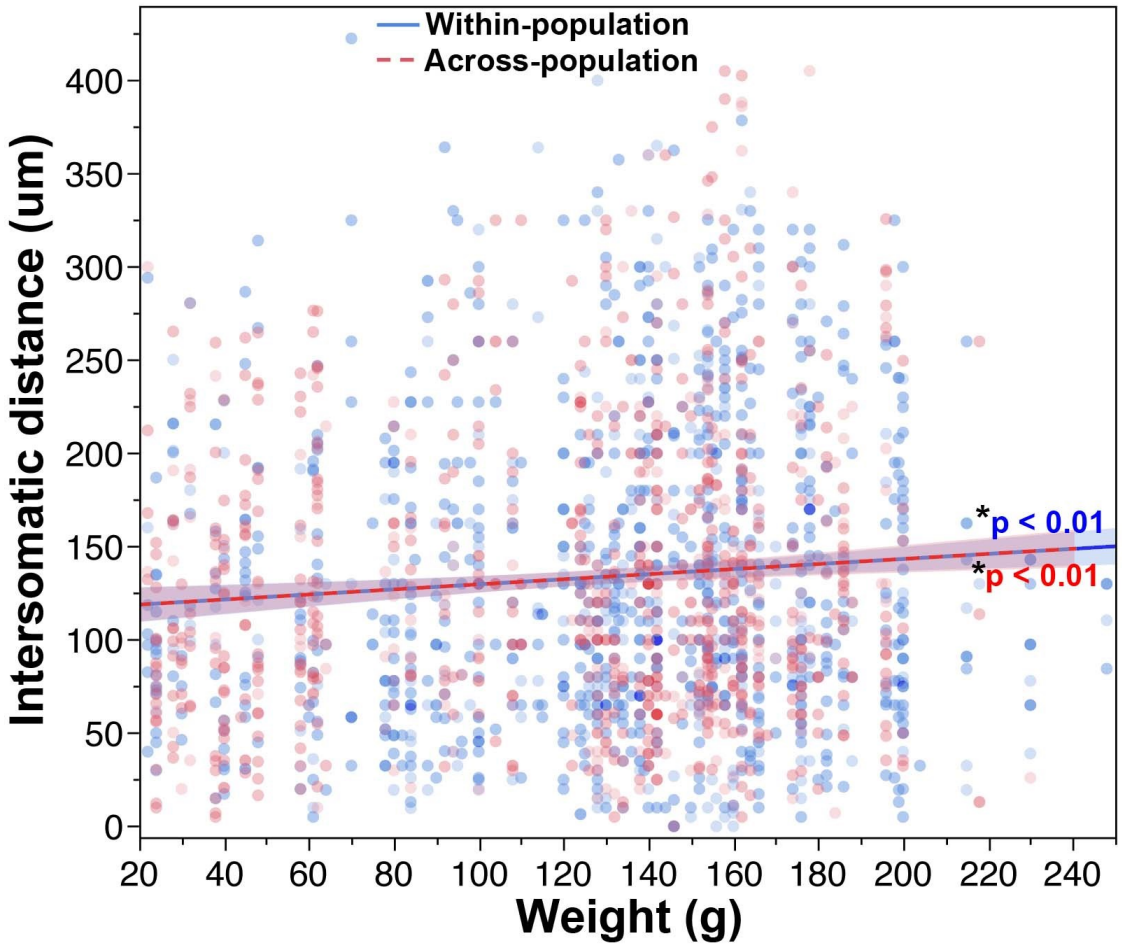

Figure 2.3. Mean intersomatic distance between recorded neurons increases with age. Dots symbolize intersomatic distance between corticospinal neurons in which synaptic connectivity was assessed. **Blue** = within-population cell pairs; **Red** = across-population cell pairs. There can be multiple cell pairs for a specific weight x distance value; these overlapping data are signified by increasing color intensity. The distance between cell pairs increased with weight similarly for within- and across-population groups. Lines and shaded area indicate the linear line of fit of the data and 95% confidence of fit, respectively.

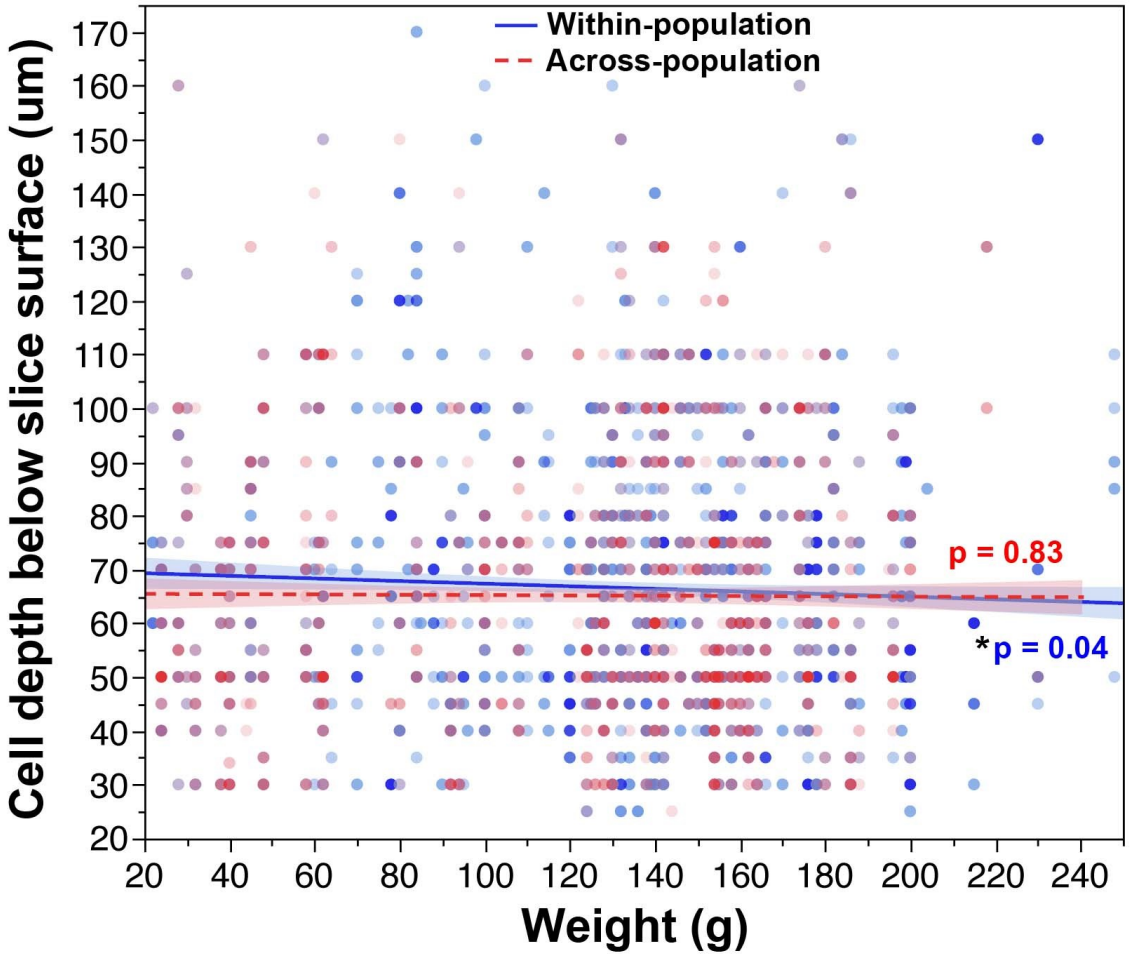

Figure 2.4. Depth below the slice surface of recorded neurons slightly decreases for within-population neurons with age. Dots symbolize corticospinal depth for individual neurons belonging to within- (**blue**) or across- (**red**) population cell pairs. Note: an individual cell can belong to both cell pair groups, as in a C8-projecting cell that was tested for connectivity with another C8-projecting cell (within-population) as well as with a C4-projecting cell (across-population). Cell depth showed a marginal but significant tendency to decrease with weight for within-population neurons, whereas across-population cells showed no such trend. Overlapping data are signified by increasing color intensity. Lines and shaded area indicate linear line of fit and 95% confidence of fit, respectively.

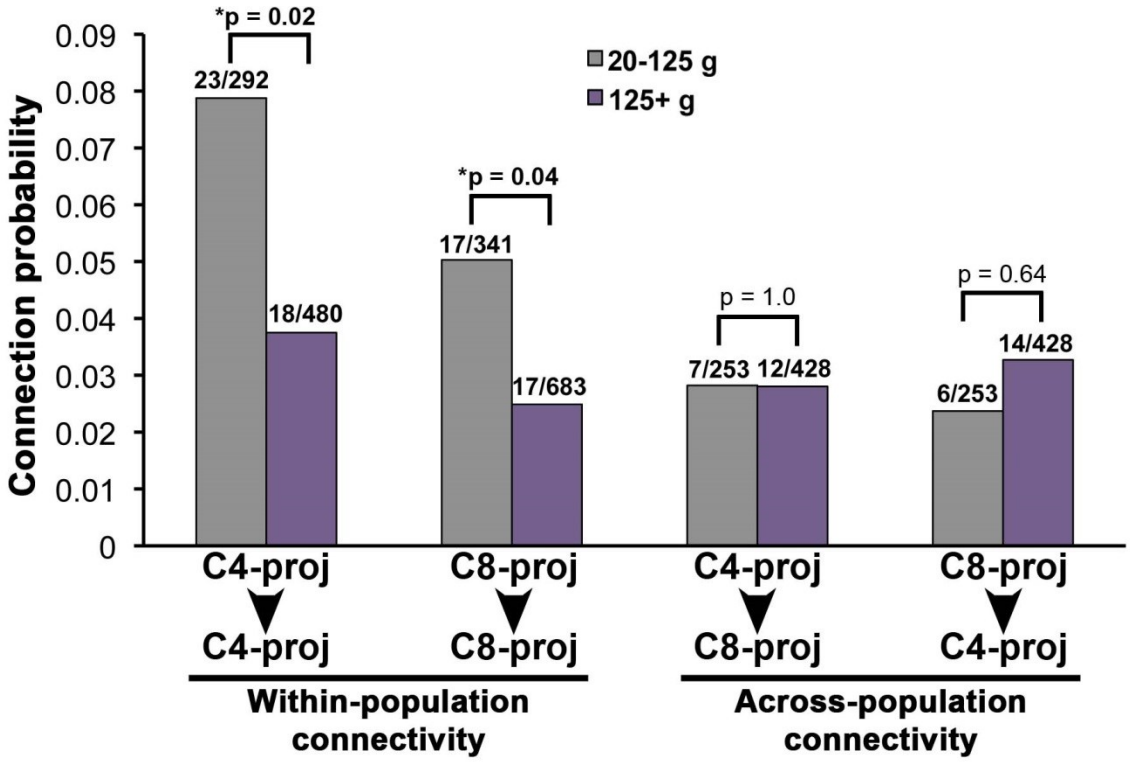

Figure 2.5. Age-related changes for within-population connectivity are jointly driven by C4-C4 and C8-C8 cell pairs. Both C4-C4 and C8-C8 cell pairs showed a significant decrease in connection probability in adulthood. Thus, elevated within-population connectivity is not due to a particular projection population, but appears to be a general property of the corticospinal system. Additionally, across-population connectivity did not change with age for either cell pair group (C4-C8 or C8-C4). The difference in connection probability between C4-C4 and C8-C8 cell pairs in developing (20–125 g) animals was not significant ($p = 0.14$, Fisher's exact test).

We speculate these data reflect innate programming that promotes synapse formation between neurons projecting to the same spinal cord segment (C4-C4, C8-C8), perhaps due to clonal relatedness of these neurons. With increasing experience, this initial overabundance of within-population connectivity is reduced as nonfunctional connections are broken (see discussion).

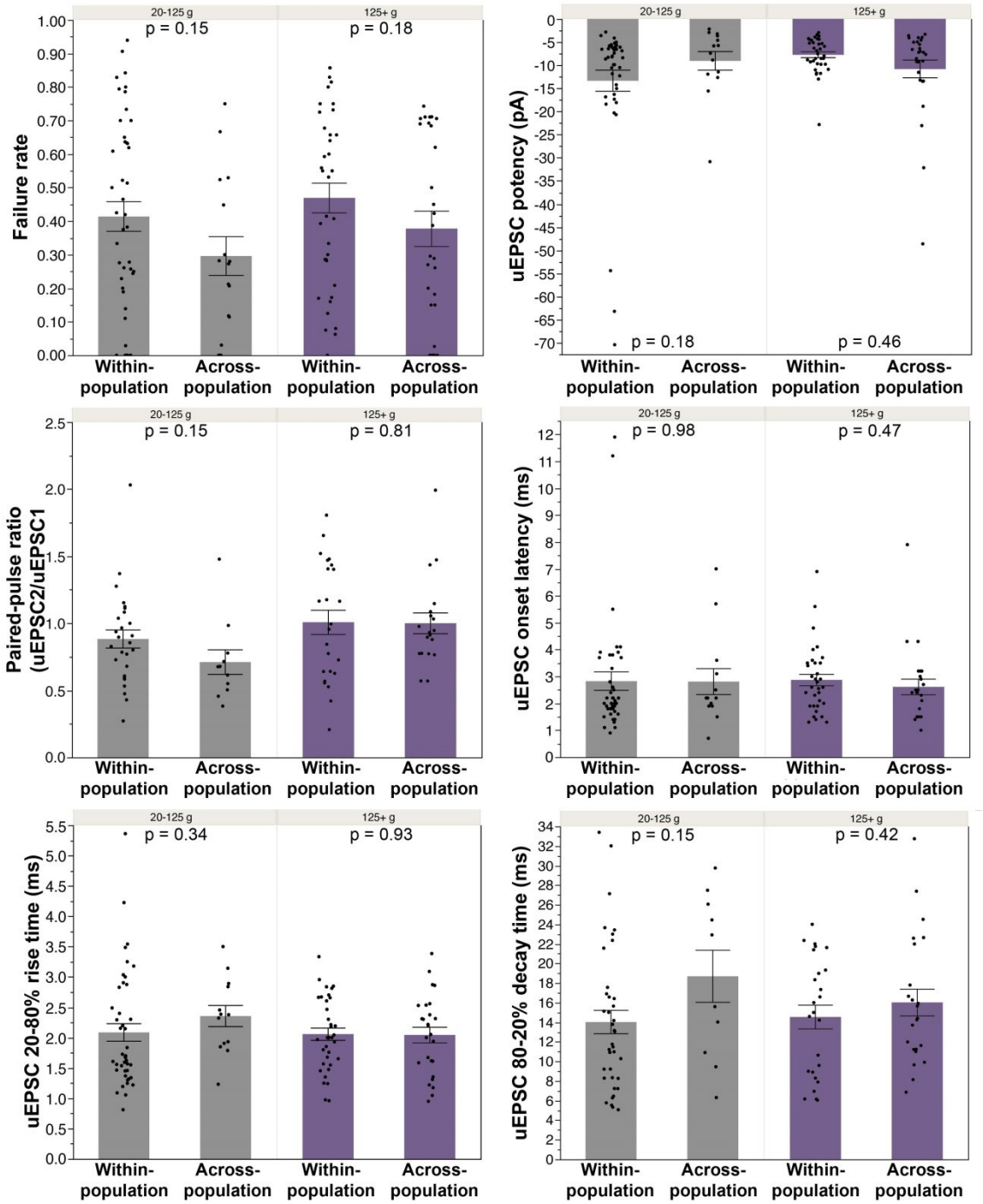

Figure 2.6. uEPSC response properties are similar for within- and across-population cell pairs. Various uEPSC properties grouped by cell-pair category (within- and across-population) and weight (20-125 g: grey bars; 125+g: purple bars). Within each weight category, there were no differences between cell pair groups for any of the response properties measured. uEPSC comparisons across weights are presented in Figure 2.7.

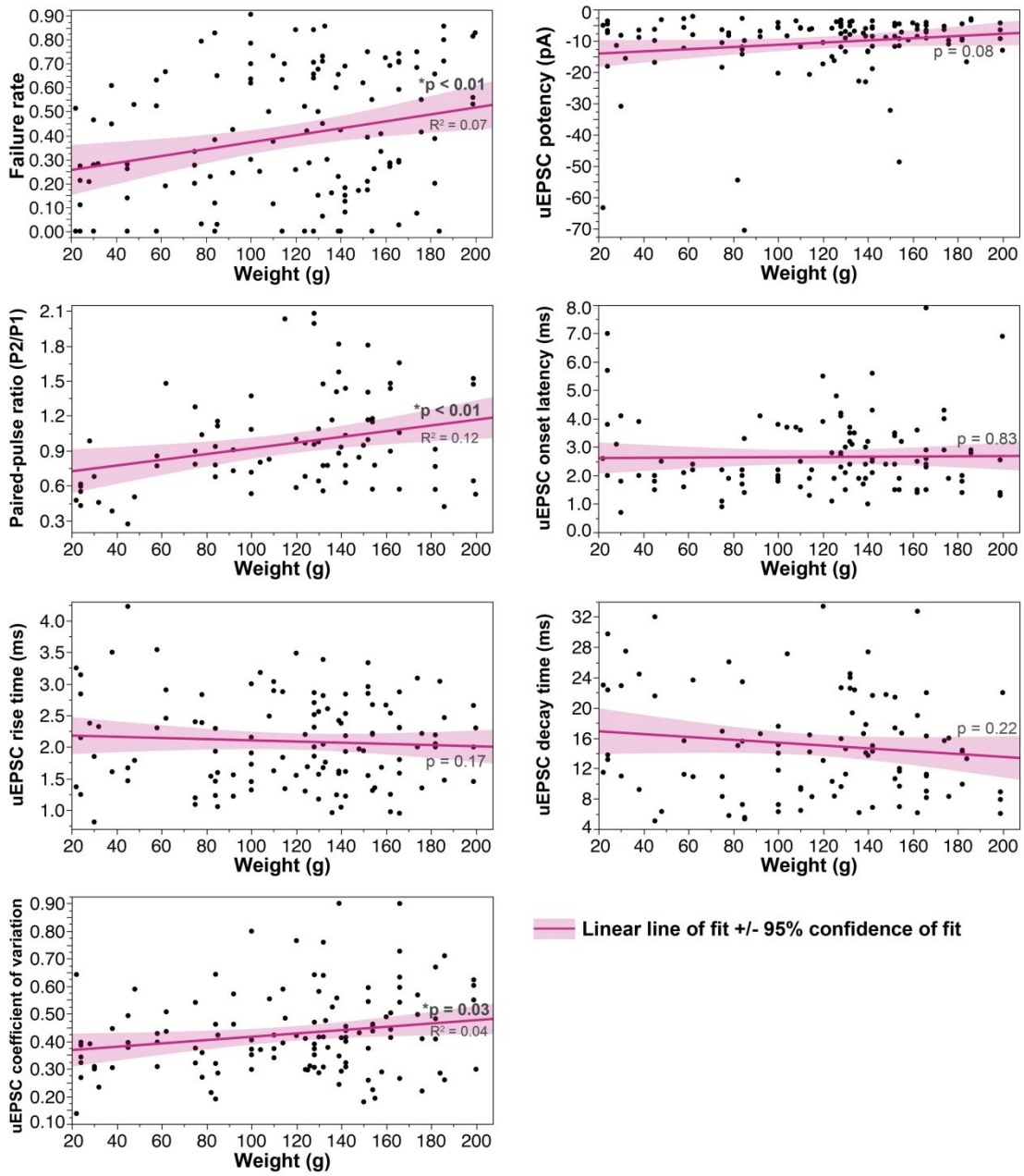

Figure 2.7. Age-related alterations in uEPSC properties suggest presynaptic release probability decreases among recurrent corticospinal connections as the motor system matures. Failure rate, paired-pulse ratio, and uEPSC coefficient of variation all showed a tendency to increase with age, suggesting a change in presynaptic function. However, note the large variability of these features in older animals, indicating corticospinal inputs show wide functional variance and likely remain highly modifiable in adulthood. Other features, including response onset latency, 20-80% rise time, and 80-20% decay time showed no change with maturation.

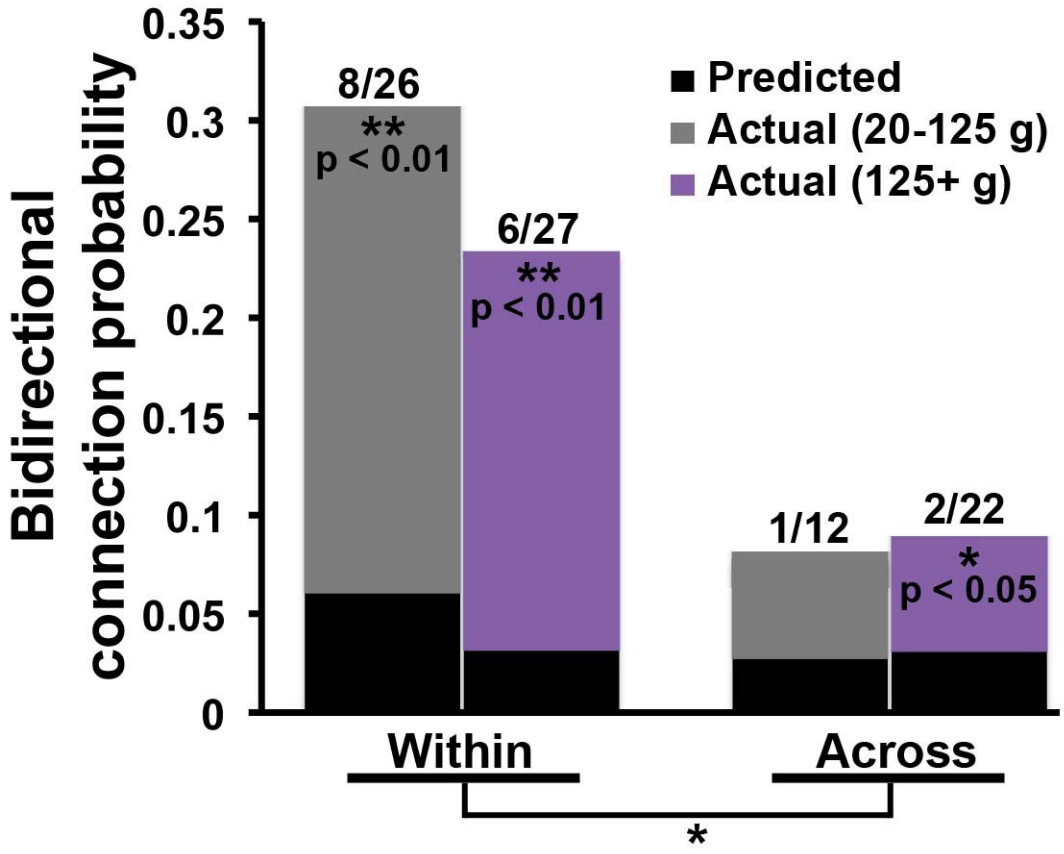

Figure 2.8. Bidirectional (reciprocal) connectivity is greater than that predicted by the overall connection probability. The probability of finding a connection between any two corticospinal neurons for each weight/cell-pair combination was used to predict the occurrence of bidirectionally connected cell pairs (black bars). The actual probability of finding reciprocally connected pairs was significantly greater than the predicted level for all groups except for across-population in 20-125 g animals (Monte Carlo simulation). When both weight groups were combined, bidirectional connectivity was more frequent than predicted by chance for both within- ($p < 0.0001$) and across-population ($p = 0.01$) cell groups. Furthermore, within-population cell pairs as a whole showed an increased rate of bidirectional connectivity when compared to across-population cell pars. Numbers atop bars denote the number of bidirectionally connected cell pairs / total number of connected cell pairs (both unidirectional and bidirectional). Cell pairs for which connectivity could only be assessed one-way (and not bidirectionally) were excluded from analysis.

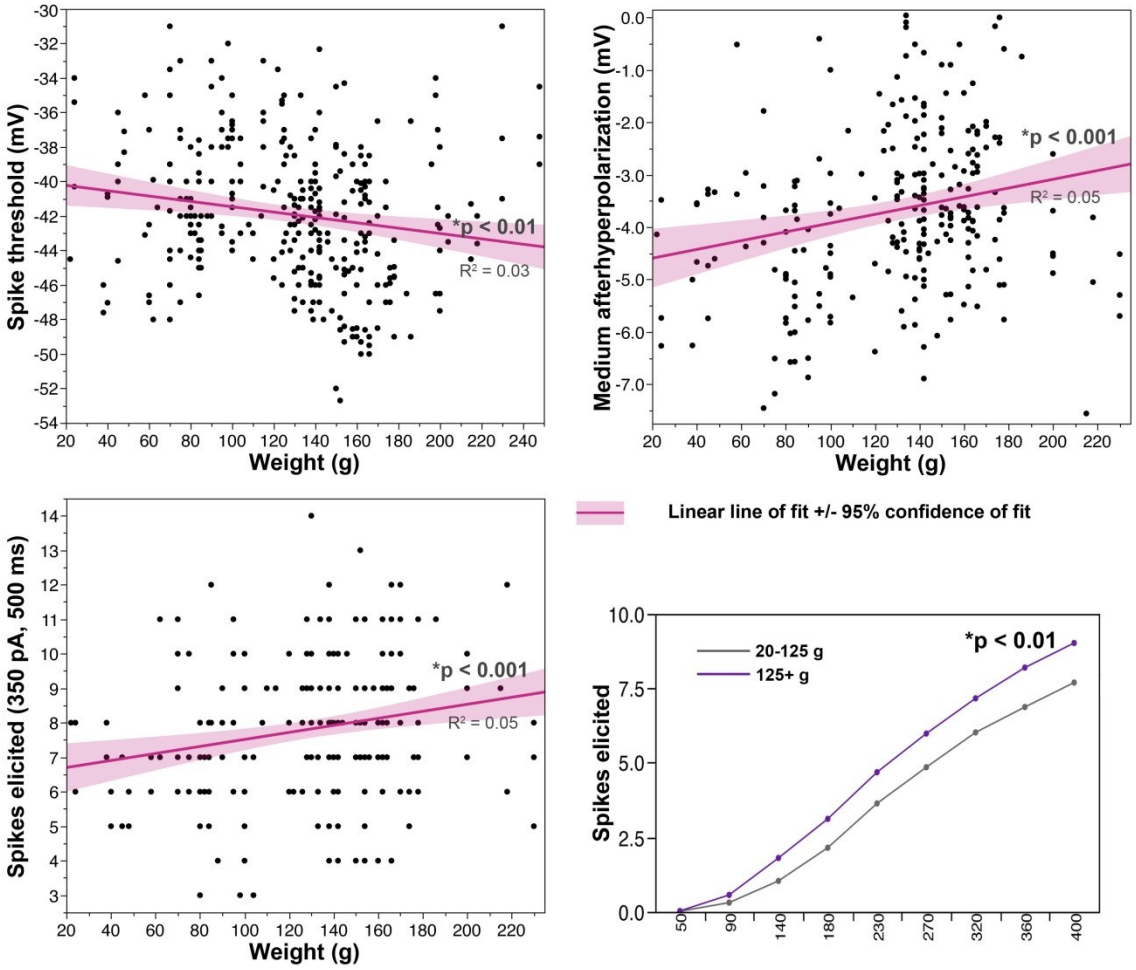

Figure 2.9. Intrinsic excitability increases over the course of development. Spike threshold and peak medium afterhyperpolarization (mAHP) both decreased with age (note the negative scale for mAHP). Spiking activity also increased with age, as shown by the age-related increase in action potentials elicited in response to a 500 ms depolarizing current injection of 350 pA. Furthermore, spiking activity was increased over multiple current injection levels for adult (125+ g) vs. developing (20-125 g) animals (repeated-measures ANOVA).

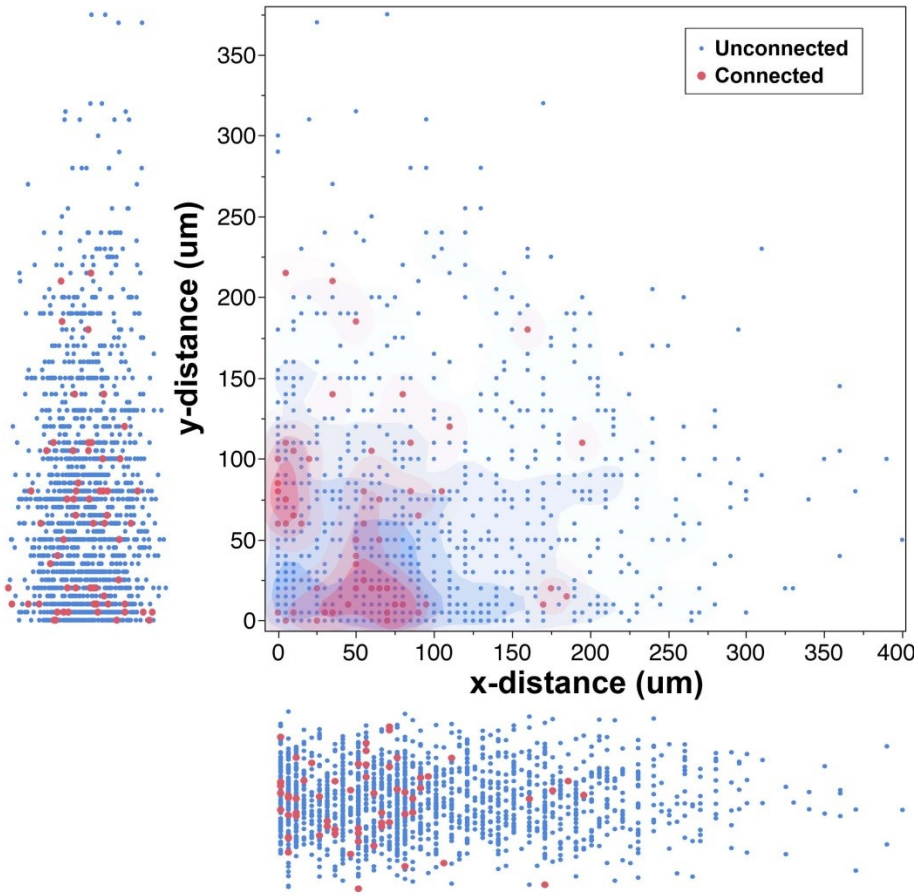

Figure 2.10. Distance between recorded cell pairs. Points represent synaptically connected (red) and unconnected (blue) cell pairs. Red dots are enlarged for easier identification. Overlaid contour plot indicates density of connected and unconnected cell pairs. Individual points within the main (boxed) graph can represent multiple cell pairs with the same intersomatic distances. Separation of these overlapping points can be seen in the plots bounding the main scatterplot. These bounding plots show individual intersomatic distances of cell pairs solely with respect to the x-axis (below main scatterplot) or y-axis (left of main scatterplot).

Along the medial-lateral dimension (x-axis), connectivity rate falls off with increasing distance between recorded cells ($p < 0.001$), and no connections were detected beyond 200 μm. Over the x-axis range of 0-100 μm, however, connection probability was unrelated to distance ($p = 0.57$). Along the dorsal-ventral dimension (y-axis), intersomatic distance did not predict connection probability ($p = 0.16$), although no connections were found for cells separated by more than 225 μm.

Table 2.1. uEPSC response properties of unidirectionally and bidirectionally connected cell pairs.

	Unidirectional	Bidirectional
Failure rate	0.42 ± 0.28 (n = 65)	0.35 ± 0.27 (n = 35) p = 0.2
Potency (pA)	-8.9 ± 7.1 (n = 63)	-12.6 ± 14.8 (n = 33) p = 0.16
uEPSC coefficient of variation	0.43 ± 0.13 (n = 63)	0.43 ± 0.16 (n = 33) p = 0.95
Paired-pulse ratio (P2/P1)	0.93 ± 0.43 (n = 50)	1.1 ± 0.4 (n = 23) p = 0.18
Latency (ms)	2.6 ± 1.1 (n = 60)	2.3 ± 0.9 (n = 34) p = 0.16
Rise time (ms)	2.2 ± 0.77 (n = 62)	1.8 ± 0.61 (n = 33) *p < 0.01
Decay time (ms)	15.7 ± 7.1 (n = 53)	12.8 ± 6.1 (n = 26) p = 0.07

Table 2.2. Intrinsic electrophysiological properties are similar across C4- and C8-projecting cell pairs in adult animals.

	C4-projecting 125+ g	C8-projecting 125+ g
Resting membrane potential (mV)	-65.9 ± 1.9 (n = 59)	66.3 ± 2.5 (n = 70) p = 0.21
R_{input} (MΩ)	79 ± 21 (n = 70)	72.9 ± 19.9 (n =90) p = 0.19
Threshold (mV)	-42.8 ± 3.6 (n =70)	-42.8 ± 4.2 (n =87) p = 0.94
Capacitance (pF)	142 ± 47 (n =75)	146 ± 54 (n =94) p = 0.68
Spike half-width (ms)	2.3 ± 0.4 (n =67)	2.2 ± 0.4 (n =76) p = 0.24
Spike height from threshold (mV)	78.5 ± 6.1 (n = 80)	79.2 ± 7.7 (n =87) p = 0.52
AHP (mV)	3.4 ± 1.4 (n =70)	3.5 ± 1.6 (n =77) p = 0.62
Spike count (350 pA, 500 ms)	8 ± 2.1 (n = 71)	8.2 ± 1.9 (n = 76) p = 0.57

Table 2.3. uEPSC response properties are similar across C4- and C8-projecting cell pairs in adult animals.

	C4-projecting 125+ g	C8-projecting 125+ g
Failure rate	0.4 ± 0.29 (n = 32)	0.46 ± 0.26 (n = 32) p = 0.46
Potency (pA)	-9.4 ± 9.2 (n = 31)	-8.2 ± 4.2 (n = 30) p = 0.74
EPSC coefficient of variation	0.42 ± 0.16 (n = 31)	0.48 ± 0.17 (n = 30) p = 0.14
Paired-pulse ratio (P2/P1)	1 ± 0.43 (n = 19)	1.1 ± 0.39 (n = 22) p = 0.27
Latency (ms)	2.9 ± 1.3 (n = 27)	2.6 ± 1.3 (n = 29) p = 0.35
Rise time (ms)	2.1 ± 0.57 (n = 30)	2 ± 0.64 (n = 29) p = 0.56
Decay time (ms)	14.9 ± 6.3 (n = 20)	15.8 ± 6.3 (n = 26) p = 0.65

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CHAPTER 3

SYNAPTIC MECHANISMS OF LEARNING: INCREASED CONNECTIVITY AND EXCITABILITY IN TASK-SPECIFIC NEURONAL SUBNETWORKS

Alterations in the strength and patterning of synaptic connectivity are believed to underlie learning, and substantial effort has been devoted toward understanding how new knowledge and abilities are represented in adult neural circuits. While progress in this field has been substantial, the inability to identify and study the properties of *single* neurons that actively participate in the learning process has been a limiting factor. Using a combination of retrograde tracing and paired cell recordings in adult rat brain slices, we show that local synaptic reorganization is confined to task-relevant neurons as rats acquire a new skilled motor grasping behavior. Skilled motor learning was associated with three cardinal sets of modifications that were unique to learning neural circuits. First, there was a 3-fold increase in the number of intracortical excitatory connections among the specific subnetwork of "learning" neurons associated with the new motor behavior. Second, we identify a targeted increase in inhibition within the same learning subnetwork. Third, "learning" neurons exhibited significant increases in intrinsic excitability compared to their baseline, untrained state ($p<0.05$). In contrast, adjoining neurons of the same class

but not involved in learning exhibited no significant change in any of these parameters. Thus, learning results in highly specialized changes that fundamentally alter the pattern of interneuronal communication through the construction and biased activation of circuits encoding behaviorally relevant information.

While acquisition of a skilled motor behavior requires the coordinated activity of multiple brain structures [71, 72], converging evidence implicates the primary motor cortex (M1) as the main locus of stable representations for learned fine motor behaviors [73, 74]. Supporting this view, long-duration (> 500 ms) stimulation of M1 evokes stereotypical and behaviorally complex movements [75-77], functional MRI in humans performing overlearned motor tasks exhibits predominant activation of the M1 region [71, 78, 79], and ablation of M1 across species, including humans, abolishes previously learned skilled motor abilities [22, 24, 34, 80, 81]. Previous efforts to understand mechanisms underlying adult learning in the motor cortex and other cortical regions have identified augmented synaptic transmission [82-85], increases in neuronal firing rate and synchrony [86-88], spinogenesis [35-37, 39], and a reorganization of representational cortical maps [32-34, 89-91] as features of adult learning. While providing useful insight, these studies used relatively non-specific neuronal sampling methods because individual neurons actively participating in the learning process could not be identified in the adult brain slice or during *in vivo* recording. As a consequence, clear understanding of synaptic mechanisms utilized specifically by *learning* neurons in the cortex remains elusive. Indeed, unless sampling

specifically from neurons engaged in a new learning task, there is a risk that essential mechanisms underlying learning in adulthood might be underestimated, misinterpreted, or actually escape detection.

We developed methods for identifying individual neurons of the motor cortex that are activated in the course of learning a new, skilled motor task to enable study of synaptic modifications in actively learning neurons [35] (Fig. 3.1). This was accomplished by labeling individual corticospinal (CS) motor neurons of layer V motor cortex that are associated with either proximal or distal forelimb control, in the same animal, after injecting different colored retrograde latex microspheres into dorsal gray matter of C8 spinal segments, controlling distal muscles for skilled grasping, or C4 spinal segments, controlling proximal muscles for shoulder movements, [16, 34, 35, 73, 92-95]. Previously we reported that this approach identifies a specific subpopulation of layer V CS cells of the motor cortex that elaborate significant increases in dendritic architecture and spine number as a function of motor learning; these structural elaborations are restricted to the C8-projecting neuronal population that is activated when learning a skilled grasping task [35]. In contrast, C4-projecting neurons that are not required to execute the skilled grasping procedure underdo no structural change [35]. Corroborating the selective activation of the C8-projecting population in learning the skilled grasping task, only C8-projecting CS cells in M1, and not C4-projecting CS cells, significantly increase the activity marker c-fos [35]. These findings highlight the restriction of potentially important plasticity mechanisms to

“learning” neurons: here we study the synaptic and network properties of these active cells.

Rats first underwent injections of retrograde tracers into C8 to label subsets of layer V corticospinal cells that subsequently “learn” the skilled grasping task [43, 44] (Fig. 3.1a, b); the same animals received injections of different retrograde tracers into C4 to label non-learning CS cells of the motor cortex [35, 45, 46]. Rats then underwent skilled grasp training over 10 consecutive days. All rats exhibited significant increases in skilled pellet grasping performance (Fig. 3.1d; $p<0.001$, repeated measures ANOVA). Within five days of training completion, we prepared cortical slices of M1 and targeted C8- and C4-projecting corticospinal neurons for up to four simultaneously whole-cell recordings (Fig. 3.1e, f). In total, 2,892 connections were tested in 129 animals of average age 56 ± 2 days.

Notably, training resulted in a highly significant, 3-fold increase in the number of excitatory connections among the “learning” subnetwork of layer V motor cortex neurons projecting to C8 ($p<0.001$, Fig. 3.2a). In contrast, excitatory connectivity among layer V neurons projecting to C4 that control the shoulder was entirely unchanged ($p=1.0$; Fig. 3.2a). This increase in excitatory connectivity among layer V, C8-projecting neurons was highly specific, as excitatory connections across functional populations of neurons (C8-projecting to C4-projecting, or C4-projecting to C8-projecting) did not change significantly ($p=1.0$ and 0.4, respectively). These results were not influenced by depth of

recorded cells from the slice surface or the distance between cells (Fig. 3.5-3.7). Therefore, skilled motor learning is associated with a selective increase in excitatory connectivity among task-relevant neurons.

In addition to the monosynaptic excitatory connections observed among corticospinal neurons, inhibitory postsynaptic currents (IPSCs) were also often detected in response to single action potentials applied to corticospinal neurons. These events had an onset latency that was significantly longer than excitatory responses (excitatory latency = 2.7 ± 1.2ms, inhibitory latency = 6.3 ± 1.5ms; $p<0.001$), and was blocked by the AMPA receptor antagonist DNQX ([20 μM], bath applied) (Fig. 3.2d), indicating this inhibitory pathway was likely disynaptic in nature. This disynaptic inhibition is present to a substantially greater extent than previously identified [96-98], and may be a function of greater prominence of inhibitory circuits in the motor cortex compared to other regions, or the more advanced age of animals from which our slices were obtained

Previous reports suggest inhibitory interneurons globally inhibit surrounding neurons via dense, unspecific innervation of neighboring pyramidal cells [99-101]. In general, our data support this model (see Fig. 3.2e). Interestingly, however, we found that learning was associated with a specific increase in inhibition onto the C8-projecting population ($p=0.05$; Fig. 3.2c), while no changes were identified for the non-learning, C4-projecting network of layer V neurons ($p=0.9$; Fig. 3.2c). Unlike the excitatory condition, increased inhibitory input to the C8-projecting network was jointly driven by both C8- and C4-

projecting cells (C8→C8 and C4→C8), and not solely from other C8-projecting neurons (C8→C8) (Fig. 3.8). These findings suggest that, despite the broad innervation pattern of interneurons, inhibitory synapses can be upregulated in a neuron-specific manner during learning, perhaps by way of an instructive signal provided by the “learning” population of excitatory cells [102, 103].

Skilled grasp learning resulted in increased monosynaptic excitatory interconnectivity between C8-projecting cells; we next investigated whether learning also altered the strength of synaptic connections in these cells. We hypothesized that learning could induce two possible outcomes: synaptic efficacy could increase as a result of LTP-related phenomena [31, 85, 104], or synaptic efficacy could decrease overall due to the presence of newly created, immature synapses that are typically weaker than mature synapses [105, 106]. Measures of synaptic efficacy included synaptic potency (average peak response amplitude, omitting failures), failure rate (frequency with which presynaptic activation fails to elicit an EPSC between connected neurons), and paired-pulse ratio (amplitude ratio of the second to first EPSCs in response to a pair of presynaptic action potentials). Overall, the results supported the second hypothesis: learning resulted in a significant *reduction* in synaptic potency among the interconnected population of layer V, C8-projecting neurons (Wilcoxon test; $p=0.03$, Fig. 3.3d), and a trend toward an increase in failure rate ($p=0.09$, Fig. 3.3e). However, the distributions of synaptic potency in untrained and trained animals were significantly different (Kolmogorov-Smirnov, $p<0.05$), due to higher variance in trained animals that might reflect the co-existence of both weaker,

newly formed synapses and strengthened, preexisting synapses that frequently substantially exceeded synapse potency of untrained animals. Paired-pulse ratio did not change following grasp training ($p = 0.9$; Fig. 3.3). We examined several additional synaptic properties including uEPSC rise time, decay time, half-width, and coefficient of variation (Fig. 3.3 and Table 3.1) that showed little change as a function of training in the C8→C8 population. Further, all remaining cell-pair groups (C4→C4, C4→C8, C8→C4), displayed no significant differences after skilled grasp learning for any of the excitatory response properties measured (Table 3.1), indicating that observed changes in synaptic efficacy as a function of training were restricted to the "learning", C8-projecting network of neurons. Finally, there were no significant changes to inhibitory response properties following training (Fig. 3.9 and Table 3.2), indicating that training-induced inhibitory synapses resemble preexisting synapses.

The preceding findings indicate that there is a selective increase in both excitatory and inhibitory inputs to learning neurons, and a significant reduction in excitatory synaptic efficacy likely reflecting the presence of new synapses, but no change in inputs to neighboring neurons of the same class that are uninvolved in learning. We next examined the intrinsic excitability of neurons, which has been associated with learning among functionally ambiguous neuronal populations [107-111], and could greatly affect how information is spread throughout the corticospinal system. We hypothesized that excitation could be biased toward the newly learned, behaviorally relevant movement via augmented excitability specifically within the C8-projecting network. Indeed, skilled grasp training was

associated with a significant increase in spiking in response to depolarizing current injection (repeated measures ANOVA, $p < 0.05$; Fig. 3.4c), and reduced medium after-hyperpolarization (mAHP) (two-way ANOVA, $p = 0.02$; Fig. 3.4b). C4-projecting cells, on the other hand, exhibited no significant changes in spiking ($p = 0.3$) or mAHP with training ($p = 0.3$; Fig. 3.4). Thus, skilled grasp training results in both within-network connectivity and intrinsic changes in neuronal state that are unique to the learning population of corticospinal neurons and may facilitate both the activation and stabilization of learning networks.

Finally, alterations in intrinsic excitability measures within the C8-projecting population following training were not accompanied by differences in input resistance ($p = 0.6$; Table 3.3), spiking threshold (rheobase; $p = 0.4$), spike height ($p = 0.5$), and spike half-width duration ($p = 0.12$), indicating learning-induced changes in membrane conductance are limited. Furthermore, these measures did not significantly differ with training for the C4-projecting population (Table 3.3).

In total, the results of this study extend our understanding of how knowledge is represented in the brain by providing the first experimental evidence that learning in the adult cortex alters neuronal connectivity and intrinsic excitability specifically within a subpopulation of neurons functionally related to the learned behavior. That learning takes place through scaling of synaptic weights is widely accepted [112]. However, our data suggest that perhaps more important among sparsely connected populations is the establishment of new

associations linking previously uncoupled neurons (although one cannot rule out potentiation of weak or silent synapses below detection threshold prior to learning). New excitatory associations likely network corticospinal neurons controlling cooperative, yet novel aspects of the learned movement, and may thus provide a basis for the formation of a motor engram within M1 [74] (see model in Fig. 3.10).

While newly functional synapses associated with learning are relatively weak, thus challenging the spread of excitation through the network, we also find intrinsic excitability to be specifically increased within the learning-related population of neurons. Such changes may aid in propagating activation throughout the nascent network, “priming” the circuit for action [113]. Additionally, heightened intrinsic excitability can facilitate information storage via long-term potentiation and spike-timing-dependent plasticity [113, 114]. Indeed, this may be an important step for strengthening newly formed synapses to establish a local memory trace [115, 116].

Finally, we identify previously unknown inhibitory plasticity that is targeted to learning-related neurons. This increased inhibition may sharpen the temporal window for neuronal and network activation, thereby increasing the precision of network activity and thus motor control [117-120]. Additionally, inhibition may reduce undesirable motor outputs and/or offset the increased excitatory drive that accompanies training [99].

The current findings provide a framework for integrating a variety of changes associated with skilled-grasp training, but with a level of specificity previously unappreciated. For instance, augmented excitatory interconnectivity among C8-projecting neurons may account for increases in neural synchrony [87], spinogenesis [35, 39], and expansion of the distal forelimb representation following learning [32]. Increased intrinsic excitability may boost firing rate during motor performance [86], while heightened inhibitory signaling could explain observed decreases in background noise [86]. Furthermore, increased excitation specifically among functionally related cells provides a mechanism by which complex behaviors can be evoked by long-duration stimulation of the motor cortex [75, 76].

METHODS

Neuronal labeling: Male F344 rats, weighing approximately 85 g (~ PD 35), were anesthetized with a cocktail (2 ml/kg) containing ketamine (25 mg/mL), xylazine (1.3 mg/mL), and acepromazine (0.25 mg/mL). In rats, the C8 spinal cord segment contains lower motor neurons that activate muscles controlling distal forelimb movements required for grasping [43, 44]. Lower motor neuron pools located in the C4 spinal segment are associated with control of proximal forelimb, shoulder, and neck musculature [43, 45, 46]. To label corticospinal neurons projecting to the C8 cervical spinal cord, the overlying dura between C7 and T1 was resected and a glass micropipette (tip < 40 μm) containing red or green fluorescent latex microspheres (Lumafluor, Durham, NC) was inserted into

the dorsal horn of spinal cord (depth 0.75 mm, 0.55 mm lateral to midline). Using a Picospritzer II (General Valve), ~250 nL of fluorescent latex microspheres was injected into each side of the spinal cord (Figure 3.1). To label corticospinal neurons projecting to the upper cervical spinal cord, the same procedure was repeated between C3 and C4 spinal vertebra, using a different colored dye (green or red) than that used for C8 injections. In all cases, tracer diffusion was assessed postmortem in 50 μm coronal slices of the spinal cord (see Figure 3.1b). Animals with tracer diffusion into the dorsal columns were excluded from further study. In total, 7 animals were excluded, while 129 were included for analysis. Because the exact tracer (red or green) injected into C4 or C8 was counterbalanced and varied from animal to animal, the experimenter was blind to the exact projection target of labeled cortical cells during recording. All procedures and animal care adhered to American Association for the Accreditation of Laboratory Animal Care, Society for Neuroscience, and institutional guidelines for experimental animal health, safety, and comfort.

Skilled grasp training: Two to five days after bead injections, animals were acclimated to the experimenter and testing chamber. The animals were handled for a total of 5 days before initiating reaching. Animals were weighed and food restriction was initiated 2 days prior to starting reaching. Animals were required to reach through a small opening to obtain a single 45 mg sucrose pellet (Test Diets, St. Louis, MO) located on an indented platform approximately 2 cm beyond the reaching chamber. Reach training was carried out across 10 consecutive days and animals performed 40-60 reaching trials per day. A

successful trial was scored if animals successfully retrieved the pellet and consumed it. To control for potential effects due to food restriction, handling, or exposure to a novel food (reward pellets), control animals were similarly food restricted, handled, spent an equal amount of time in the reaching chamber, and consumed an equal number of reward pellets as did reach trained animals. However, controls were manually fed reward pellets with forceps, thus not allowing the animal to reach or grasp reward pellets.

Slice preparation: One to five days following completion of training, rats were anesthetized and perfused for 3 minutes with ice-cold, oxygenated, modified sucrose ACSF containing (in mM) 75 NaCl, 2.5 KCl, 3.3 $MgSO_4$, 0.5 $CaCl_2$, 1NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 52.6 sucrose, 10 HEPES, 10 choline chloride, 1 pyruvate, 1 L-ascorbic acid (~300 mOsml, pH 7.4). The brain was rapidly dissected and 330 μm-thick slices spanning the motor cortex were cut at 15° anterior to the mid-coronal plane to match the projection pattern of layer 5 corticospinal neurons [35]. Cortical slices were cut and collected in ice-cold, oxygenated, modified sucrose ACSF. Slices were transferred to an interface chamber containing the same modified sucrose ACSF solution and incubated at 34° C for 30 min. Slices were then held at room temperature (23° C) in the interface chamber for at least 45 min before initiating recordings. Recordings were made in a submersion-type recording chamber and perfused with oxygenated ACSF containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 1.3 NaH_2PO_4, 26.0 $NaHCO_3$, 20 glucose (~300 mOsml) at 23° C at a rate

of 2-3 ml / minute. Postnatal age at time of recording was 56 ± 2 days, an age where the motor system has fully matured [18, 42].

Electrophysiology: All recordings were performed within the cortical hemisphere contralateral to the preferred reaching paw during training. In the case of untrained animals, the hemisphere recorded was selected randomly. The experimenter was blinded to the training status of the animal being recorded. Neurons were selected based on emission spectra (red or green, reflecting tracers injected at either the C8 or C4 spinal segment) and then visualized under infrared differential interference contrast videomicroscopy (Olympus BX-51 scope and Rolera XR digital camera). Whole-cell voltage and current clamp recordings were made at room temperature using pulled patch pipettes (4-7 MΩ) filled with internal solution containing (in mM) 150 K-Gluconate, 1.5 $MgCl_2$, 5.0 HEPES, 1 EGTA, 10 phosphocreatine, 2.0 ATP, and 0.3 GTP. Post-synaptic data were analyzed exclusively from cells with a resting membrane potential ≤ -55mV, with drift less than 6 mV over the entire recording period, with access resistance ≤ 35 MΩ, with the ability to evoke multiple spikes with >60 mV peak amplitude from threshold, and with a holding current of > -500 pA to keep the cell at a “native” resting membrane potential of -65 mV. Series resistance was not compensated, but was continuously monitored via negative voltage steps. In a minority of cell pairs (~10%), the “presynaptic” neuron was fired in cell-attached mode and was not reciprocally tested for synaptic input.

Data acquisition and analysis: Whole-cell patch clamp recordings were obtained using Multiclamp 700A patch amplifiers (Molecular Devices) and data analyzed using pClamp 10 software (Molecular Devices). To characterize basic membrane properties, a series of hyper- and depolarizing current steps were applied for 500 msec in 10-45 pA increments at 5 sec intervals. Rheobase was determined as the lowest level of depolarizing current required to evoke at least one spike. Action potential spike measurements were taken from the first action potential on the first sweep to reach threshold. Spike height was measured as the overall peak membrane voltage and half-width was measured at the half amplitude of the action potential. Input resistance (R_{in}) was determined from the slope of the linear regression taken through the voltage-current relationship in the hyperpolarizing range.

To determine connectivity among C4- and C8-projecting cell populations, simultaneous whole-cell recordings were made in groups of 2-4 retrogradely labeled cells. In many cases, a recorded cell in one pipet was replaced with a new cell while keeping the other patched cells intact. Repeating this procedure allowed us to test many (up to 9) synaptic partners for a particular cell. The distribution of C4- and C8-projecting cells, which are almost entirely intermingled across the primary motor cortex [35], enabled all types of paired recordings to be obtained within a single field (C4→C4, C8→C8, C4→C8, and C8→C4). Data were collected from cells greater than 30 μm below the slice surface (mean ± SD = 67 ± 26 μm). Connectivity was determined by evoking paired action potentials

in a presynaptic cell spaced 50ms apart while monitoring responses in postsynaptic cells held at -65 mV in voltage clamp. Presynaptic action potentials were evoked by a 7 ms depolarizing current injection of 2 nA. Individual sweeps were separated by 5 s. Postsynaptic response properties were measured in response to the first pulse.

The responses to 30-50 evoked action potentials were measured for each paired recording. Connectivity was inferred if the average peak response in the postsynaptic cell was > 2 SD above noise. In connected cell pairs, failure rate was calculated as the percentage of single trials in which the postsynaptic peak current was < 2 SD below baseline current noise. All traces were manually inspected for signal consistency, including monotonic rise and decay and reliable onset latency. Postsynaptic response amplitude was calculated as the averaged current over a 1.5 ms time window of peak response current compared to baseline, which was defined as the average current in a 17 ms window prior to presynaptic firing. Response potency, latency, rise time, decay time and half-width were calculated using only traces where a postsynaptic response was detected (failures omitted). Response latency was measured from the peak of the presynaptic spike to the onset of EPSC/IPSC (onset was defined as 5% of peak signal). Rise time was calculated as the time between 20 and 80% peak EPSC/IPSC amplitude. Decay time was calculated as the time between 80 and 20% of peak EPSC/IPSC amplitude. Response half-width was measured as the time between points that were 50% of the peak amplitude relative to baseline. For paired pulse analysis, the peak response to each pulse was averaged over

all trials (that is, failures were not omitted), and the average response of the first response was divided by that to second pulse. To preserve the relative differences in magnitude for ratios above and below a value of 1, the logarithm of each ratio was used for statistical comparisons.

To assess whether concurrent inhibitory input innervated postsynaptic cells independent of their long-range spinal cord projection target, we tested the hypothesis that the postsynaptic identity of a cell receiving concurrent inhibitory input followed a binomial distribution, where the probability (p) that inhibition is received by a C4-projecting cell is 0.5 (and, conversely, the probability that the inhibitory cell innervates C8 is also p = 0.5). A chi-square goodness-of-fit test was then run on the expected and experimentally observed distributions.

Current-spike relationship and maximum afterhyperpolarization (AHP) were measured in cells requiring less than -150 pA to hold at a membrane potential of -65mV. AHP was calculated in cells for which a 300 pA depolarizing current delivered for 500 ms evoked at least 4 spikes. Reported p-values reflect the main effect of training for a two-way ANOVA (IV1 = training; IV2 = number of spikes elicited by current injection).

Statistical comparisons were performed using JMP software, version 10.0 (SAS Institute Inc., Cary, NC). Comparisons of connectivity were made using Fisher's exact test. Pairwise comparisons utilized Student's t-test unless otherwise noted. Significant p-value was set at 0.05. In text data values are presented as the mean ± standard deviation.

FIGURES

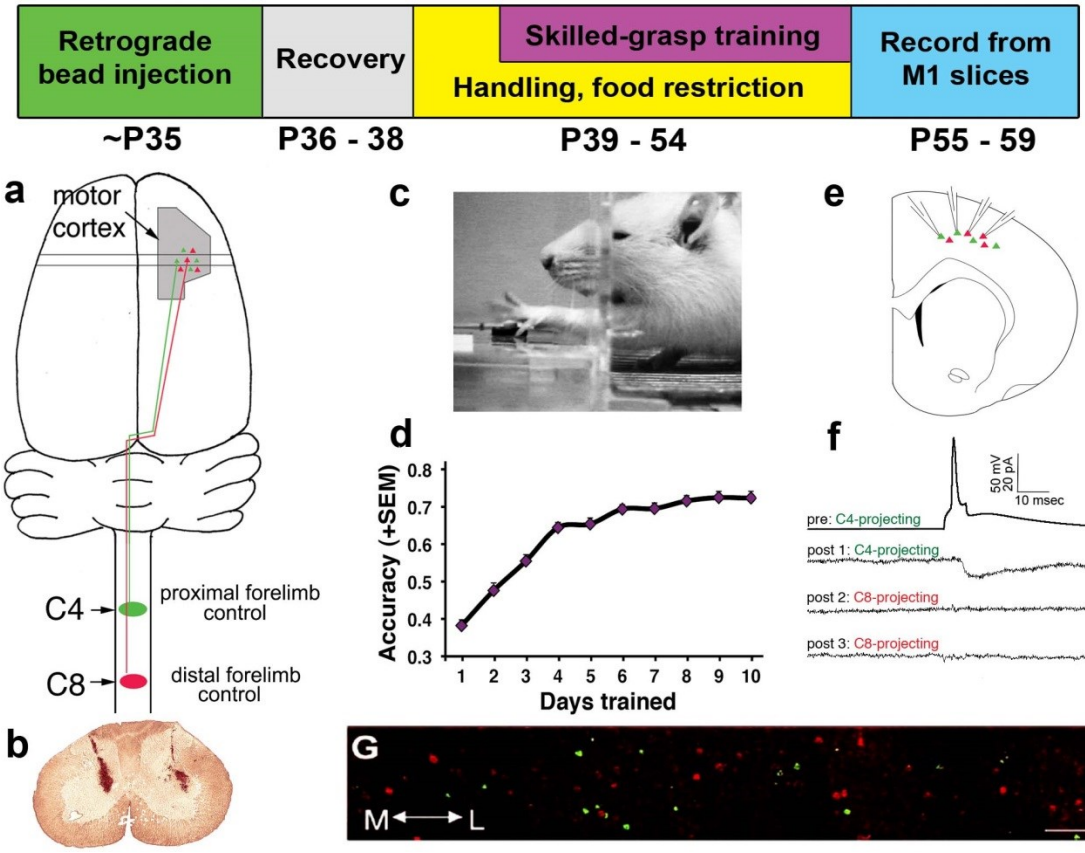
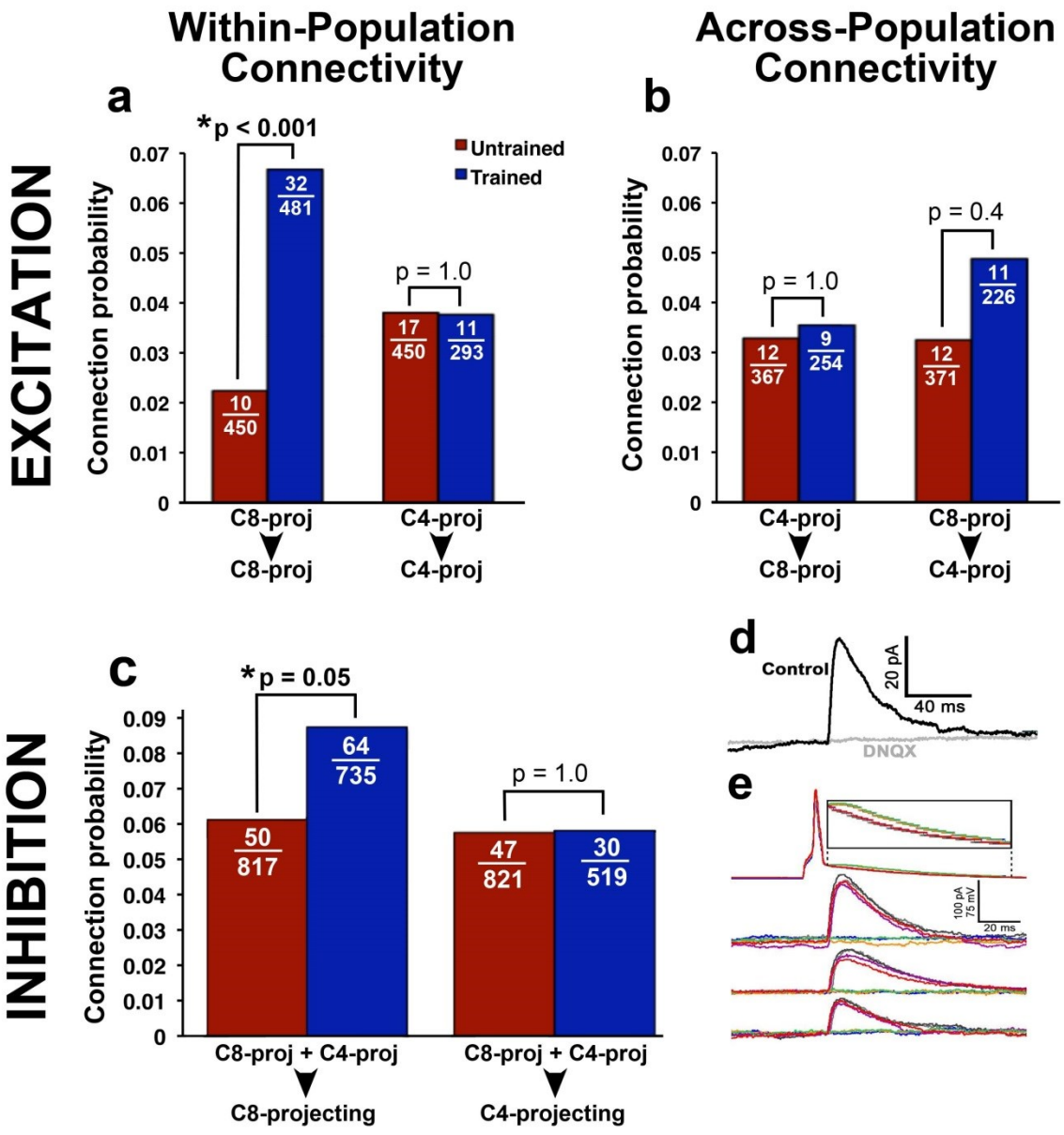


Figure 3.1. Experimental Overview. Top bar shows timeline of experiments. **a)** In rats, cervical level 8 (C8) of the spinal cord contains motor neurons that project to and control distal forelimb musculature, while level C4 controls proximal forelimb musculature, including the neck and shoulder (ref). At approximately postnatal day 35, F344 male rats received separately colored retrograde tracer injections in C8 and C4 levels of the spinal cord. **b)** Cross section of C8 spinal cord showing location of retrograde tracer injections. All animals were handled and food restricted starting approximately at postnatal day 39. Roughly half of the animals then underwent skilled grasp training **(c)** for 10 consecutive days. Untrained animals were exposed to the testing chamber and manually fed reward pellets. **d)** Animals showed a significant increase in task performance (successful trial = procurement of the reward pellet using a single forelimb) over 10 days of training. **e)** Following training, acute slices containing the forelimb area of M1 contralateral to the preferred grasping paw were collected and retrogradely labeled layer V corticospinal neurons were targeted for whole-cell patch clamp. **f)** sample quadruple recording trace showing cell pairs that either received excitatory input (post 1) or were not connected (post 2 and 3) with the presynaptic cell. **g)** Retrogradely labeled cells were intermingled across the motor cortex, enabling simultaneous recording of neighboring C8- and C4-projecting cells. Scale bar: 60 µm. Panel (**g**) taken from Wang et al., 2011.

Figure 3.2. Excitatory and inhibitory connectivity increase specifically onto grasp-related neurons following skilled grasp training. a, b) Following skilled grasp training, excitatory connectivity increased selectively within the population of corticospinal neurons containing grasp-related cells (C8-projecting). **c**) As with excitatory input, polysynaptic inhibitory input increased with training specifically onto C8-projecting neurons. Analysis of the presynaptic source of this input showed this increase was jointly driven by both C8-projecting and C4-projecting presynaptic neurons (Fig. 3.7). **d**) Addition of DNQX [20 μM] to the recording bath abolished IPSCs, indicating inhibition comprised an AMPA-receptor dependent polysynaptic pathway. **e**) Frequently, when an IPSC was detected in one cell, an IPSC could also be detected in one or more concurrently patched cells ("concurrent inhibition"). The figure shows one instance where all four simultaneously patched neurons receive concurrent inhibitory input. Individual traces are color coded. The fact that synaptic failures occur simultaneous across all cells suggests that each neuron receives input from the same inhibitory interneuron. Collectively, the mean distance between neurons receiving concurrent inhibition was significantly lower than that between neurons that did not receive concurrent input (concurrent = 115 ± 70μm, not concurrent = 147 ± 100μm; $p<0.02$). Whether two postsynaptic neurons shared inhibitory input was not dependent on their population identity (chi square, $p=0.82$). Together, these data are consistent with a model in which interneuron(s) mediating polysynaptic inhibition innervate nearby corticospinal neurons densely and unspecifically [99, 101].
The inset in panel (**e**) shows magnification of the post-action-potential trace, where non-failure traces correspond to a greater hyperpolarization of the membrane potential, indicating the presence of recurrent self-inhibition. Such self-inhibition was observed on several occasions, but was not included in analysis due to an inability to confidently classify traces in most cells.


EXCITATION
INHIBITION
Within-Population Connectivity
Across-Population Connectivity
a
b
c
d
e
*p < 0.001
p = 1.0
p = 0.4
*p = 0.05
Untrained
Trained
Connection probability
10/450
32/481
17/450
11/293
12/367
9/254
12/371
11/226
50/817
64/735
47/821
30/519
C8-proj
C4-proj
C8-proj + C4-proj
C8-projecting
C4-projecting
Control
DNQX
20 pA
40 ms
100 pA
75 mV
20 ms

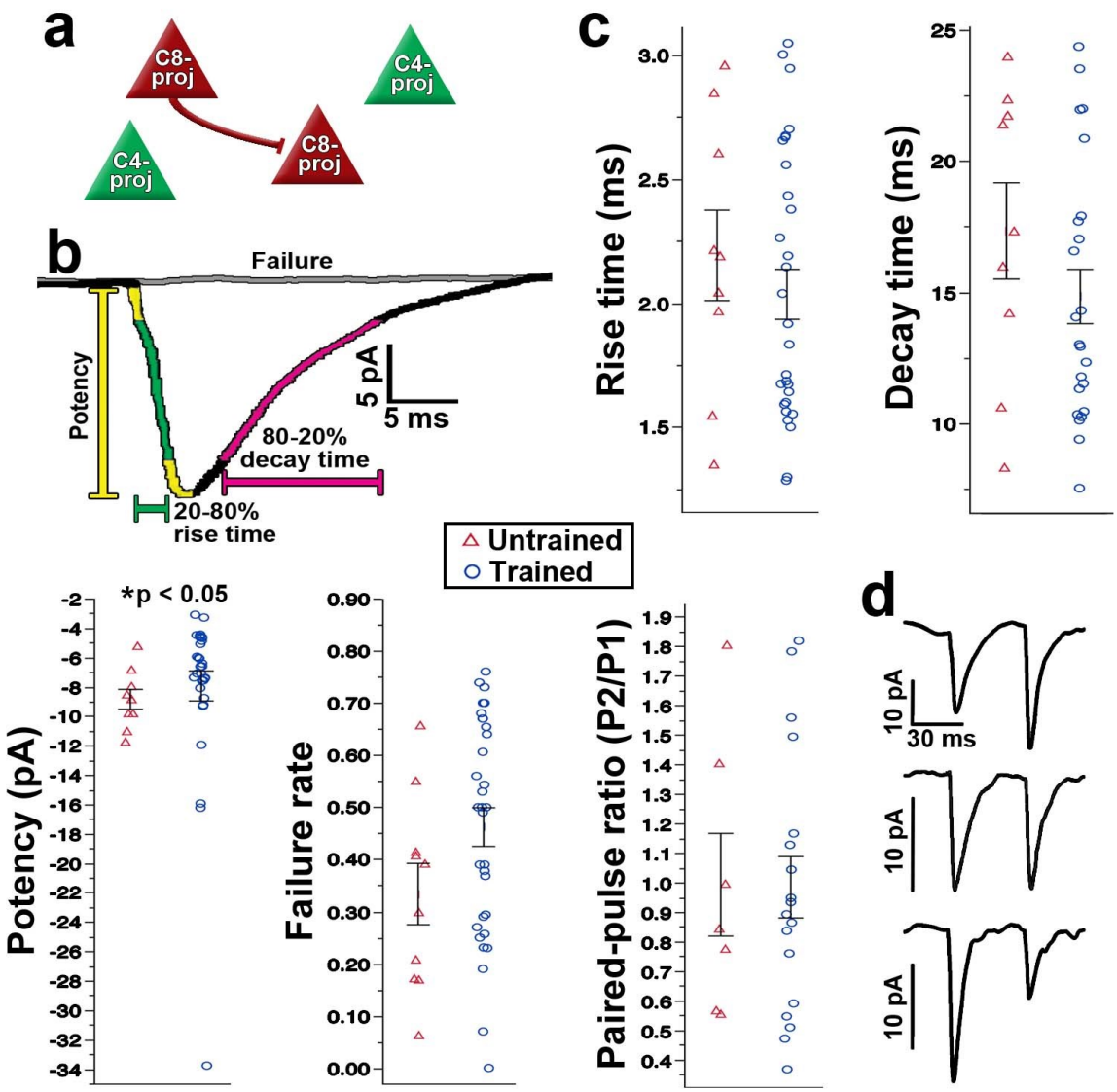


Figure 3.3. Excitatory synaptic efficacy decreases with training. a) Excitatory data in Figure 3.3 is obtained only from connected C8→C8 cell pairs; for all other cell pairs, see supplementary Table S3.1. **b**) A sample postsynaptic response (and a failure) illustrating features of the unitary EPSC used for analysis. **c**) Among all properties measured, only uEPSC potency differed with training, exhibiting lower peak amplitude in trained animals (Wilcoxon rank-sum test). **d**) Paired-pulse ratios did not change with training, and showed a high level of variability for both conditions. Traces on the right correspond to distinct cell pairs where the second pulse displayed (top to bottom) facilitation, no change, or depression.

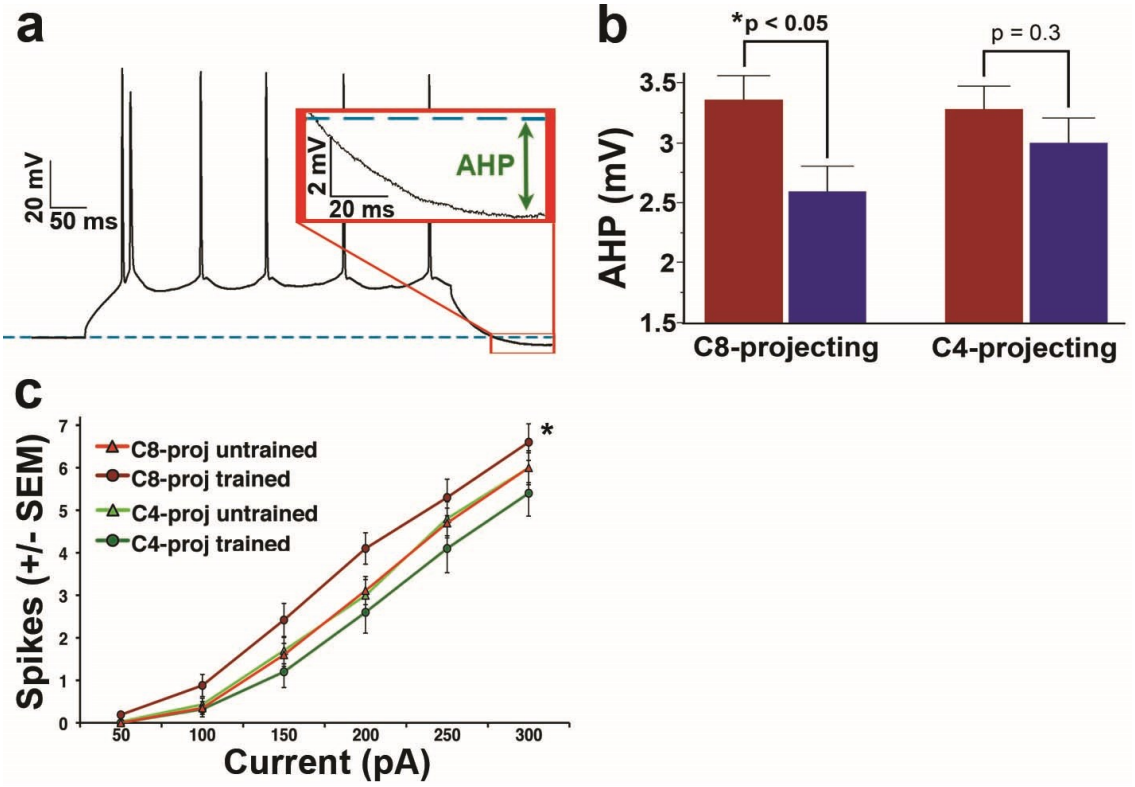


Figure 3.4. Intrinsic excitability increases with training specifically in C8-projecting, "learning" neurons. a) Sample corticospinal response to injection of a 300 pA depolarizing square pulse. The maximum afterhyperpolarization (AHP) was measured with respect to the pre-depolarization baseline (dashed blue line). **b**) A two-way ANOVA (independent variables: number of spikes, training condition) showed AHP values were lower in trained vs. untrained animals within the C8-projecting group only. **c**) Likewise, C8-projecitng neurons from trained animals showed greater spiking frequency to depolarizing current injections vs. all other groups (repeated measures ANOVA, $p < 0.05$).

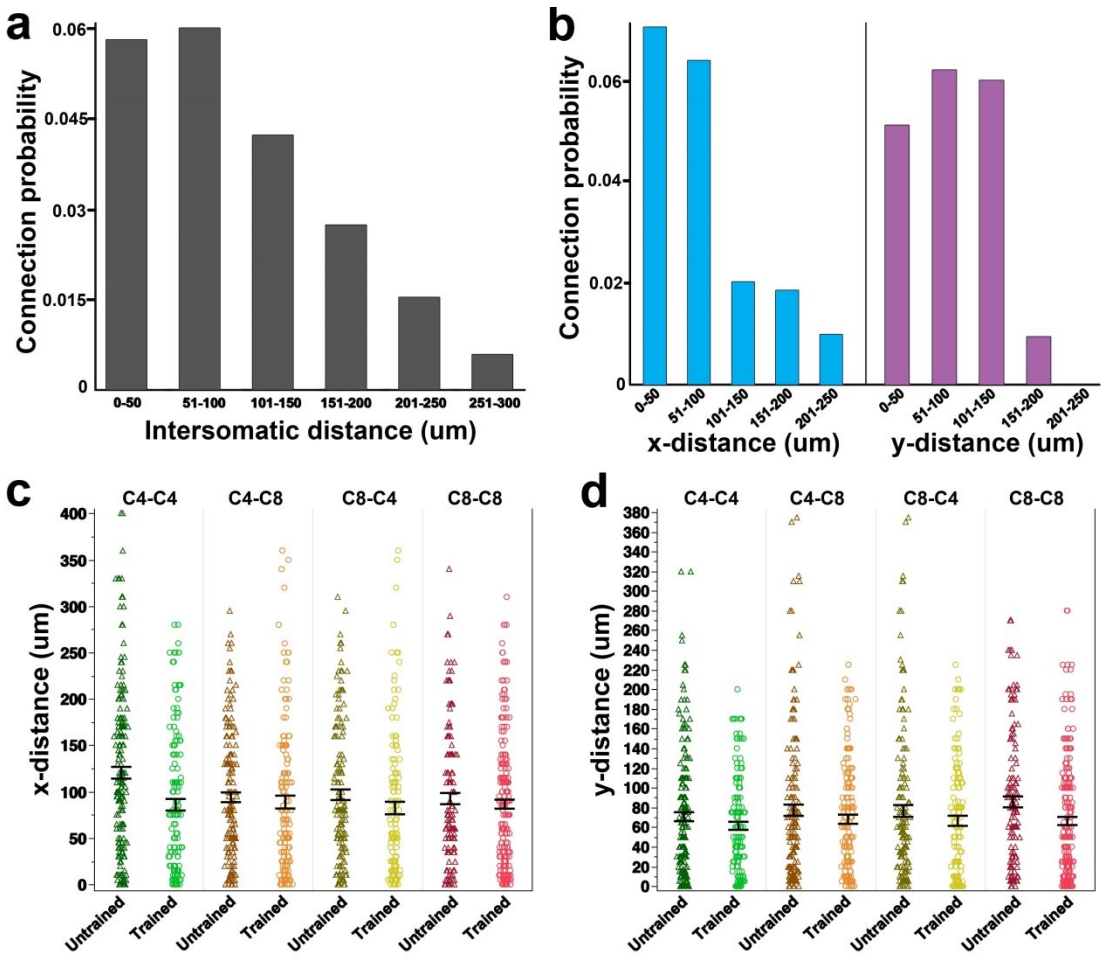


Figure 3.5. Excitatory connection probability declines with increasing intersomatic distance. a) Although intersomatic distance had little-to-no effect on connection probability at distances of 0 – 100 μm (logistic analysis, p = 0.89), beyond 100 μm the rate of excitatory connectivity decreased with distance (p < 0.01). **b**) This relationship holds most strongly for intersomatic separation along the (medial-lateral) x-dimension (0 – 100 μm, p = 0.19; 0 – 300 μm, p < 0.01). In fact, distance along the y-dimension (dorsal-ventral) was not significantly related to connection probability over 0 – 300 μm (p = 0.18). **c**) and **d**) Intersomatic distance for individual cell-pairs along the (**c**) x- and (**d**) y-dimensions. Bars = +/- SEM. Although the mean distance along the y-dimension between untrained C8→C8 cell pairs is elevated compared to trained C8→C8 cell pairs, y-distance had seemingly no effect on connectivity at distances under 150 μm, where the vast majority of C8→C8 cell pairs resided. Therefore, this increase cannot account for the increased interconnectivity among the C8 population of trained animals.

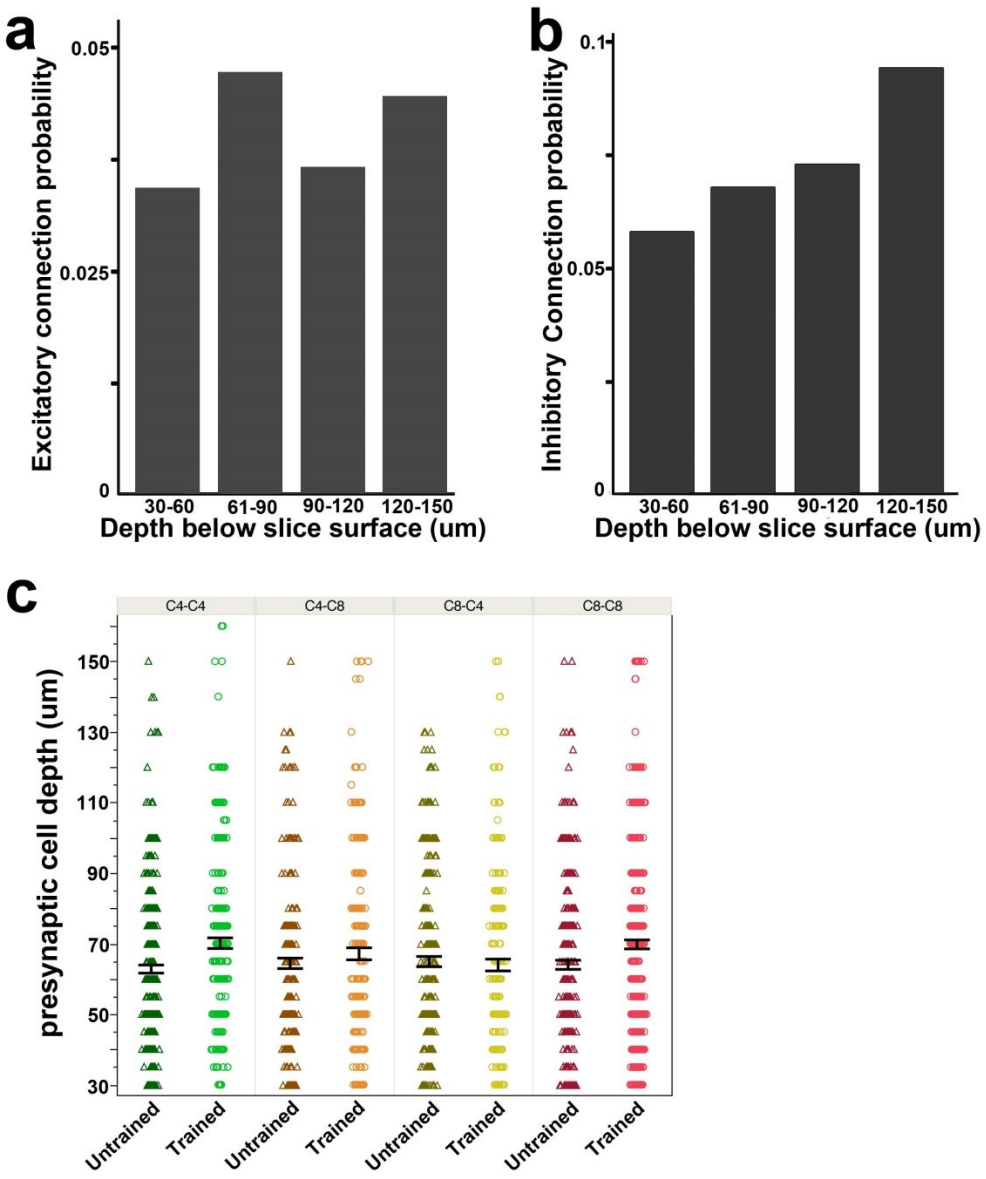


Figure 3.6. Connection probability by cell depth. a) Connection probability was not systematically related to presynaptic cell depth below the surface of the slice (logistic analysis, $p = 0.51$). **b**) Inhibitory input, however, showed a stronger directional relationship, as connection probability increased with presynaptic cell depth ($p < 0.01$) Data relating the depth of *postsynaptic* cell to connection probability mimics those for presynaptic cell depth presented above (excitatory, $p = 0.1$; inhibitory, $p < 0.05$). **c**) Presynaptic cell depths for individual cell-pairs. Both C4-C4 and C8-C8 cell pairs were significantly deeper in the trained versus untrained condition (Wilcoxon test; $p < 0.05$). However, the mean difference was minimal (5 μm for C4-C4 and 4 μm for C8-C8), and these minor differences in depth contributed negligibly to the connection probability differences observed.

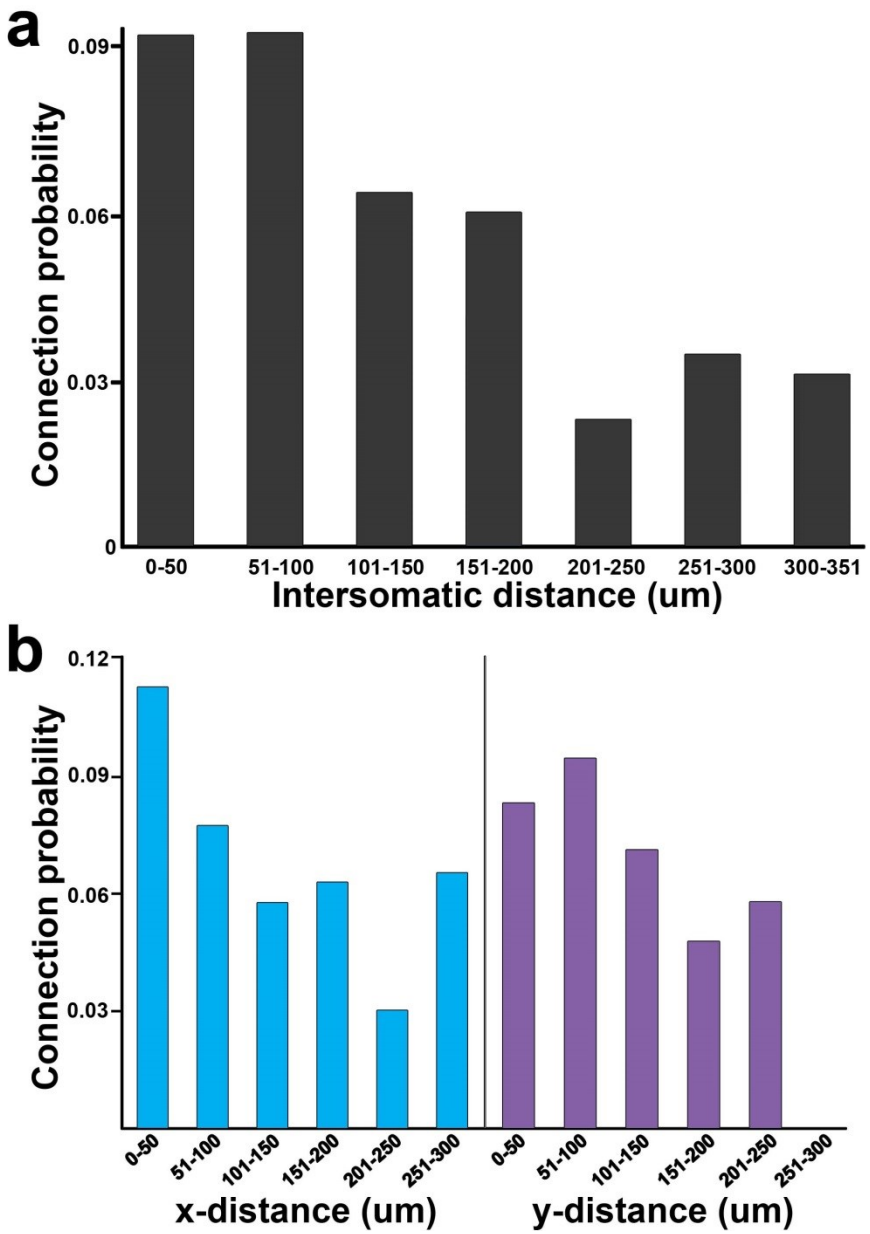


Figure 3.7. Polysynaptic inhibitory connectivity declines with increasing intersomatic distance. a) The relationship of intersomatic distance with connectivity was less pronounced for the inhibitory condition relative to the excitatory condition. However, inhibitory connection probability also dropped off with increasing intersomatic distance (logistic analysis, $p < 0.01$). **b**) This relationship was driven by distance along the x-axis ($p < 0.01$). Distance along the y-dimension was significantly related to connection probability over 0 – 300 μm ($p = 0.05$), but there appears to be no relationship at lesser distances (0 – 150 μm, $p = 0.67$). Again, increased inhibitory input onto the C8-projecting population of trained animals could not be accounted for by differences in intersomatic distance between trained and untrained cell pairs (see **Fig. S1c**)

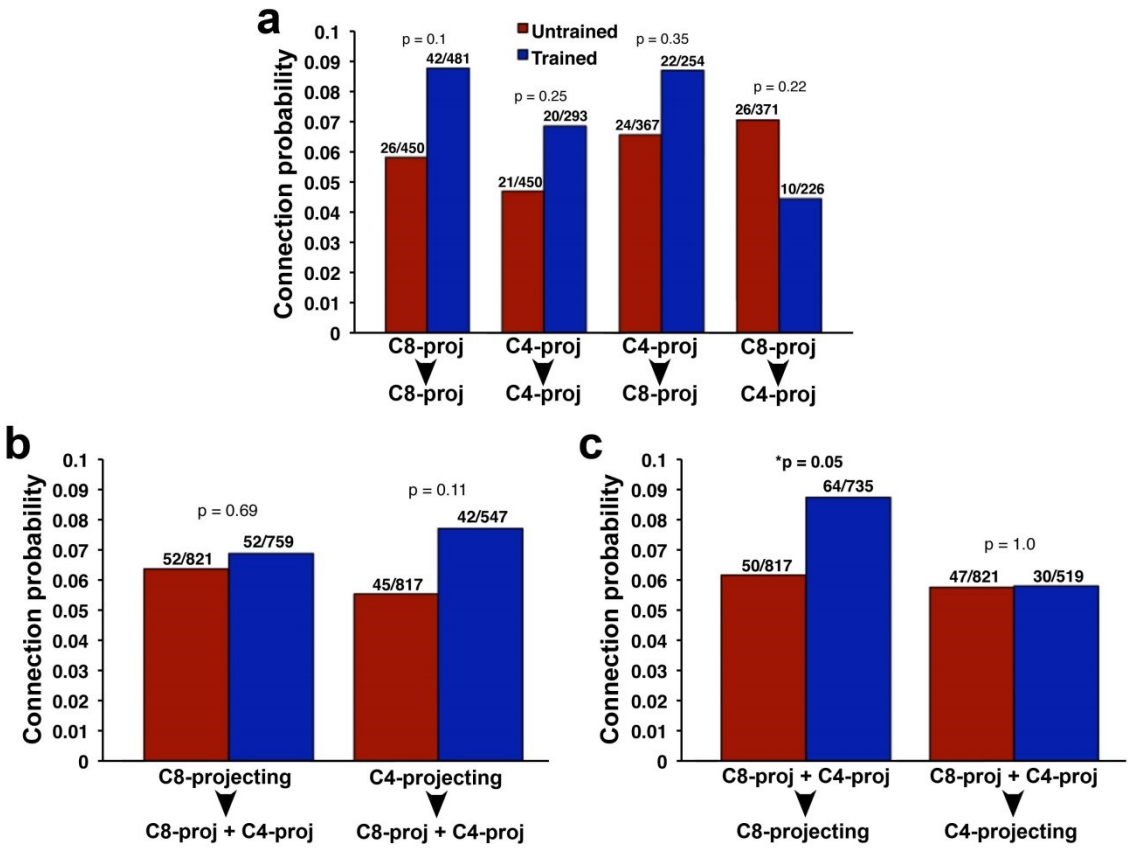


Figure 3.8. Inhibitory input increases specifically onto C8-projecting neurons following learning. a) Probability of detecting polysynaptic inhibition between specific corticospinal cell pairs. Although there was a significant, 3-fold increase in monosynaptic excitatory connectivity rate among C8-C8 cell pairs, the inhibitory rate only trended toward increasing between C8-projecting cells. **b**) To measure the total inhibition *elicited* by a specific projection population, we combined recipient populations. For example, the total amount of inhibition elicited by C8-projecting cells (C8→C8+C4) was calculated by combining data from C8→C8 and C8→C4 cell pairs. Polysynaptic inhibition was not evoked to a significantly greater extent by either projection population following learning. **c**) To measure the total inhibition *received* by a specific projection population, we combined source populations. Doing so revealed that C8-projecting neurons receive significantly more inhibition with training.

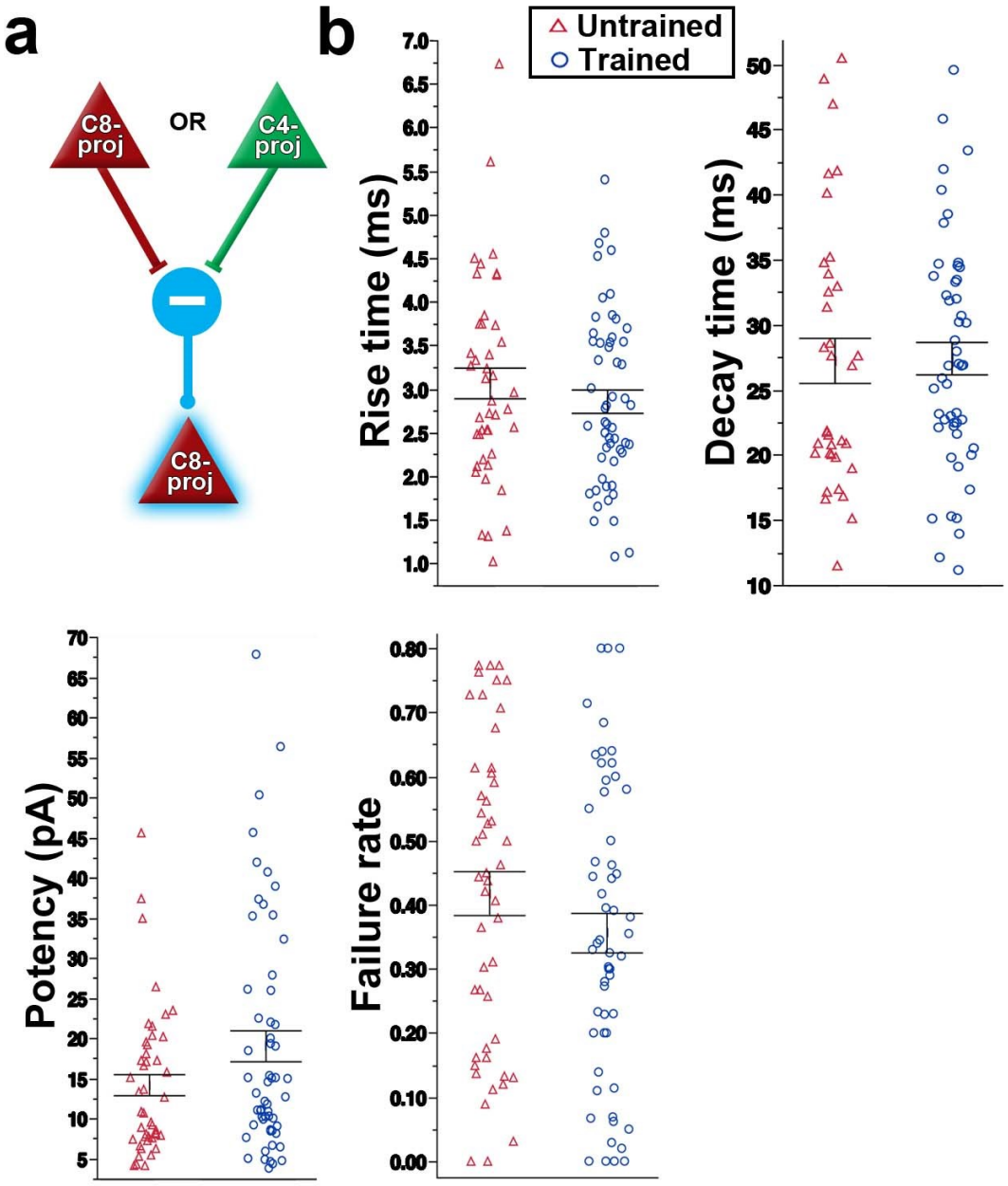


Figure 3.9. Inhibitory response properties do not change with training. a) Polysynaptic inhibitory data reflects input to C8-projecting neurons originating from evoked action potential in both C8-projecting and C4-projecting cells (C8→C8 and C4→C8). **b**) As with excitatory responses, kinetic response properties (rise time, decay time) showed no change with learning. Synaptic strength trended toward an increase in the C8-projecting population of trained animals, as evidenced by trends for increased response potency, (Wilcoxon rank-sum test; p = 0.12) and decreased failure rate (Student's t-test; p = 0.14)

Figure 3.10. A model of functional changes in corticospinal circuitry accompanying motor skill learning. a) The activation patterns of C8-projecting corticospinal neurons (red circles) controlling skilled grasping behavior is coordinated by higher-order input (yellow arrows) during initial stages of learning. **b**) Repeated coactivation of novel corticospinal ensembles during training may increase their connectivity (solid red lines) via Hebbian-like mechanisms, functionally encoding the learned motor behavior in M1. This local encoding should increase trial-to-trial movement consistency, and augmented interconnectivity should enable pattern completion within the network and facilitate activation of the motor program. These developments minimize the need for supervision by higher-order regions [71, 78, 121] (diminished yellow arrows), and allow cognitive resources, such as attention, to be allocated to other tasks [122]. Connectivity between circuits (red dashed line) may help in sequencing network activation patterns. Increased intrinsic excitation (red halos) would channel activation through this movement network. Additionally, heightened excitation increases firing rate during the target interval, enhancing reliability of downstream muscle recruitment [86], but may also generate undesirable activity outside this target period. **c**) An increase in inhibition (top blue arrows) lowers background firing, sharpening network activation patterns, further contributing to the fidelity and precision of neuronal output. Targeted inhibition between circuits (lower blue arrow) may discourage improper (premature or perseverative) activation of individual sequences. In total, these local modifications may play an important role in the automation and stereotypy associated with skilled motor behavior.

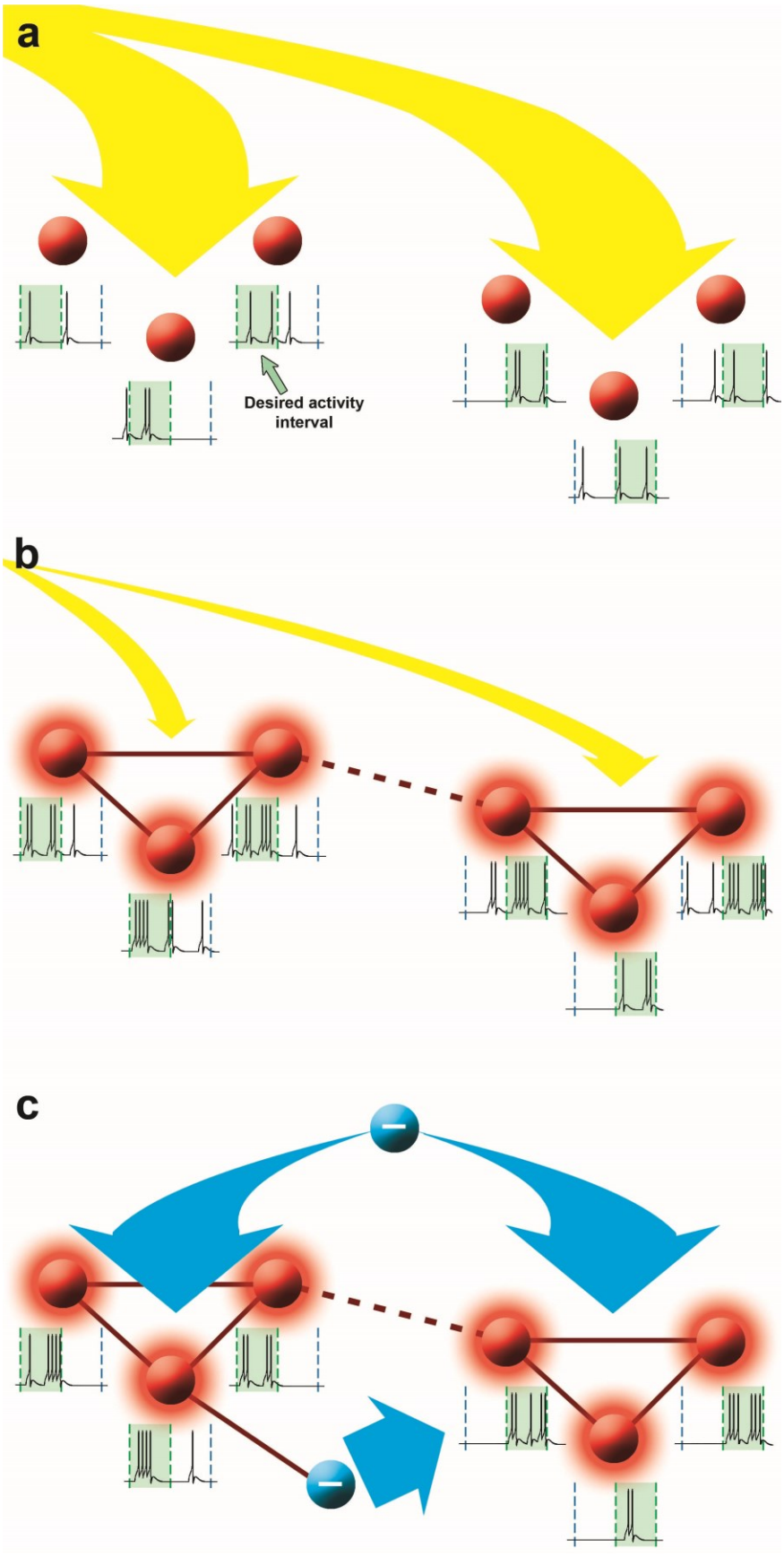

a
Desired activity interval
b
c

Table 3.1 Excitatory postsynaptic response properties. P-values reflect comparisons of trained vs. untrained conditions within each cell-pair group.

	C4→C4		C8→C8		C4→C8		C8→C4	
	Untrained	Trained	Untrained	Trained	Untrained	Trained	Untrained	Trained
Failure rate	0.54 ± 0.26 (n = 17)	0.45 ± 0.17 (n = 11) p = 0.33	0.33 ± 0.19 (n = 10)	0.46 ± 0.21 (n = 32) p = 0.09	0.47 ± 0.29 (n = 12)	0.42 ± 0.24 (n = 8) p = 0.72	0.28 ± 0.25 (n = 11)	0.41 ± 0.23 (n = 11) p = 0.2
Potency (pA)	-6.9 ± 3.1 (n = 16)	-7.1 ± 2.6 (n = 11) p = 0.81	-8.9 ± 2 (n = 9)	-7.9 ± 5.7 (n = 31) *p = 0.03	-8.1 ± 6.1 (n = 11)	-7.6 ± 3.3 (n = 8) p = 0.82	-9 ± 3.8 (n = 11)	-7.3 ± 3.9 (n = 11) p = 0.31
Coefficient of variation (potency)	0.44 ± 0.14 (n = 16)	0.41 ± 0.1 (n = 11) p = 0.45	0.4 ± 0.15 (n = 9)	0.5 ± 0.17 (n = 31) p = 0.14	0.5 ± 0.17 (n = 11)	0.38 ± 0.07 (n = 8) p = 0.08	0.43 ± 0.18 (n = 11)	0.43 ± 0.08 (n = 11) p = 0.94
Latency (ms)	3.2 ± 1.5 (n = 15)	2.6 ± 1.6 (n = 7) p = 0.39	2.6 ± 0.8 (n = 8)	2.4 ± 0.9 (n = 24) p = 0.5	3.1 ± 1.8 (n = 11)	2.9 ± 1.3 (n = 7) p = 0.87	2.3 ± 1 (n = 9)	2.9 ± 1.1 (n = 11) p = 0.22
Paired-pulse ratio	0.85 ± 2.5 (n = 12)	1.17 ± 0.39 (n = 5) p = 0.4	0.91 ± 0.46 (n = 7)	0.89 ± 0.44 (n = 18) p = 0.92	0.91 ± 1.3 (n = 9)	0.89 ± 0.22 (n = 4) p = 0.9	0.89 ± 0.35 (n = 7)	0.84 ± 0.12 (n = 5) p = 0.47
Rise time (ms)	2.17 ± 0.7 (n = 16)	2.33 ± 0.9 (n = 10) p = 0.59	2.2 ± 0.55 (n = 9)	2.0 ± 0.55 (n = 30) p = 0.46	2.01 ± 0.7 (n = 10)	2.39 ± 0.7 (n = 8) p = 0.28	2.23 ± 0.5 (n = 11)	2.5 ± 1.7 (n = 11) p = 0.62
Decay time (ms)	16.9 ± 14.1 (n = 9)	16.5 ± 10.4 (n = 7) p = 0.95	17.3 ± 5.5 (n = 9)	14.8 ± 5.1 (n = 23) p = 0.22	17.4 ± 7.4 (n = 9)	15.7 ± 5.5 (n = 6) p = 0.64	14.8 ± 6.4 (n = 11)	12.3 ± 6.3 (n = 9) p = 0.40
Half-width (ms)	11.1 ± 4.5 (n = 11)	12.1 ± 4.8 (n = 9) p = 0.74	12.7 ± 3.2 (n = 9)	11 ± 2.4 (n = 25) p = 0.1	11.1 ± 3.6 (n = 10)	11.8 ± 5.2 (n = 7) p = 0.75	12.6 ± 8.7 (n = 11)	11.8 ± 7.9 (n = 11) p = 0.82

Table 3.2. Inhibitory postsynaptic response properties by corticospinal population. p-values reflect comparisons of trained vs untrained conditions within each cell-pair group.

	C4-projecting		C8-projecting	
	Untrained	Trained	Untrained	Trained
Failure rate	0.45 ± 0.3 (n = 47)	0.41 ± 0.2 (n = 29) p = 0.47	0.42 ± 0.2 (n = 47)	0.36 ± 0.2 (n = 57) p = 0.14
Potency (pA)	17.8 ± 16 (n = 43)	20.5 ± 16 (n = 28) p = 0.24	14.1 ± 9 (n = 47)	19 ± 14.5 (n = 57) p = 0.12
Coefficient of variation (potency)	0.4 ± 0.18 (n = 45)	0.36 ± 0.2 (n = 29) p = 0.61	0.42 ± 0.2 (n = 47)	0.38 ± 0.1 (n = 57) p = 0.21
Latency (ms)	6.6 ± 1.3 (n = 42)	6.4 ± 1.3 (n = 25) p = 0.68	6.1 ± 1.7 (n = 47)	6.5 ± 1.5 (n = 45) p = 0.27
Rise time (ms)	2.99 ± 1 (n = 43)	3.13 ± 0.9 (n = 28) p = 0.52	3.06 ± 1.2 (n = 43)	2.85 ± 1 (n = 55) p = 0.34
Decay time (ms)	28.4 ± 12.1 (n = 41)	30.6 ± 7.6 (n = 28) p = 0.41	27.2 ± 10.3 (n = 35)	27.4 ± 8.8 (n = 51) p = 0.93
Half-width (ms)	22.9 ± 7.3 (n = 40)	22.4 ± 5 (n = 28) p = 0.76	21.8 ± 6.6 (n = 41)	22.2 ± 5.5 (n = 54) p = 0.77

Table 3.3. Neurophysiological properties of C4- and C8-projecting corticospinal populations.

	C4-projecting		C8-projecting	
	Untrained	Trained	Untrained	Trained
R_{input} (MΩ)	79.6 ± 21 (n = 65)	77.3 ± 24 (n = 71) p = 0.57	77.5 ± 19 (n = 64)	79.2 ± 21 (n = 88) p = 0.6
Rheobase (pA)	138 ± 60 (n = 45)	139 ± 43 (n = 33) p = 0.95	137 ± 50 (n = 39)	124 ± 48 (n = 42) p = 0.4
Spike half-width (ms)	2.29 ± 0.4 (n = 65)	2.35 ± 0.4 (n = 68) p = 0.42	2.28 ± 0.4 (n = 59)	2.39 ± 0.4 (n = 85) p = 0.12
Spike height (mV)	35.5 ± 6.2 (n = 65)	35.5 ± 5.6 (n = 68) p = 0.98	36.2 ± 8.8 (n = 59)	35.3 ± 7.8 (n = 85) p = 0.51

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CHAPTER 4

GENERAL DISCUSSION

The primary hypothesis of this thesis was that cortical function is derived from the configuration of synaptic connectivity among populations of functionally diverse neurons, and that targeted reorganization of synaptic patterning leads to transformations in behavior, sensory representation, and knowledge. As such, we endeavored to understand how the pattern and function of cortical circuitry supports learning and the expression of complex behavior. Using a recently developed model that allows identification of individual neurons controlling distinct motor behaviors, we investigated local changes in synaptic organization and neuronal function associated with the emergence of fine motor behavior (Chapter 2), and the acquisition of a specific motor skill with identifiable neuronal correlates (Chapter 3). As predicted by previous studies, we find that alterations to the behavioral repertoire of an animal are accompanied by substantial synaptic reorganization [35-39]. However, we have shown that such reorganization is remarkably focused and contingent on the functional relationship of neurons. In the case of the development and maturation of fine motor control, interconnectivity between neurons controlling the same or similar muscle groups declines with maturity. For motor learning, synaptic networks are constructed

between neurons associated with control of the newly learned behavior. Although the synaptic and network mechanisms that underlie learning and behavior have been widely theorized and modeled, these findings provide the first experimental characterization of learning-related modifications among neurons of known behavioral relevance, extending our understanding of how knowledge is represented in the brain.

At face value, the results of these two studies may appear contradictory, as the ability to perform fine motor behaviors is associated with *decreased* connectivity during development, while acquisition of a new motor behavior is associated with *increased* connectivity in adulthood. However, the underlying contexts are fundamentally different. Developmental changes in cortical circuitry involve breaking synaptic bonds that were initially formed with respect to innate cues and therefore may not be of functional relevance in adulthood. For example, our studies of development show that connectivity is initially elevated between corticospinal neurons innervating the same spinal cord segment and thus controlling functionally related muscles. Communication between such neurons is certainly important for motor control, as coordinated activity of neighboring muscles is essential for many fine motor movements [123]. Therefore, having such connectivity innately programmed almost certainly confers an advantage for the development of motor behavior. Nevertheless, many of these initial connections may prove counterproductive, such as associations between neurons controlling muscles that are antagonistic or, at best, irrelevant for

functional behaviors. Only after these bonds are severed can fine motor control emerge. Additionally, reduced interconnectivity results in a greater number of independent networks, which allows for enhanced resolution and fractionation of movement. The end result is dissolution of unfavorable contacts and the construction of functionally relevant assemblies.

On the other hand, acquisition of a fine motor skill is slowly built following repeated training over the course of days, and even years in the most complex of tasks in humans (such as playing a musical instrument at an advanced level). Repetition of a behavior results in repeated coactivation of the neuronal ensembles controlling its execution. We hypothesize that, through Hebbian-type plasticity, this leads to the formation of synaptic contacts between these ensembles, thus increasing connectivity within the learning network. As with development, the process of motor learning probably involves breaking preexisting connections as well. However, this is likely to be on a smaller scale compared to development, as synaptic associations in adulthood are more "purposeful" than during development. Additionally, the loss of synapses may take place at a later time point than investigated in the current study, as *in vivo* experiments show that dendritic spine elimination lags behind learning-induced spine proliferation [36, 38].

Limitations of the experimental method

Two important caveats of the retrograde labeling technique should be considered when interpreting the results of these studies. First, the collection of lower motor neurons located within a particular spinal cord segment innervates a variety of downstream muscles. In the case of segment C8, this includes multiple muscles of the forearm and paw that conduct the trained movement (skilled grasping), and other muscles that may not be related to skilled grasping, such as the triceps [43, 44]. Accordingly, the C8-projecting population includes learning neurons, but it also contains non-learning neurons that are not predicted to change with training. This suggests that learning-related alterations are rather robust to be detected among this mixed population. Unfortunately, no technique currently exists for labeling a subpopulation of corticospinal neurons that innervate a specific muscle without damaging the labeled neurons, as multisynaptic retrograde tracers such as rabies virus are cytotoxic and it would prove challenging to separate the learning effects from cytotoxic effects

Second, whether rats possess a direct corticospinal pathway to lower motor neurons in the spinal cord is debated [124-128]. Notwithstanding, the majority (if not all) of descending corticospinal axons synapse upon intermediate neurons within the spinal cord that in turn form local propriospinal circuits that innervate motor neurons potentially located throughout multiple spinal segments [127, 129]. This adds a layer of uncertainty for mapping corticospinal output to actual muscle control, although it does not fundamentally alter the conclusions of these studies.

Supplemental conclusions – Developmental study

In addition to the mechanisms proposed in Chapter 2 to explain the observed developmental changes, there are other interpretations that are consistent with our findings. For example, we observed that intrinsic excitability increased globally with age during development. In our learning study, however, increased excitability was specific to the "learning" network of trained animals. Since our development study assayed cells at a time when cortical control of movement becomes established, the cortical system is likely learning a variety of motor skills that engage much of the corticospinal network. Consequently, it is possible that the global increase in excitability during development is a reflection of this widespread learning, instead of signifying a fundamental and persistent shift in the nature of neural behavior, as proposed. The fact that variability was so high indeed suggests we may have been recording from multiple populations – that is, learning and non-learning cells. Expanding recordings from animals where the development of fine motor skills has largely subsided (~p100) would address this alternate interpretation; if intrinsic excitability is decreased at a time when the motor system is not undergoing extensive learning, it may be reasoned that heightened excitability is a reflection of learning neurons.

Additionally, we speculated that the decrease in within-population interconnectivity (e.g., C4→C4 and C8→C8) during development both enables fine motor behavior by segregating large, interconnected networks into smaller,

independent circuits, and is a reflection of the animal's increasingly complex motor repertoire. However, during the timeframe of our first recordings (~p20), corticospinal input to the spinal cord is relatively weak, as spinal terminals are not fully developed [10, 15]. Increased interconnectivity at this time may serve to amplify descending signals to the spinal cord by stimulating a larger network of corticospinal neurons. Thus, heightened connectivity in young animals may actually serve a functional purpose and not simply be a vestige of innate programs for initial synapse formation that must be refined.

Supplemental conclusions – Learning study

Interestingly, acquisition of the skilled reach-to-grasp behavior does not produce detectable changes in synaptic or intrinsic properties in the C4-projecting population, which control muscles of the upper forelimb and shoulder. Despite the obvious involvement of these regions in the reaching behavior, it can be argued that the reaching movement required for this task: 1) is not being refined with training; and 2) does not constitute a novel movement for the animal, as opposed to the single-paw grasping behavior. As such, corticospinal neurons controlling reaching are not likely activated in a novel manner, and although these neurons will be repeatedly coactivated with training, these circuits are already established.

Although excitatory connectivity was significantly greater following learning specifically between C8-projecting cell pairs, there was a noteworthy increase in

connectivity from C8-projecting cells to C4-projecting cells (C8→C4) following learning (1.5-fold increase; Fig. 3.2). Although speculative, it is plausible that this modest increase could be caused by a learning-induced process whereby active, learning neurons blindly upregulate presynaptic contacts with surrounding dendrites, perhaps with the goal of finding new synaptic partners in support of the learned behavior. Through random sampling, connections that are functionally relevant to the behavior will be stabilized (perhaps through Hebbian-type mechanisms), while inappropriate synapses will not persist. Therefore, connectivity remains high among C8-projecting neurons controlling the grasping behavior (C8→C8), while C8→C4 synapses do not endure, although C8→C4 connectivity will still be higher compared to untrained animals due to newly formed synapses that have yet to disappear.

Prospective mechanisms of increased inhibition during learning

Somewhat surprisingly, polysynaptic inhibition was frequently detected in our recording preparation in response to a single presynaptic action potential. Fast polysynaptic inhibition similar to that reported here is believed to be mediated by basket cells with depressing excitatory synapses [97, 130, 131]. We found a specific increase in polysynaptic inhibition onto C8-projecting neurons following training.

An increase in disynaptic inhibitory connectivity could be due to alterations at the first synapse (presynaptic corticospinal neuron to inhibitory interneuron)

and/or the second synapse (inhibitory interneuron to postsynaptic corticospinal neurons; Fig. 4.1). Modifications at the first synapse could take place either through formation of new synapses [132], or by potentiation of preexisting synapses such that presynaptic activation that generated a subthreshold response in the inhibitory neuron prior to learning now subsequently causes a suprathreshold response after training. Both of these scenarios, however, seem unlikely. Previous studies indicate inhibitory interneurons promiscuously innervate surrounding pyramidal neurons, seemingly without regard to the function of downstream neurons [99-101, 133]. Dissection of our recording characteristics supports these prior findings, as disynaptic inhibition was observed simultaneously across neighboring postsynaptic cells without regard to their functional classification (i.e., spinal cord target). Therefore, increasing activation of interneurons should lead to an overall increase in inhibition in both C4- and C8-projecting neurons, which was not observed.

On the other hand, alterations at the second synapse could increase connectivity among a select population of postsynaptic targets. For example, inhibitory neurons could form new synapses specifically onto C8-projecting neurons during learning, or learning could potentiate weak preexisting inhibitory synapses, amplifying IPSCs that might have been below detection threshold previously (Fig. 3.9). Indeed, LTP-like mechanisms have been reported at inhibitory synapses [102, 103, 134], representing a mechanism through which selective changes in inhibition could take place. In our learning study, IPSC potency in C8-projecting cells trended toward increasing following learning ($p =$

0.13, Fig. 3.9), suggesting inhibitory synapses may have been potentiated. Thus, increased inhibitory connectivity onto the C8-projecting population may be due to a combination of new synapse formation and potentiation of preexisting synapses. Finally, it is important to recognize that the fast disynaptic inhibition evident in our recordings represents only one of many inhibitory systems, each of which may undergo vastly different modifications during learning.

The motor engram

In his landmark 1950 paper, “In search of the engram,” Karl Lashley concluded from 30+ years of memory research that, “It is not possible to demonstrate the isolated localization of a memory trace anywhere within the nervous system. Limited regions may be essential for learning or retention of a particular activity, but within such regions the parts are functionally equivalent. The engram is represented throughout the region.” [135] However, Lashley’s experiments fell short in two key areas: 1) the use of deceptively complex tasks that engage multiple systems of the brain; and 2) in the case of simple tasks, failure to examine the truly relevant brain region for memory formation. Consequently, subsequent work has challenged Lashley’s assertions, demonstrating that a memory trace may indeed be confined to a small, localized collection of neurons [136-138]. Notably, the mechanism often invoked for the establishment of these memory circuits comes from Lashley’s own student, Donald Hebb, and the theory of Hebbian plasticity [139].

Numerous studies identify the primary motor cortex as the potential storehouse of skilled motor representations, or motor engrams [74], and a model of how such engrams may be established within the corticospinal system of M1 was presented in chapter 1. Briefly, increased connectivity between corticospinal neurons controlling a motor behavior, forged following repeated training, generates a local copy of the newly learned skill, thereby automating its execution. The present work provides the first experimental evidence that learning indeed increases connectivity between neurons functionally related to the learned behavior, providing a basis for the formation of a motor engram within M1.

It could be argued that hardwiring a sequence of motor outputs would lead to a motor behavior that is too rigid for practical use. Indeed, a corticospinal engram is unlikely to be self-sufficient and would require trial-to-trial adjustments based on changes in the environment, posture, or muscle fatigue. However, feedback can be built into the circuit to automatically compensate for such intertrial variables. For example, muscle stretch receptors provide feedback to lower motor neurons regarding the status of their target muscle, and can modulate the activity of lower motor neurons around the muscle's equilibrium point [140]. That is, overstretching of the muscle will cause activation of the innervating motor neuron, inducing contraction to bring the muscle back toward the equilibrium length; the farther away from the equilibrium point, the larger the activation. In this way, unexpected deviations in limb position can be compensated. Thus, there are mechanisms within the motor system that can

compensate for trial-to-trial variations, making seemingly fixed movement networks of the motor cortex much more versatile.

There are several benefits a motor engram could confer on the motor system: precision of movement, reliability of movement, and independence of movement. Greater precision is of obvious importance as it increases the capabilities of the motor system. As previously shown [86], skilled-grasp training increases the fidelity of muscle recruitment among layer V neurons in M1, such that muscle activation is more strongly correlated with neuronal firing. This is conceivably caused by boosted coactivation of corticospinal neurons controlling the same muscle, or by increased neuronal bursting during movement, both of which could be produced by augmented recurrent connectivity between functionally related neurons. Thus, creation of a local movement engram increases the accuracy and temporal resolution of muscle activation, thereby enhancing the precision of motor output.

Movement independence refers to the ability of a skilled motor program to be executed without the need for higher-order cognitive processes. That is, once a movement has been locally encoded within M1, upstream inputs that coordinate the pattern of corticospinal output can be minimized. As such, movements becomes automated, or become so-called “muscle memory”. Assuming higher-order coordination of motor activity requires attention, as well as extensive feedback as to the success of varying movement patterns, local

encoding of a movement will free up these processes to be utilized elsewhere, although optimal performance may still require some level of concentration.

Movement reliability is important as it increases the brain's predictive abilities by enhancing its accuracy for modeling intended movements. For example, hard wiring the constitutive movements of the grasping behavior will promote activation of this particular pathway whenever a grasping movement is "requested" by the brain. Consequently, once the grasping behavior has been stably encoded in M1 (i.e., a grasping engram created), an animal can be reasonably certain of the movement pattern that will be executed. With subsequent experience, the animal can predict the probability of obtaining a sugar pellet reward whenever the grasping engram is called upon, and use this information for decision making. For instance, if the animal is hungry and success rate is normally low, it may choose to allocate more attention to its grasping behavior to boost pellet retrieval success. Conversely, if that giant animal in the lab coat feeding it pellets is behaving suspiciously, the animal may be more attentive to its surroundings.

One potential drawback of locally encoding motor behaviors is it could increasingly constrain the output of the motor system as more and more neuronal ensembles become hardwired within M1 [141]. Taken to the extreme, one can imagine a scenario where every available corticospinal neuron has become incorporated into a movement network. Novel motor behaviors would need to exploit these preexisting networks, perhaps to the detriment of performance, as

“motor resolution” would be reduced [142]. However, this radical scenario is unlikely, as synaptic strength between corticospinal neurons is low, suggesting individual neurons are not rigidly fixed to a particular network but are likely available for inclusion in multiple independent motor behaviors.

Lingering questions and future directions

To reiterate, the hypothesis examined in this thesis is that the strength and pattern of corticospinal connectivity shapes the capabilities of the motor system. We have supported this view by showing that the emergence of fine motor behavior and acquisition of motor skills are associated with distinct reorganization of synaptic topography. Our ultimate goal is to build upon this knowledge to better understand how information is acquired and represented in the brain across multiple levels. As such, many questions remain.

Of obvious interest is the specific role of the different modifications observed during these studies. Is intrinsic excitability elevated during learning to support synaptic plasticity, or does it enhance network transmission (and/or muscle recruitment) by increasing neuronal firing rate? Is inhibition necessary for learning and behavioral refinement, or does it simply offset increases in intrinsic and synaptic excitatory drive? Is the production of recurrent corticospinal contacts actually necessary for formation of motor memory? The most straightforward method of addressing these questions is selective elimination of each system. Although it may be feasible to inhibit elevated excitability by

knocking down molecules such as CREB [115, 143], silencing the disynaptic inhibitory pathway must await identification of the population of interneurons that mediate this signal. Furthermore, it is currently not possible to eliminate synapses specifically between select populations of neurons, such as recurrent corticospinal connections. Absent the ability to selectively eliminate these systems, insight into the role and mechanisms of learning-related changes can be realized by investigating their individual time courses. Although we examined many different time points for our developmental study, our learning study investigated cortical properties only after the skilled grasping task had been well learned. Determining the time of onset, pace, and relative order of changes will help address the questions above.

A strong inference made in this thesis is the central role of C8-projecting corticospinal neurons for performance of the skilled grasping behavior. There is ample evidence to support this assertion: the motor cortex and corticospinal tract are required for this behavior [25, 92, 94], C8-projecting neurons project to the area of the spinal cord that controls grasping musculature [43, 44], C8-projecting neurons selectively undergo structural modifications following learning [35], and now we have shown that this population is also the exclusive target of network and cell intrinsic modifications.

However, to truly test the necessity of this projection population would require its elimination, either through targeted lesions or reversible silencing. This has been a goal of our lab for some time, and we are currently piloting a study to

address this question. If C8-projecting neurons are indeed required for learning, the next step would be to perturb their local circuitry to probe whether memory traces are in fact stored in the synaptic contacts between neurons. An ideal experiment would be to selectively eliminate every new synapse formed during learning, perhaps by tagging all synapses constructed within a specified time window with an inducible toxic substance. Before triggering synaptic destruction, learning would be intact. Afterward, the newly learned skill should be ablated, while preexisting motor skills remain.

In this thesis, we restricted our analysis of synaptic reorganization to recurrent corticospinal inputs. Although recurrent connectivity among layer V neurons is among the strongest local signaling pathways in M1 [144, 145], these connections represent only a fraction of total input to corticospinal neurons. There is great opportunity, therefore, to address how input from other motor systems is modified during development and motor skill acquisition. This includes all inputs to corticospinal neurons, such as projections from the basal ganglia and cerebellum (via the thalamus), frontal cortex, somatosensory cortex, contralateral M1, and even local interlaminar connections, such as layer II/III inputs which comprise a strong signaling pathway to layer V neurons [145, 146]. Again, investigating the synaptic modifications associated with learning (and development), as well as the time course of these changes, will elucidate their individual roles in the expansion of the motor repertoire. For example, we speculate that the frontal and somatosensory cortices play an important role in

the initial stages of learning. Accordingly, changes in synaptic signaling for these populations may precede those observed for recurrent corticospinal inputs.

There is a dearth of information regarding how inhibition changes with learning. Our system, however, provides an excellent substrate for such inquiry. Numerous local inhibitory populations should be investigated to determine how both their inputs and outputs change with learning, and how motor performance is affected in their absence. A dimension of this question includes whether changes in inhibition are orchestrated by external influence, or are regulated locally, perhaps by activity levels. As alluded to earlier, the population(s) of interneurons mediating the disynaptic inhibition observed in the current work also needs to be characterized. One area of interest is how the strong coupling between corticospinal neurons and inhibitory neurons is achieved.

Finally, several features of recurrent synapses should be explored more thoroughly during development and learning. These studies might include anatomical characterization of synapses, such as their location along the dendrite, postsynaptic density (PSD) size, and presynaptic vesicle properties. Further, pharmacological and electrophysiological methods can be employed to classify postsynaptic receptor composition before and after learning; this should be applied to both excitatory and inhibitory conditions. Another question of interest is the extent to which increased connectivity during learning reflects *de novo* synapse formation versus conversion of silent or weak synapses. Additionally, comparing modifications in gene expression between C4- and C8-

projecting cells during learning should prove particularly insightful, not only for genes related to synaptic function, but for various cellular processes that may support learning. These genetic studies are already underway.

Conclusion

The work presented here indicates that functionality of the motor system is regulated by the specific pattern of local corticospinal connectivity, and suggests that behavioral experience both influences and is influenced by corticospinal circuitry. That is, repeatedly performed movements that are behaviorally relevant are encoded within corticospinal networks (behavior influencing circuitry), and the pattern of local connectivity can bias the spread of excitation through the system, enabling – and possibly facilitating – certain movements, while suppressing others (circuitry influencing behavior). We hypothesize that local encoding of ethologically relevant behaviors confers several advantages for survival, including automation, stability, and enhanced precision of movement.

While our experiments specifically examined the corticospinal system of the motor cortex, repeating motifs of structure and function are preserved across the cortex. Therefore, we speculate that these findings identify global mechanisms of neuronal-intrinsic and network-specific modifications applied throughout the cortex during development and learning.

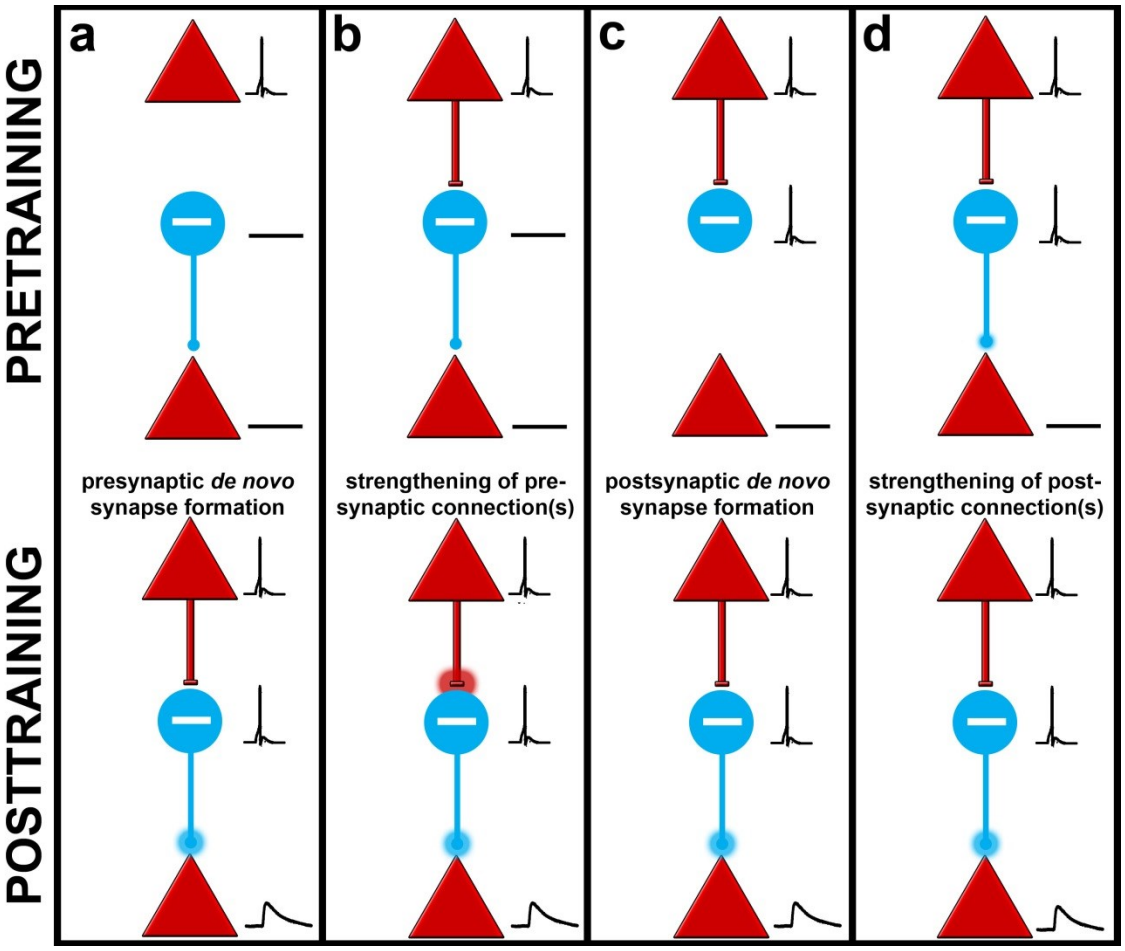

Figure 4.1. Potential mechanisms of increased disynaptic inhibition. Red triangles symbolize corticospinal neurons that are presynaptic and postsynaptic to inhibitory interneurons (blue circles).

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