UNIVERSITY OF CALIFORNIA, SAN DIEGO

Population activity in the motor cortex and its relationship to movement across motor learning

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Neurosciences with a Specialization in Computational Neuroscience

by

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I have pursued a career in science because I have always loved learning about the natural world and because I consider it a remarkable privilege to be involved in generating new knowledge. These fundamental drives can be obscured by the business and politics of doing science, the commonality of failures and slow progress, and the looming presence of ever-increasing competition. I therefore owe every step I have made along the way to people that helped me clear the dirt of transient problems from the bedrock of my fascination with science. Every contribution that I have made or will make to science would not be possible without them.

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ABSTRACT OF THE DISSERTATION

Population activity in the motor cortex and its relationship to movement across motor learning

by

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Professor Takaki Komiyama, Chair

The fundamental function of the brain is to produce useful movements. Movement has become progressively more directly controlled by descending systems through evolutionary history, culminating in the mammalian motor cortex which contains projections to the spinal cord. The activity of neurons within the motor cortex is highly heterogeneous, with different neurons being correlated with different aspects of movement. Consequently, activity within the motor cortex is most comprehensively described at the level of large neuronal populations. The motor cortex is also capable of substantial plasticity after injury and during learning, suggesting that the role of activity in guiding movements is flexible. A key component of understanding the function of the motor cortex

therefore is to monitor changes in activity of motor cortex neuronal populations across learning.

The experiments described within this dissertation follow this approach primarily through the use of *in vivo* two-photon calcium imaging. Mice were trained in a forelimb motor learning task over the course of two weeks, and the activity of a consistent population of hundreds of neurons was recorded during each training session through calcium indicator fluorescence. Movements of the forelimb during each training session were continuously monitored by displacement of a lever, allowing for a comparison between activity patterns and corresponding movements.

Three separate neuronal populations were analyzed. These include excitatory pyramidal neurons in layer 2/3, inhibitory interneurons within layer 2/3, and corticospinal neurons within layer 5b. Layer 2/3 was found to be active primarily around movement, while corticospinal neurons were active either during movement or quiescence. Inhibitory interneurons were found to be relatively stable across recording, being primarily correlated with local excitatory neuron activity. Excitatory neurons in layer 2/3 and corticospinal neurons, on the other hand, were modulated by learning. This included an initial increase in the number of movement-related cells, followed by a decreased but stable population. The temporal activity of individual cells also changed across time but became more consistent late in learning. These dynamics resulted in a differing relationship between activity and movement across learning, suggesting that the influence of motor cortex activity on movement is shaped by learning.

Chapter 1. Introduction

Life exists at an intersection between organisms and their environment. The Earth provides an incredible array of resources, all of which are utilized by different forms of life expanding into an equally incredible array of niches. This fundamental give-and-take relationship has molded life through evolution to productively interact with the world around it. In most branches of life, these interactions are slow and inflexible. Single-celled protists can move towards food with flagella; plants can bend towards the sun through asymmetric growth; fungi can coalesce to form fruiting bodies for reproduction. The appearance of the brain in animals, however, ushered in a new era for how life could respond to the surrounding environment. After three billion years of life without brains, the appearance of the first nervous systems progressed rapidly from the nerve nets of the cnidarians to the structured central brains of the arthropods[1]. For the first time, the organisms demonstrated an ability to quickly react to the outside world in varying and complex ways.

This evolutionary development provides an important perspective for assigning the essential function of the brain. Each animal is equipped with sensory organs to provide relevant information about the world, and each animal is likewise equipped with movable body parts to act on that information to benefit survival. The brain is the link between these two capacities; it serves as a transformer of sensation into action. Not all external information is equally important to every animal, and hence all animals have developed different collections of sensory apparatuses. The sensory world that each animal samples

from contributes to the gestalt of that animal's experience, termed *umwelt* by the biologist Jakob von Uexkϋll. The sensory input remains only half of an animal's *umwelt*, however; the information collected by the brain is only as useful as the ways in which it can be acted upon. In many animals, this takes the form of reflexive and stereotyped movements. In some instances, the underlying neural circuits which drive these movements have been deduced; for example, moving the body away from tactile stimuli is a common movement achieved in different ways by sea slugs[2], leeches[3], and fish[4]. In other instances, behaviors can be complicated and not yet ascribed to specific neural processes, like honey bees signaling food sources through their waggle dance or graylag geese retrieving misplaced eggs through egg-rolling with their beaks. In these types of behavior, the animals have developed innate neural architectures for performing complex movements to interact with the world. They do not learn these behaviors from conspecifics, nor do they develop these behaviors through experimentation and practice. The ability to learn movements beyond hard-wired behaviors, termed motor learning, therefore represented another leap forward in organism-environment interaction by the animal kingdom. Animals endowed with this ability could adapt within their lifetimes to the demands of their surroundings, affording them the flexibility to modify innate behaviors or create new ones entirely.

Motor learning at a general level is not a recent evolutionary feat. For example, sea slugs are able to create novel associations between stimuli and movements[5], and fish can control the strength of swimming in response to visual

cues[6]. These forms of motor learning primarily act towards modifying existing behaviors and have limitations, famously demonstrated by the inability of frogs to adjust fly-catching behavior after altering visual input[7]. The ability to coordinate muscles in novel ways and improve the execution of a movement through behavioral exploration, on the other hand, appears to be a more phylogenetically restricted skill. Some notable examples of this exist outside of the mammalian clade, including vocal learning by birds and skilled tentacle manipulation in octopuses; however, motor learning of this sort is universally present in mammals.

Motor learning operates through existing substrates of general motor control. Muscles are controlled directly only through activity of neurons within the spinal cord. The spinal cord, and homologous structures in invertebrates, is itself capable of organizing certain complex behaviors. This can be demonstrated when connections between the spinal cord and upstream structures are severed, yet intact behavior or structured neural activity are still observed, for example with flight in locusts[8] and coordination of muscles for defensive behaviors in frogs[9,10]. Control over behavior therefore relies on input into spinal cord circuitry from descending pathways, which follows a common pattern for all vertebrates but is more extensive in animals which exhibit more sophisticated aptitude for motor learning[11]. Specifically in mammals, the major brain structures which contribute to these descending pathways include the cerebellum, the basal ganglia, and the motor cortex. While likely an oversimplification due to anatomical and functional interdependence[12–15], these structures are

traditionally associated with separate aspects of motor learning: the cerebellum is linked to fast corrective and adaptive control of movements, the basal ganglia are implicated in action selection, and the motor cortex is involved in novel skill learning[16,17]. The motor cortex in particular has undergone marked changes in connectivity with the spinal cord across phylogeny, with more dexterous species having more extensive[18] and direct[19] cortical control over spinal motor circuits compared to less dexterous species. The motor cortex therefore represents a prime site of interest for elucidating the mechanisms by which motor learning occurs. The aim of this dissertation is to offer evidence towards understanding how the motor cortex contributes to motor learning.

Discovery and early characterization of the motor cortex

The antiquity of the cortex is rich in being dismissed as a support organ. Highly vascularized and largely visibly homogenous without staining, the outermost layer of the brain was generally held to be glandular and "inexcitable", unable to elicit movements[20]. It was the dawn of electricity in the laboratory that finally refuted this view. In 1870, Gustav Fritsch and Eduard Hitzig strapped dogs to Fritch's wife's dressing table and methodically applied electrical stimulation from a battery to restricted areas of the cortex. The now famous result demonstrated conclusively that not only could electrical stimulation of the cortex drive movements, but that movement generating portions of the cortex were restricted to anterior regions and acted upon the contralateral side of the body[21].

The pathway by which the cortex drove movements was relatively quickly attributed to a direct connection between the cortex and the spinal cord. Historically called the pyramidal tract due to its outward appearance as pyramid-shaped bulges along the medulla, it is now synonymously called the corticospinal tract. Even before Fritsch and Hitzig's experiments, the existence of a telencephalic projection to the spinal cord was shown by observing degeneration in the spinal cord after cortical lesions. The site of origin for the pyramidal tract was initially debated, but the cortex became the primary candidate following demonstrations of excitability. In the following years, more degeneration studies were conducted which both verified the corticospinal projection and established that it crossed the midline in the medulla. This decussation resulted in each hemisphere of the cortex being connected to the contralateral side of the spinal cord, therefore explaining the contralateral action of motor cortex stimulation[22]. Delineating the precise location of the corticospinal tract paved the way for targeted manipulation to confirm its role as mediator between cortex and movement, where the effects of cortical stimulation in the spinal cord were abolished following corticospinal tract transection[23,24].

The organized distribution of movement centers in the brain had been hypothesized a few years prior to Fritsch and Hitzig's experiment by the neurologist John Hughlings Jackson. A pioneer in the study of epilepsy, Hughlings Jackson described a form of seizure in which movements would "march" across areas of the body[25]. The procession of Jacksonian seizures coupled with the

excitable cortex demonstrated in dogs lead the medical student David Ferrier to embark on tying these two phenomena together. Ferrier showed that prolonged electrical stimulation of the cortex could reproduce seizure-like movements which progressed along the body, and that these marches occurred through a chartable layout of the body on the brain which he termed the "motor map". Ferrier carried out these experiments on a number of different species, showing that the map of bodily movements elicited by electrical stimulation was generally consistent across animals. Importantly, he also demonstrated that destruction of a particular area of the cortex resulted in impairment in movements of the corresponding area of the body[26]. The topography of bodily movements across the primate cortex was then methodically charted in the laboratories of Victor Horsley[27] and Charles Sherrington[28], robustly delineating the motor cortex and establishing a roughly somatotopic organization. This work not only established the existence of the motor cortex, but also significantly contributed to the burgeoning theory of localized function within the brain[26].

In the following decades, the cortical areas which drove movements were further subdivided. This began by noting cytoarchitectural differences across excitable cortex, particularly in the presence and density of very large cell bodies in the deep layers. These histological findings were later coupled with functional mapping, including the electrical threshold for stimulating movement, the types of movements produced, and the effects of lesions[29]. The motor cortex was subsequently divided into a posterior "motor" and anterior "premotor" area. In the "motor" area (now called primary motor cortex, or M1), movements could

be elicited from low threshold stimulation, evoked movements were simple, and lesions caused a general but transient paralysis. In contrast, the "premotor" area required higher levels of stimulation to drive movements, the resulting movements were complex, and lesions produced deficits over control of movement, including lack of movement coordination and increased reflex vigor[30]. These observations suggested a hierarchy of the cortical motor system, in which the premotor region organized movement at a higher level but ultimately went through the motor region as the final output.

Early work supported a hierarchy because the premotor area was found to project to the primary motor area, and importantly it was thought to contain fewer projections to the spinal cord than the primary motor area. However, the development of more sophisticated retrograde labeling techniques in the second half of the twentieth century allowed for more direct methods for visualizing pathways, and it was found that the premotor and primary motor areas in fact both had substantial connections with the spinal cord[31]. Those techniques also allowed motor areas to be more precisely segregated through their connectivity with the primary motor cortex and the spinal cord. In this case, for a given area of the primary motor cortex which drove movements of particular muscles, incoming fibers would originate from distinct premotor areas, and each of those areas likewise projected to the level of the spinal cord which drove the same muscles. In this way, the motor map of the body on the brain was in fact represented multiple times. It was thereafter established that the primate motor cortex contains roughly seven total motor areas: the primary

motor cortex anterior of the central sulcus, the dorsal and ventral premotor areas immediately anterior, the supplementary motor area medial to the premotor areas, and three cingulate motor areas on the medial wall[32]. In rodents on the other hand, the motor cortices were separated into just two parts: a lateral area considered primary motor cortex and a medial area considered potentially non-primary motor but with unclear homology to the primate brain[33,34]. While the functional differences between the motor and premotor areas are still not clear today, the primary motor cortex is conserved across mammals as the primordial excitable cortex and therefore represents a homologous target for studying cortical control of movements[11].

Modern definition, comparative anatomy, and evolutionary considerations of the motor cortex

The motor cortex today is defined through four primary characteristics. First, stimulation evokes movements. The ability to evoke movements through artificial stimulation continues to be a common method for identifying the motor cortex, both through classical electrical microstimulation and more recently with optogenetic stimulation[35]. Second, it projects to the intermediate and sometimes ventral lamina of the spinal cord. While the excitable portions of cortex have long been thought to act through projections to the spinal cord, it is also now known that the somatosensory cortex also contributes heavily to the corticospinal tract[36]. The spinal projections from cortex are segregated such that somatosensory cortical inputs terminate in the dorsal lamina of the spinal cord and the motor cortical inputs terminate in the intermediate and ventral lamina of

the spinal cord, and therefore these projections are likely functionally specific to sensation or movement respectively[37]. Third, the motor cortex is histologically agranular, lacking a medial layer of dense small cells. These cells are the primary sensory thalamic recipients in sensory areas of cortex, and make up a prominent fourth layer in other cortical areas. The lack of these cells in motor cortex has traditionally been viewed as a lack of a functional layer 4, although recent work suggests that it may in fact be present but diminutive[38]. Finally, the motor cortex receives inputs from the ventrobasal thalamic nuclei. The ventrobasal nuclei of the thalamus receive input from other movement-related structures including the basal ganglia and cerebellum and project in turn to the motor cortex, making input from the ventrobasal thalamic nuclei a key component of integrating the motor circuits of the brain[39,40]. Aside from these characteristics, the motor cortex is similar in form to the rest of the cortex. It is a laminar structure, where layer 1 is sparsely populated by cell bodies but densely packed with dendrites from deeper layers, a graded layer 2/3 contains neurons that project mostly within the cortex, layer 5 is large and nominally divided into 5a and 5b sublayers with corticospinal neurons being restricted to 5b, and layer 6 contains neurons which project to the thalamus[41]. Excitatory and inhibitory neurons are spread throughout the layers in approximately a 4:1 ratio, with the exception of layer 1 which contains exclusively inhibitory neurons[42].

The unique signatures of the motor cortex have been studied across a wide range of species, providing an evolutionary and comparative perspective of the motor cortex. The presence of a motor region of the pallium, including

descending pathways to the spinal cord, appears to be absent in reptiles and is therefore possibly a novel mammalian feature[43]. It is notable that birds are often studied for their impressive motor abilities and do possess a motor pallial region[16,44]; however, it is likely that this is the result of convergent evolution and may have different functional properties from the mammalian motor cortex[16,43]. Within the mammalian lineage, the motor cortex is not always present as a distinct area but instead is overlapping to differing degrees with the somatosensory cortex, which itself was probably present in reptilian ancestors[43]. The degree of cortical overlap between the somatosensory and motor areas is also paired with a corresponding degree of overlap in inputs from the sensory and motor thalamic nuclei[45]. The origins of the motor cortex may be best seen in the marsupial *Monodelphis*, in which a complete somatosensory map is present that also holds motor functionality restricted to the facial regions, potentially arising with the control of whiskers[46]. Interestingly, a complete motor map is also present in monotremes, making the homology of cortical control of the entire body unclear but emphasizing that some form of motor cortex has likely always been present in mammals[47]. The motor and sensory cortical areas then exist as mirrored but partially overlapping maps on the rodent cortex, and are fully separated in the primate cortex[11].

The emergence of the motor cortex for control of the body is hypothesized to have coopted spinal circuitry of locomotion to perform other types of movements[48,49]. While early novel skills included walking on complex terrain and manually manipulating food as seen in rodents, this progressed to

more complex abilities like visually-guided reaching and individual finger use in primates[50]. Forelimb movements in particular have been a major focus of motor cortex research because of the ubiquitous and wide array of available behaviors; differences in forelimb motor skill are notable even between rodents and marsupials[51]. Development of the motor cortex across phylogeny is also closely associated with motor skill and dexterity, particularly as it pertains to forelimb use[52]. Interestingly, increased motor abilities do not necessarily relate to the number of corticospinal cells or corresponding size of the corticospinal tract, but are more correlated with other features within the cortex and spinal cord. Specifically, in the cortex, the number of corticospinal cells is correlated within a lineage but not globally with motor skill, where for example raccoons have even more corticospinal cells than monkeys. Instead, expansions of the motor cortex are accompanied by a relative increase in non-corticospinal neurons, suggesting that intrinsic computations within the cortex may be more important than the mere number of outgoing projections[53]. Indeed, the amount of corticospinal neurons relative to the total cortex is uniquely consistent in primates and less so in other lineages, suggesting that a balance between corticospinal and non-corticospinal neurons is particularly important in primates[54]. In the spinal cord, on the other hand, the location of corticospinal terminations appears to be the strongest predictor of dexterity. Here, more ventrally-terminating fibers closer to spinal motor neurons associate with increased dexterity[18,55]. This is even true among closely related species, where cebus monkeys exhibit finer hand control and likewise have denser corticospinal projections to the ventral horn of the

spinal cord compared to squirrel monkeys[56]. In certain cases, these ventral lamina-projecting cells contact motor neurons directly, establishing a disynaptic link between the cortex and muscles[57]. These cells, termed corticomotoneuronal neurons, are only present in certain animals and are either incredibly sparse or completely absent in the adult rodent, although corticospinal cells do transiently contact motor neurons during development[58,59]. Corticomotoneuronal neurons are in fact thought to be unique to primates, and correlate with degree of manual dexterity[19]. Moreover, they cluster in an area which is hypothesized to be an evolutionary amendment to the primary motor cortex[60]. The functionality of the motor cortex therefore seems to critically relate to both circuits within the cortex and the targets within the spinal cord.

Connectivity of the motor cortex

The capabilities of neural circuits are endowed by their connectivity. Specifically, the input defines what information the circuit can act upon, the local connectivity defines the computations within an area, and the output defines how locally processed information is relayed to other areas. Each of these three features has been seen to relate to the role and evolutionary development of the motor cortex: inputs from the thalamus became segregated and connections with anterior motor regions emerged, separation between sensory and motor areas developed while retaining a large number of non-corticospinal neurons, and projections to the spinal cord extended their area of influence and reduced their synaptic distance to muscles. The anatomy of the

motor cortex therefore provides an essential component towards understanding its role in movement.

One primary input to the motor cortex arises from the thalamus. The thalamus in the case of the motor cortex acts much as it does for sensory cortex by providing a waystation from subcortical structures. Both the cerebellum and the basal ganglia are crucial for normal motor function, and they are likewise heavily interconnected with the motor cortex by way of the thalamus. The output nuclei of the basal ganglia and cerebellum target the ventrobasal aspect of the thalamus, which are distinguished to differing degrees in different species as the ventral anterior, ventromedial, and ventrolateral nuclei. It was initially debated whether the basal ganglia and cerebellum target separate or overlapping populations within the thalamus; however, it is now accepted that they are segregated in both rodents and monkeys to the ventral anterior/ventromedial and ventrolateral nuclei, respectively[61–63]. Surprisingly, considering that outputs from the basal ganglia are inhibitory while those from the cerebellum are excitatory, the activity of these separate recipient populations are very similar under most conditions[64]. In fact, increased basal ganglia activity does not even yield decreased recipient thalamic activity, making the nature of the thalamic relay an unresolved matter[65]. The information which the thalamus provides the motor cortex from these subcortical structures is therefore still an open question[66].

Despite the enigma of information sent by the thalamus, there are notable differences in how thalamic nuclei project to the motor cortex. The

separate subcortical targets within the thalamus bias their projections on a generally rostrocaudal gradient; the primary motor cortex receives more numerous fibers from the cerebellum-recipient thalamus, while the premotor and supplementary motor areas receive more input from the basal ganglia-recipient thalamus[67,68]. The thalamic nuclei also project with unique laminar profiles, where the cerebellum-recipient thalamus projects primarily to layer 2/3 and layer 5 to a lesser extent[69], while the basal ganglia-recipient thalamus contacts primarily the layer 1 apical dendrites of layer 2/3 and layer 5 somata, including those of corticospinal neurons[69]. The basal-ganglia recipient thalamus likewise includes both the ventral anterior and the ventromedial nuclei, in which the latter is even more spatially restricted to layer 1 projections[70]. These thalamocortical projections also differ greatly with regard to their sensory counterparts in their degree of axonal arborization, in that they are widely spread throughout their target regions and single thalamic neurons can often target multiple cortical areas[71]. The function underlying the differences in cerebellum- and basal ganglia-recipient thalamic projections remains unknown; however, they strongly hint at separate actions on the motor cortex from each structure.

In addition to receiving subcortical information though the thalamus, the other major input to the motor cortex comes from other areas of cortex. One main site of input is from the contralateral primary motor cortex, which originates from all layers[68,72]. Interestingly, however, anterior motor regions including the supplementary motor area are more highly connected with their contralateral equivalents than the primary motor cortex, suggesting a possible difference in

the role of coordinating bilateral movements[73]. The motor areas are also interconnected with each other, though with anterior motor regions preferentially targeting layer 5b neurons of the primary motor cortex[74]. The primary somatosensory cortex also projects heavily to the motor cortex, although more laminarly restricted to superficial layer 2/3 and layer 5a[75,76]. These inputs can also combine with thalamic inputs in single neurons[77]. A prominent input from the secondary somatosensory cortex is also present, largely confined to layer 2/3[78]. These sensory inputs provide tactile receptive fields to neurons in the motor cortex, which are widely disseminated though functionally restricted by local inhibition[79]. The gradual evolutionary separation of the somatosensory and motor cortices combined with substantial functional interconnections suggests an important role for the integration of somatosensory information in motor cortical control of movement[80]. Prefrontal non-motor regions also substantially project to the motor cortex, although they uniquely target layer 6 neurons within the primary motor cortex[81].

Finally, there is a strong input to the motor cortex from subcortical neuromodulator sources. These include dopaminergic inputs from the ventral tegmental area[82], cholinergic inputs from the basal forebrain[83], and noradrenergic inputs from the locus coeruleus[84]. Dopaminergic inputs are distributed in a decreasing rostrocaudal gradient in the motor cortex though with largely specific targets[82] and have varying effects on neuronal activity[85,86].

Connectivity within the motor cortex is extensive but organized[87]. Axon collaterals of somata within layer 2/3 and layer 5 are incredibly broad,

encompassing multiple functional domains and providing a substrate for highly recurrent connectivity[88,89]. In the deeper layers, however, the level of reciprocity in connections is asymmetrical across cell types, where corticostriatal neurons synapse on to corticospinal neurons but the reverse connection is only minimally present. These horizontal connections are complementary to the predominant flow of information in the motor cortex, which is situated vertically from layer 2/3 to layer 5[90]. These connections include specific connections from layer 2/3 neurons to deep corticospinal neurons[91]; however, corticospinal and corticostriatal neurons receive input from segregated populations of layer 2/3 neurons[92]. This general layout has been proposed to function through layer 2/3 acting as a "preamplifier" to subsequently drive deeper layer 5 activity.

The motor cortex, in accordance with the rest of cortex, consists of roughly 20% inhibitory interneurons. While in the minority, interneurons have been demonstrated to exert powerful and important effects on sculpting cortical activity[93]. Inhibitory neurons are diverse and associate with separate circuit functions[94]. Specifically, layer 1 interneurons receive input from the thalamus and oppositely engage layer 2/3 and layer 5 activity[95,96], parvalbumin positive (PV) interneurons target somata and axons, somatostatin positive (SOM) interneurons target apical dendrites[97], and vasointestinal peptide positive (VIP) interneurons are associated with inter-areal disinhibition[98]. These interneurons integrate the activity of local excitatory neurons and locally feed back onto the majority of excitatory neurons[99], acting functionally to restrict the large axonal domains of excitatory neurons[100]. However, separate classes of interneurons receive

separate sources of laminar input in the motor cortex; for example, layer 5 PV interneurons are driven by layer 5 excitatory cells while layer 5 SOM interneurons are driven more by layer 2/3 excitatory neurons[101]. Inhibitory input also varies by excitatory cell type, where corticospinal cells preferentially receive PV over SOM interneuron input[102]. This architecture is suggested to have similarly important functional implications in the motor cortex[103] as it does for the visual cortex[104,105], though few studies have breached this topic for frontal cortical areas[106].

The outputs of the motor cortex are largely separated into three classes which are shared across the cortex, being composed of pyramidal tract, intratelencephalic, and corticothalamic types in accordance with their long range targets[107]. Despite these broad classes, neurons within each category can exhibit substantial heterogeneity[108]. Of these, the pyramidal tract neurons, or corticospinal cells, are presumed to mediate the motor functions of the motor cortex. It was originally presumed that these cells histologically corresponded to gigantic deep layer neurons termed Betz cells; it was later revealed that Betz cells are in fact a small minority of all corticospinal cells[109]. Corticospinal cells, in addition to their size variance, also display a widely heterogeneous set of electrical properties[110,111]. However, they all share a projection to the spinal cord, where single axons can branch extensively throughout multiple spinal laminae[37,112]. These wide projections do appear to be organized by functional domains[113], and in monkeys are also more spatially restricted specifically for distal forelimb musculature both anatomically[114] and functionally[115]. Notably, corticospinal cells primarily target interneurons within the spinal cord[58], although

a minority do synapse directly on to motoneurons in the ventral laminae in primates[56]. Although the specific connectivity of cortical input in the spinal cord and spinal circuits themselves are still a matter of study[116], one exemplar target is the inhibitory propriospinal neurons[24] which are involved in dexterous movements[117]. The variety of corticospinal targets also allows for both facilitative and suppressive impacts on muscle activity[118], which can be coordinated by single spinal neurons across multiple spinal segments[119] to effect many combinations of muscles[120]. Importantly, however, the spinal cord is not the only target of corticospinal neurons, and axon collaterals can be found throughout the brain including the striatum, superior colliculus, and brainstem[121].

The remaining outputs of the motor cortex arise from corticothalamic neurons which reside in layer 6, and intratelencephalic neurons dispersed throughout layers 2 to 5, which in addition to mediating long-range connections within the cortex also strongly bilaterally connect with the striatum[122–124] As with thalamic input to the motor cortex, output from the motor cortex to the thalamus remains largely uncharacterized, although corticothalamic neurons are unusually isolated in their local connectivity[71] and appear to avoid those nuclei which project to the motor cortex[125]. Collectively, the connections of the motor cortex are elaborate and complex, yet there are patterns among the clutter which are progressively being attributed to function.

Function of the motor cortex through manipulation

The comparative features of the motor cortex across species strongly suggest that the motor cortex, at least as part of a larger system, supports motor

skill. Correlational evidence for this role can be found even within an animal through the relative size of the motor map. The amount of cortex which can elicit movements of a given body part is highly biased towards more skill-related muscles, which was famously illustrated by Wilder Penfield and Edwin Boldrey as the "motor homunculus"[126]. Their drawing of an unusually shaped man is meant to imply that a much larger amount of human motor cortex is devoted to the hands, for instance, than expected from their relative size on the body. This is also true of the rodent[34], emphasizing the universal importance of forelimb movements.

The role of the motor cortex in skilled movement is more causally supported by a century of manipulation studies, where disruption of the motor cortex produces motor deficits. Acute pharmacological or optogenetic inactivation of the motor cortex is highly effective in reducing motor aptitude[127,128]. It has been clear even from the earliest chronic lesion experiments, however, that the motor cortex is not necessary for the production of all movements[20]. In fact, even though complete motor cortex lesions induced temporary paralysis in primates, animals generally regained function as long as other motor areas, including premotor cortex, were still present[30,129]. Certain forms of movement, including walking, are spared even if the entire cortex is removed[130]. Instead, motor cortical lesions have more nuanced effects, which interestingly correlate with the motor skill of the species. Damage to corticospinal pathways in both primates and rodents produces a permanent deficit in the production of dexterous movements, particularly in the arm and fingers[130–133]. A

higher degree of impairment following motor cortical lesions is evident in primates compared to cats and rodents, and differences are even notable between apes and monkeys[134]. The relationship between motor skill and the impact of motor cortical lesions has been hypothesized to result from tradeoff between different descending tracts across evolution. A number of other structures project to the spinal cord, prominently including the red nucleus to form the rubrospinal tract and the reticular formation to form the reticulospinal tract, among others. These descending pathways are present in all mammals and are likely also involved in dexterous movements, but possess potentially less functional import in primates as compared to cats and rodents[135,136]. Since the presence of skilled movement predates mammals and therefore includes animals without a corticospinal tract, the control of complex movement may have shifted from these extrapyramidal tracts to the pyramidal tract[137]. The gradient of motor cortex involvement in driving movements across species therefore suggests that the circuitry within the cortex confers an advantage in the descending control of the spinal cord.

Any benefit of tying cortical circuitry to movement crucially depends on the method of control and functional output of motor cortex activity. Throughout the original reports of the motor cortex, a consensus did not emerge on how the motor cortex acted to move the body. Specifically, although a motor map could be inferred through electrical stimulation and later retrograde labeling, it was not the dominant view that specific points of the cortex existed explicitly and uniquely to drive contractions of specific muscles[29]. The motor map was

instead known to have very indistinct borders between elicited movements, and in fact the same site could yield different movements depending on which sites had been stimulated immediately prior[28]. The nature of elicited movements was also dependent on the type of stimulation being used, where Fritsch and Hitzig saw twitches with short stimulation while Ferrier observed coordinated movements with prolonged stimulation[26]. The output that the motor cortex actually controlled, either muscles or movements, was debated throughout the 20th century[138] and remains an active research question today[139].

It has long been clear that the motor cortex generally does not work through control of individual muscles, although at there might be more specific control of distal compared to proximal muscles[114]. This is even true of corticomotoneuronal cells, the most direct connections, which can have both inhibitory and excitatory effects on multiple muscles[118] . At the most basic level, the motor cortex is suggested to operate on muscle synergies, in which groups of muscles operate together as functional units. Evidence for synergies can be extracted merely by recording electromyographic (EMG) signals from muscles; the simultaneous activity from many muscles often exhibit less degrees of freedom than are possible, instead utilizing repeated patterns across muscles[140]. While the flexibility of these synergies and their role as a fundamental unit of motor cortex control is currently debated[141,142], spinal circuits which receive cortical input and coordinate multiple outputs from the spinal cord are known to exist[119].

The motor cortex could therefore potentially operate by controlling small groups of muscles in an impromptu fashion. This muscle-centric view of the motor cortex may have been largely embedded in the neuroscientist psyche from Penfield's "homunculus", which superficially suggested a point-to-point control scheme of the motor cortex[29]. Some evidence for this control scheme has also come from modern stimulation studies. Deep low-threshold microstimulation for mapping the output of a local pool of neurons has demonstrated fine-grained and columnar-organized sections of the motor cortex which are associated with specific muscles[23,143]. These output zones moreover can be functionally combined, where for example simultaneous microstimulation of multiple sites can yield a movement which is a linear combination of the movement elicited from each site individually[144]. This suggests the possibility that the motor cortex may execute behaviors by combining a limited set of outputs, defined perhaps through synergies, to produce a wide variety of movements as needed. One hypothesized feature of this puppet-like output is equilibrium-point control, where the motor cortex may direct specific combinatorial magnitudes of muscle activation to achieve a desired position, either with sensory correction (the lambda model) or without (the alpha model)[145]. A hallmark of this scheme is elicited movement being dependent on the current position of a limb; if a given cortical input induces a particular set of muscle activity, the same input will bring the arm down or up if the arm is initially higher or lower than the set point, respectively. This dependence of body position on stimulation outcome has been observed in primates with motor cortex stimulation[146,147] and has even

been shown in frog spinal cord stimulation[9]. It is unlikely that the motor cortex acts in a purely equilibrium-point fashion though, as it is neither supported by properties of natural movement[148] nor the impacts of corticospinal input on natural movement[149]. Instead, it appears to integrate and work in conjunction with input from the body[150–152].

An alternative level of control from the motor cortex is that of movements, not being concerned with the action of muscles independently but instead driving varying patterns of muscle activity within the context of discrete behaviors. The idea of a movement-centric motor cortex dates back to the earliest investigations of the motor cortex, famously being championed by Hughlings Jackson[153]. These ideas took further root in the movements elicited by prolonged stimulation originally observed by Ferrier, which have been recently resurrected. Most early reports of motor cortex stimulation relied on very brief periods of current. Longer stimulation requires proper balancing of current; direct stimulation from a battery can quickly damage tissue. Once these parameters are set, however, long-duration stimulation reveals much more complex movements than the simple twitches associated with traditional mapping studies. It was first shown in monkeys that not only could a wide variety of movements be driven by long-term stimulation, but that these movements were ethologically relevant to the behavior of the animal and included bringing the hand to the mouth, making defensive movements, and reaching to grasp different locations in space[154]. This work was then extended to rodents[155] and it was demonstrated that reversible inactivation of an area yielded an impairment

in natural use of the elicited behavior[156], implying a universal action of the motor cortex in driving discrete movements. Distinguishing between short twitches and long movements moreover allowed for a new approach to defining the motor hierarchy between the premotor and motor cortices. It was found that even though the premotor areas project to the spinal cord, they require an intact primary motor cortex to generate full complex movements during stimulation, suggesting circuitry within the primary motor cortex as critical for the output of such behaviors[157,158]. Complementary work also highlighted the importance of circuitry within the primary motor cortex towards eliciting complex movements, where the effects of stimulation were significantly reduced if synaptic activity within the motor cortex was blocked[159]. It is worth noting, however, that muscle activity driven by long-duration stimulation is different from that in natural movements[160], and that electrical stimulation as a technique is precise neither in the efficiency or location of activated neurons[161]. Regardless, the results collectively suggest that points within the motor cortex are not dedicated to specific muscles, but instead are organized into networks that drive specific and meaningful behaviors.

It can be strongly argued that the storied debate between muscle- and movement-centric views of the motor cortex in fact presents a highly oversimplified, potentially meaningless distinction[162]. It is clear from manipulation experiments that the motor cortex has many properties simultaneously: it is associated with both specific muscles and specific movements, it can operate on few muscles or many muscles together, it is roughly organized

somatotopically but it is also highly fractionated and graded. It is likewise clear that however the motor cortex controls the body, it does so towards the end goal of driving certain types of dexterous, skilled movements. These types of manipulation experiments rely on disrupting the motor cortex to infer function through altered states, forgoing examination of what the motor cortex does during normal movement. A separate and complementary approach to studying the motor cortex is to monitor to the activity of neurons during voluntary and natural behavior. This approach has expanded the scope of experimental inquiry and ignited a quest for the code between cortical activity and movement.

Neuronal activity and coding of movement within the motor cortex

The ability to record the electrical activity of neurons in intact and behaving animals ushered in a revolution for neuroscience. The earliest use of passive electrical recording of the brain generally accompanied the earliest use of electrical stimulation by Fritsch and Hitzig, although with more distributed credit[22]. Electrical recording of the nervous system, under the general field of electrophysiology, then exploded in three domains: the study of electrical properties within single neurons, the study of neuronal activity via action potentials in intact animals, and the study of large-scale electrical fluctuations of broad regions. The middle of these three levels, examining the spiking activity of neurons *in vivo*, initially required anesthesia or immobilization to achieve stable recordings. This type of recording remarkably allowed for eavesdropping on individual neurons evidenced by a consistent shape of voltage deflections,

cautiously termed single-unit recordings as opposed to the broadband multi-unit activity of many neurons. This work produced prodigious results even under the given constraints, famously including the characterization of visually-responsive neurons in cats[163]. Single-unit recording was then quickly adapted to awake and mobile cats[164], which was subsequently carried forth by Edward Evarts to study neuronal activity within the motor cortex of actively behaving monkeys during both spontaneous[165] and conditioned[166] movements. Evarts' early work even pressed beyond arbitrary neuron recording by identifying pyramidal tract neurons through antidromic responses to stimulation within the medullary pyramid. These initial studies laid the foundation for decades of motor cortex research centered on relating the activity of single neurons within the motor cortex to trained movements. Furthermore, they originally established that activity in the motor cortex began before movement, evidencing a causal relationship between the motor cortex and behavior, though even the activity of one class of neuron could be heterogeneous from cell to cell.

The primary strategy to deciphering motor cortex activity focused on defining relevant properties of movement that appeared to be represented by the motor cortex. In this regard, it was not assumed that activity in the motor cortex was mirrored by spinal cord and muscle activity, which has since been experimentally confirmed[167]. This yielded a class of experiments in which monkeys were trained to perform a selection of arm and hand movements that varied independently in multiple dimensions. The first of these, carried out by Evarts, drew the distinction between displacement and force. The motivation for

this work was to determine the aspects of a movement that the motor cortex controlled. If signals sent from the motor cortex to the spinal cord were associated with the displacement of the arm, then it would appear that descending commands were targeted to positional configurations of muscles without regard to their ongoing engagement. Conversely, if motor cortex output was related to force irrespective of displacement, then it would indicate that the motor cortex directed the level of muscle contraction irrespective of how the limb would subsequently move. These two possibilities represented a fundamental dichotomy for motor control which continues to be a matter of investigation. Evarts' approach was to dissociate these two features by requiring monkeys to move a lever while cleverly adding stable weights which pulled the arm towards the left or right. This effectively uncoupled the direction of movement from the force required to move the arm; if the monkey wanted to move left and was being pulled to the left, it would simply have to release tension and allow the weight to do the work, whereas if the monkey wanted to move left but was being pulled to the right, if would have to increase tension to overcome the weight. What Evarts found in this first study was twofold: pyramidal tract neurons were largely related to force over displacement, and specifically those neurons were often related to changes in force as opposed to simply being more active with more force[168]. Moreover, motor cortex activity was more concentrated with movement onset than offset. This principle was later confirmed and contrasted with activity in the cerebellum, which oppositely was more active around movement offset[169]. However, the same work also disputed

the ratio of force- and direction-selective neurons, instead finding an incredibly diverse relationship between activity and movement relating to every parameter measured. This early work suggested that the motor cortex is involved with issuing orders for the force exerted by muscles, but simultaneously awoke a sleeping giant by showing that this trend was far from absolute.

A defining experiment was conducted in the following years that would influence dogma on motor cortical activity for decades. In an effort to conduct a more comprehensive examination of the effect of movement direction on activity, monkeys were trained to reach in eight directions evenly spaced across a circle. It was found that neurons of the motor cortex exhibited bell-shaped tuning curves to these directions akin to tuning in visual neurons[163], where a given cell exhibited the most activity at a single preferred direction and progressively decreasing activity for adjacent directions[170]. This demonstrated that movement was represented in a graded way across neuronal activity; specific movement directions were not driven by specific subsets of neurons, but instead were associated with particular combinations of neuronal activity. These combinations were proposed to contribute a "population code" of movement, with every individual neuron being loosely related to movement but all neurons together providing a unique activity profile for every movement. This construct was formally demonstrated as movement being predictable by a "population vector" of motor cortex activity, which even extended to a three-dimensional workspace for movement[171]. Presciently, it was noted in this work that while activity could predict muscle activity, the reverse was not necessarily true;

muscles were observed to be active during behavior without accompanying activity in corresponding motor cortical neurons.

In the ensuing years, it was debated whether population vector coding represented some abstract, limb-independent scheme, or whether it ultimately related to the engagement of muscles[172]. A number of experiments provided evidence for directional tuning being heavily influenced by limb position and kinematics, suggesting that at least extrinsic direction itself was not a consistently represented parameter in the motor cortex. For example, the population vector accurately coded direction within a given three-dimensional space, but it was not consistent across spaces when the arm was shifted to the left or right in both primary motor[173] and premotor[174] cortex. Even within a given space, changing the orientation of the arm produced significantly modified activity[175]. Furthermore, when the kinematics of the arm were rigidly controlled and measured, it was found that the preferred directions and resulting population vector of motor cortex neurons were actually highly skewed instead of symmetric across the range of movement[176]. This skew was hypothesized to originate from the mechanical properties and arrangement of muscles within the arm; some directions of movement required different levels of muscle activity than others[177]. It was also found that activity during movement was not temporally constant but exhibited different epochs of phasic and tonic activity, related to, although not necessarily directly, reflecting the acceleration and deceleration of the arm[178]. Together, these results signified that activity in the motor cortex did not primarily

issue abstracted commands and instead was very much tied to the physical components which it controlled.

Concurrent studies suggested, however, that activity was not merely related to direct and consistent modulation of muscles[179]. When a multidirectional reaching task was coupled with opposing loads for example, as in Evarts' original work, neuronal activity reflected a complex mixture of force and direction[180]. Attempts were also made to reduce motor commands to their basic levels and eliminate the kinematics of movement by utilizing isometric tasks, where monkeys were trained to apply force to an immovable lever. One such study involved monkeys moving a cursor on a computer screen using an isometric lever, but there was an added implied "bias force" where the cursor would drift on its own in a given direction if left unattended. Astoundingly, neurons in the motor cortex were often decoupled from the force provided by the monkeys, and instead were related to the net of the arm and bias forces, or to the changes in force across time[181]. However, as with reaching movements, motor cortical activity accompanying isometric force was influenced by limb position in addition to direction, underscoring the importance of the physical effector[182]. It was further demonstrated that when directional reaching was examined independently from muscles involved in the movement, both direction-specific and muscle-specific neurons were observed, providing evidence for mixture of extrinsic and intrinsic features in the motor cortex[183]. A common conclusion from this slew of studies was that there was no single movement parameter that the motor cortex controlled, and in fact, multiple

encoding schemes likely existed simultaneously[184]. Even if the motor cortex did exert control over certain parameters of movement, it was clear that the association between movements and activity was not consistent and highly context dependent. In this case, activity of a neuron may be related to a particular muscle, but the neuron could be active without corresponding muscle activity[185] and the muscle could be active without corresponding neuronal activity[186,187].

One notable offshoot of finding overlapping movement features in the motor cortex was the theorized computation of sensorimotor transformation. Primates are highly visually-oriented animals and often move according to visual cues. Reaching the hand to a desired spot would therefore require a conversion from coordinates in visual space to appropriate muscle activity, which is an inherently arbitrary mapping and likely requires specialized neural circuits. In fact, it was suggested that a major evolutionary drive for the expansion of motor regions in primates was for the purpose of coupling vision to movement[48]. One suggested aspect of this sensorimotor transformation was the presence of preparatory activity. This activity was apparent when cue and movement stimuli were uncoupled in time; if a monkey was instructed on the direction of arm movement but was awaiting instruction to actually move, substantial motor cortex activity was observed in the absence of any movement[188]. Interestingly, this preparatory activity was not predictive of activity during the actual movement, precluding the explicit use of a gating mechanism to shunt activity downstream[189,190]. One possibility was that activity related to extrinsic features of

space was being transfigured into activity relating to intrinsic features of movement. Studies examining this proposal often relied on direction and movement decoupling through visuomotor rotation, where the movement of a monkey's arm caused the movement of a cursor on a screen but with a systematically rotated configuration. It was found that the direction expressed through the population vector rotated from extrinsic to intrinsic coordinates during this type of task[191], although it was later remarked that this process might occur across separate neurons, instead of within single neurons[192]. Moreover, it was found that a higher amount of preparatory and extrinsic-related activity was found in premotor areas as compared to the primary motor cortex, alluding to a potential progression of sensory- to motor-related activity[193,194].

Despite clever experimental designs, many potential trends of organization, and a river of literature debating results, decades of attempts to classify activity primarily exposed the chaotic and obscure nature of the motor cortex. Motor cortex activity was found to relate to almost every movement parameter studied, presenting a thoroughly uninterpretable view of function. More recently, however, the complexity of activity has begun to be embraced and freed from the restraints of parameterization. When activity was related to movement in a more unbiased fashion, it was found that single neurons actually related to complex trajectories in space, which are consistent across limb positions[195]. It was suggested from this work that motor cortex activity does not direct single components of movement, but instead contains the driving apparatus for small fragments of movement which can combine to produce the

natural repertoire. An even more recent series of studies has proposed what would amount to a complete paradigm shift in the way motor cortex activity is understood. This line of research began by quantifying the temporal complexity of neurons within a movement, in which single neurons displayed highly varying activity across time that was not directly coupled with movement parameters[196]. It was then noted that even though preparatory activity did not resemble activity during movement, the two epochs of activity did exhibit a consistent relationship within individual neurons[197]. Finally these components were coupled to suggest that the incredible diversity of activity observed in the motor cortex could be reduced to a small number of temporally complementary patterns[198]. The implications for these results suggested a number of potentially important features of motor cortex activity. First, activity in the motor cortex could be diversely coupled to movement but reliably coupled to itself; many types of activity could produce movement, but the relationships between the activity of individual neurons would be maintained. Second, motor cortical activity could be very temporally complex yet predictable; activity at one point could be predicted from immediately prior activity. Finally, this temporally complex activity could be capable of producing observed muscle activity in a consistent and experimentally validated manner. Theoretical work following these initial reports suggested that this control scheme may be a natural result of neural networks controlling movement under restraints of parsimony[199], and that the observed temporally complex activity may be an inherent feature of neural networks given proper inhibitory circuitry[200]. These new approaches to deciphering motor cortex

activity are in their relative infancy, but they provide a solid foundation for a completely novel interpretation of the motor cortex.

A conspicuous aspect of research on motor cortex activity is the favor of primates, specifically macaque monkeys, as the model organism. Motor control has been studied in various other animals, including frogs, birds, and cats, but monkeys offer a highly-trainable skill set in addition to being the most similar to humans. The recent rise of genetic tools, flexibility of use, and growing recognition of behavioral abilities of rodents, however, has led to a burgeoning field of motor control research in both rats and mice. The entry of rodents into the fray of motor cortex activity research was initiated by Jan Bureš not long after the technique had been initiated in cats and monkeys. Bureš used a method of training in which hungry rats had to reach through a narrow opening to retrieve a food pellet, which resulted in a highly stereotyped movement that had previously been demonstrated to rely on an intact motor cortex[201]. It was found that activity in the motor cortex of rats, like that of monkeys, began slightly before the initiation of movement and was active in a largely phasic manner that corresponded with reach onset[202]. It was further established, again as in monkeys, that motor cortex activity in rats contained preparatory activity between cue and movement signals[203] and was more specifically related to the phasic rather than tonic features of movement[204]. Following these initial reports, there was a dearth of research in rodent motor cortical activity towards movement encoding. One insightful study in the next decade highlighted the important feature of activity timing, emphasizing that although activity in the rat

motor cortex began before movement, much of the activity actually occurred after reach onset, implicating control over ongoing features of movement[205] . In the ensuing years as the technical toolbox began to grow, rodents began to take on a more prominent role in the field, with researchers taking full advantage of their experimental strengths. The relationship between activity to laminar depth, transmitter type, and morphology were examined in rats, a feat that would be otherwise impractical in primates[206]. The specialized rodent motor system of whisking was studied and linked to sensorimotor localization of objects[207]. The newly developed technique of two-photon imaging, then limited to rodents, demonstrated that different neurons were involved in different behaviors, yet were clustered in the brain[208]. Optogenetic tools for neuronal identification were used to show that control of directional licking was accompanied by a shift from a bilateral to a contralateral-specific activity pattern[209]. Where rodents really delivered impressive and complementary results to monkeys, however, was in the field of plasticity[210]. Alongside all research of the motor cortex, every manipulation study, every activity recording study, lurked the omnipresent capacity for change. Far from being a wrench in attempts to understand how the motor cortex operated, the mutable nature of the motor cortex offered fundamental insights into its role in movement.

Plasticity of the motor cortex and motor skill learning

Reorganization of the brain to support motor functions has been documented since the earliest studies of the motor cortex. Specifically, it was noted that while primary motor cortex lesions resulted in paralysis in monkeys, this

paralysis was only temporary, but recovery required an intact premotor cortex[30]. This initial result suggested that the function of the primary motor cortex could be shifted across the brain, though this shift was limited to other motor areas. The coopting of motor function by premotor areas after primary motor cortex lesions has since been confirmed in both primates[129] and rodents[155,211], and it has additionally been demonstrated that functional recovery is aided by rehabilitative training[212]. Importantly, however, this plasticity is not restricted to the damaged brain. Instead, it is a normal but latent process which is thought to underlie the capacity for motor learning[213].

Motor learning is a broad term that encompasses any process of developing, refining, or adjusting movement. While many regions of the brain are undoubtedly involved in motor learning, prominently including the basal ganglia and cerebellum, each of these areas are thought to contribute in different domains, with the motor cortex being implicated in the learning of novel motor skills[16]. The development of motor skills occurs when animals are driven by their environment to create new movements, and learning of this sort is generally implemented in an initial fast improvement phase followed by a slow optimization phase[214]. One hallmark of motor learning is stereotypy, in which movements directed towards a task are highly variable early in learning but become increasingly similar over time. Variability may be an intrinsic feature of the motor system[215,216], but the ability to explore movement space is suggested to be an active process which paves the way for a trial-and-error generation of new movements[217,218]. The primary motor cortex has been shown to be crucial for

the process of consolidating novel movements into effective motor memories, where animals become reliably able to execute movements that were successful towards some task. Temporarily disrupting motor cortical activity after practice, for example, deteriorates the ability to reproduce successful movements later. Lesioning the motor cortex before training similarly abolishes the ability to improve performance, but surprisingly lesions may not affect stereotypy after the movement has already been learned[219]. It has been proposed that generation of motor memories is due to changes within the motor cortex, as opposed to the motor cortex merely being a waystation for changes occurring in other areas. Recently, powerful causal evidence for this principle has emerged, in that selectively eliminating new connections within the motor cortex effectively erases improvements acquired from recent training[220]. Characterizing changes within the motor cortex during learning therefore represents a vital factor in determining the function of the motor cortex. Plasticity of the motor cortex has consequently been a subject of much investigation across all scales, including motor maps, neuronal activity, and the structure and connectivity of neurons[221].

Procedures for using electrical microstimulation to chart a map of movements in the motor cortex had been in use for a century, though it was often assumed that such maps were a fixed property[222]. When applied to a damaged system, however, these techniques provided the first demonstrations of large-scale reorganization. Damage in this case could originate either from the periphery[223] or within the cortex itself[212], but in both cases it was paired with a

reconfiguration of the stimulated motor map. Brain damage itself was also not explicitly necessary; limb amputation[224] or even simple restraint of a limb was also shown to modify the motor map on an impressively rapid time scale of minutes to hours[225]. Such reorganization also appeared to be the basis for recovered function[211]. The first demonstration that learning could alter the motor cortex map was then carried out in squirrel monkeys[226]. When monkeys were trained to execute skilled retrieval of a food pellet, it was found that the amount of cortex capable of driving task-relevant muscles expanded into territory previously associated with extraneous movements. Moreover, the site of plasticity depended on the particular movement being learned; skilled grasping expanded the finger map, while skilled reaching expanded the forearm map. These expansions not only consisted of new territory which could drive a muscle, but it was found that groups of muscles that were engaged in a task began to be co-activated by given cortical sites. Finally, it was noted that if monkeys were removed from training for many months and then participated in the task, their skill level and corresponding motor map had reverted to pre-training condition. This whirlwind first characterization of learning-related changes in the motor cortex strongly suggested that the motor cortex undergoes active reorganization during learning, in particular by creating new networks to drive newly learned movements.

Changes in motor map topography during learning were then generalized to other animals. Rats were shown to exhibit similar task-specific map expansions as in monkeys, although interestingly these fluctuations were limited

to the caudal "primary" forelimb motor cortex and were absent from the rostral "secondary" forelimb motor cortex[227]. The recent development of functional magnetic resonance imaging (fMRI) had also previously indicated motor cortex plasticity in humans during learning[228], which suggested that the area of active neurons during movement and not just the electrically-induced map actually changed during learning [229]. Interestingly, however, an increased map representation does not appear to be ultimately necessary for executing learned movements. It can be noted, for example, that somatosensory representations of the hands of string instrument musicians can be asymmetric with regard to the more skilled hand[230]. In the motor cortex, however, the relationship between topographic range of activity and skill level are actually inversely related, where more skilled players demonstrate more consolidated regions of task engagement[231]. Despite a potential lack of an expanded movement representation, when the motor cortex of string players is stimulated, they appear to produce movements more akin to instrumental skills than non-musicians, implicating the existence of skill-specific circuitry within the motor cortex[232]. These results suggest that motor map plasticity itself does not represent the storage of motor memories. More direct evidence for this principle has recently been collected which suggests that changes in motor maps are a more epiphenomenal result of underlying processes of plasticity. When rats were trained in a reaching task and implanted with a chronic array to allow for repeated stimulation mapping across days, the expansion of motor maps was shown to be transient even though the motor skill persists[233]. However, the initial

extent of expansion was correlated with performance within each animal, suggesting that motor map plasticity does reflect some aspect of the learning process. A similar function of map expansion has also been suggested in the auditory cortex, where perceptual learning of tone discrimination is facilitated by but not reliant on changes in maps of auditory parameters on the cortex[234]. Instead, it has been proposed that map expansion represents an initial explorative process to allow for the formation of new connections, which is followed by a stabilization of the newly constructed circuit and closing of a window for plasticity. Supporting evidence for such restructuring was found in humans, where learned movements were more accurately decoded from fMRI signals in humans and implied a more permanent albeit local novel organization of function[235]. Furthermore, monkeys show a decrease in motor cortical metabolic activity during skilled movement following long-term training, suggesting that movement execution increased in efficiency across learning[236]. This would not be expected if more neuronal activity was associated with learned movements, instead signifying that volume of associated motor cortex is not the pertinent manifestation of motor learning.

All things considered, the motor map is an artificially induced construct which may or may not accurately portray functions of the motor cortex. The meaningful output of the motor cortex instead stems from the activity of its neurons; action potentials are the currency of information that is ultimately translated into the muscle contractions. Any plasticity underlying motor learning must therefore be expressed at some level in the spiking pattern of individual

neurons. Attempts to investigate this essential aspect of motor learning have been fraught with technical challenges, as it requires monitoring neuronal activity during the process of learning. Despite this, activity specifically within the motor cortex had been suggested to reflect motor skills from the earliest studies by Evarts. When monkeys were trained to reposition their hand to a particular spot following a perturbing force, a substantial amount of motor cortical activity was associated with the resulting movement, suggesting that such corrective movements were generated by the motor cortex and not elsewhere like the spinal cord[152]. Recently, a much more convincing demonstration was made towards this point, where skilled movements were shown to be associated with specialized activity. In this experiment, monkeys were trained in a sequential reaching task, but also engaged in a cued movement task with the same motor requirements but in arbitrary order. Motor cortical activity was considerably different between the two types of tasks even though they the movement kinematics were the same, suggesting that the learned sequence task utilized cortical circuitry in a different manner than the cued task[237].

More direct investigations of the effects of motor learning on motor cortex activity have been possible within the last decade, which have been fruitful in capturing ongoing changes. Using rodents in particular, subjects can be trained in tasks on a scale of days to weeks while simultaneously monitoring activity changes. Moreover, these changes can be observed both within single neurons and at the population level. One approach towards this end has been to examine changes of activity within a day as it pertains to learning. It was found

that when rats were trained in a reaching task, they exhibited a selection and tuning of relevant muscle activity. Activity in the motor cortex was then related to groups of coactive muscles, which were proposed to be synergies, and the change in synergy use across learning was directly reflected in the activity of associated neurons[238]. This within-day sculpting of activity and corresponding movement provided an initial window into the process of flexible motor control early in skill learning. It was subsequently shown that during the late stages of learning, there was also a change in the quality of activity. Specifically, it was found that the variance of activity within single neurons decreased with learning which was coupled with a decrease in the variance of muscle activity[239]. Concurrent work conversely proposed that there was no relationship between activity of single neurons and movement variability, although suggested that this relationship instead emerged when considering a large population of neurons[240]. Importantly, however, not all changes in activity were directly attributable to changes in movements. It was also noted for example that the number of neurons which were correlated with muscle activity increased during learning[239]. This finding was very shortly replicated and expanded in an experiment utilizing chronic electrophysiological recording in rats. Instead of reaching, rats in this case were trained to run on an increasingly quickly spinning rod, termed the accelerating rotarod. The accelerating rotarod is a paradigm that is well grounded in studies assessing motor deficits[241] and involves learning on both a short and long time scale[242]. It was found that learning to run on the rotarod was accompanied by two main changes in the activity of the motor cortex: first, the

number of neurons exhibiting task-related activity substantially increased early in learning, and second, the variability of activity on a trial-by-trial basis decreased late in learning[243]. Notably, it was also observed that even though the number of neurons activated during early learning increased, the total amount of activity within the motor cortex did not change. Together, this work provided intriguing clues towards understanding how activity in the motor cortex related to learning. Namely, it hinted at a process of initial exploration and subsequent refinement of motor cortex activity to drive learned movements, reminiscent of what had been observed during motor map plasticity.

Capturing changes in activity during learning in primates, in contrast to rodents, is often difficult due to the length of time required for behavioral training and the impracticality of such longitudinal electrophysiological recordings. A very interesting line of research has instead emerged from an alternate route, examining how animals are able to control motor cortex activity independently from how that activity acts towards movement. This broad field, alternately known as brain-computer interfacing, brain-machine interfacing, or neuroprosthetics, investigates the extent to which single neurons or populations of cells can be volitionally controlled by the subject[244,245]. A significant practical application towards controlling and reading activity directly is that of allowing paralyzed patients or amputees to interact with the world without the requirement of muscles. On a more basic scientific level, however, these studies allow for a deeper understanding of the extent to which motor cortical activity is tied to movement. Experiments exploring this domain began as soon as

electrophysiological recordings in behaving monkeys were first developed, demonstrating that monkeys could learn to increase the activity of a targeted neuron, potentially by executing a correlated behavior[185]. It has since been shown that monkeys can in fact learn to control the activity of populations of neurons in a stable manner, not unlike learning novel movements[246]. A number of important principles have emerged from this work. First, motor cortical activity can be decoupled from movement, where animals become able to modulate activity to control an external source while not exhibiting any movement themselves[247]. Structured activity in the absence of movement has also been shown to occur normally during movement preparation, and this has been formalized into a potential space of activity which collectively negates downstream movement[190]. Second, it is not just the targeted neurons which change their activity, but the activity of surrounding neurons as well, implicating network-wide interdependence instead of the volitional control over single neurons[248]. Third, the patterns of activity used for controlling brain-machine interfaces are most successful when they are based on naturally occurring patterns of activity, suggesting that the correlational structure of activity across neurons is a property of the network and not a function of many independently active neurons[249,250]. Finally, the ability to volitionally control motor cortex activity is dependent on plasticity within other structures, prominently including the striatum[251,252]. The combination of studying activity during learned movement and learned activity in the absence of movement has yielded a view of the motor cortex that is flexible in function yet with defined features of plasticity.

A critical restriction on research of motor cortex activity during motor learning has been the inability to record changes in individual cells over the entire course of learning. Additionally, the activity across cells is markedly heterogeneous, and it has not been well characterized how population activity relates to subsequent learning-induced changes[253]. Comprehensively investigating plasticity in motor cortex activity therefore requires recording the activity of large populations of neurons simultaneously. Both of these technical challenges have recently been addressed through the use of two-photon calcium imaging. In this method, neurons are made to contain indicators that fluoresce upon binding to calcium, which enters through voltage-gated calcium channels during action potentials[254]. The fluorescence of neurons is then imaged through a window in the skull, allowing for visualization of hundreds of neurons. The first application of this technique in the motor cortex during learning demonstrated that the correlation structure of neuronal populations changes over time, where neurons that exhibit similar patterns of task-related activity become more correlated with each other[255]. This initial work utilized an acutely injected calcium indicator, precluding collecting data from the same neurons over days. Subsequent studies, however, have utilized the genetically encoded calcium indicator GCaMP[256–258], where the same large population of neurons could be imaged every day across learning. This work has shown that while individual neurons can change their activity during learning, they are unlikely to change the type of movement which they are associated with[259]. Nevertheless, given neurons can alter their relationship to movement in a lamina-dependent

manner, where the same population of neurons can better predict movement over learning in layer 5 but not in layer 2/3[260]. These types of experiments open the doors for tracking changes in motor cortex neurons throughout learning, thereby providing an unprecedented opportunity for understanding the complex and changing interaction between activity and movement.

The mechanisms that serve motor cortex plasticity lie in the structure and connectivity of neurons. When it was initially discovered that the map of the motor cortex was susceptible to reorganization, the search began for the substrates of such change, primarily using rodents as the experimental animals. The first of these experiments involved charting a map of movements across the cortex and then repeating those measurements after local injection of the inhibitory antagonist bicuculline. It was found that upon suppressing inhibition at a given site, movements relating to adjacent sites were suddenly also able to be driven by stimulation[261]. These associations were manipulated too acutely to represent any changing of connectivity. Indeed, it was shown that long-range connections were rife within the motor cortex, therefore implying that these connections were always present but functionally shunted by local inhibition[100]. This pioneering result uncovered a potential dormant network of neurites ready to be engaged as necessary after damage or learning. The connection between horizontal connections and motor map plasticity was more firmly drawn later in cats, where it was shown that map reorganization following peripheral nerve cuts was confined to connected areas[262]. In this case, if the nerves controlling the whiskers were cut, the forelimb representation expanded

into what was previously vibrissa territory. This expansion, however, was only found to extend to areas of vibrissa motor cortex that contained lateral connections from the forelimb motor cortex. It has since been suggested that these horizontal connections do not necessarily coopt the function of a given site in cortex when they are utilized, but instead can exist in parallel towards the creation of multifunctional sites[155].

The existence of latent connections, while supporting rapid reorganization, did not represent the full potential for motor cortex plasticity. Techniques had previously been developed for probing plasticity by experimentally manipulating connection strength between neurons[263], which proved highly effective in the motor cortex. These methods involved stimulation of an input pathway while recording the electrical activity within a neuron, where different stimulation parameters could evoke an increase or decrease in connection strength, respectively termed long-term potentiation (LTP) or depression (LTD). The horizontal connections across the motor cortex were surveyed using this approach, and they were found to be capable of significant change instead of representing latent but fixed networks. Specifically, LTP could be induced from intralaminar layer 2/3 projections, but remarkably required the application of the inhibitory antagonist bicuculline[264]. The role of inhibition in LTP therefore paralleled its masking properties in existing connections of the cortex, effectively restricting communication across the motor cortex. This work was then extended to include interlaminar interactions, where it was found that stimulation of superficial layers could similarly induce LTP in deep layers[265] and vice versa[266].

Stimulation in the interlaminar pathways also was found circumvent the requirement to suppress inhibition, where intralaminar layer 2/3 LTP could be successfully induced with simultaneous deep layer stimulation[267]. Conversely, horizontal layer 2/3 connections were also capable of LTD, though without the added requirement of inhibition suppression[268]. All of these mechanisms were thought to support motor learning, although direct evidence had not been presented. Consequently, horizontal connections across layer 2/3 were shown to be increased in strength after motor learning, and these changes were specific to the hemisphere associated with the trained forelimb[269]. This work proposed that not only did the capacity for connectivity changes exist within the motor cortex, but this plasticity provided a mechanistic basis for motor learning. It should be noted that these results were disputed[270]; the absence of large-scale connectivity changes would not necessarily preclude the importance of connective plasticity in the motor cortex during learning.

Inputs from other areas to the motor cortex were also shown to be plastic. It was found that LTP could be induced in the motor cortex following stimulation of the somatosensory cortex, demonstrating that connections from other cortical regions into the motor cortex were malleable[271]. Another prominent source of input was from the ventrolateral thalamus, which had been suggested to present another adjustable pathway[272]. Interestingly, it was found that while stimulation of the somatosensory cortex could induce LTP in the motor cortex, stimulation of the ventrolateral thalamus alone could not. If these two pathways were stimulated together, however, the combined effect successfully potentiated the

thalamic input to the motor cortex[273]. Moreover, this "associative" LTP was restricted to layer 2/3 which received input from both structures, and did not occur in layer 5 neurons which were presumed to receive thalamic input but not somatosensory cortex input[274]. The requirement of facilitating factors to induce LTP both within the motor cortex and for motor cortex input therefore suggested restrictive factors for plasticity that may prevent aberrant changes but allow for controlled and beneficial changes in connection strength. These factors moreover were not limited to glutamatergic inputs, but also were found to include neuromodulators like dopamine[211,275] and acetylcholine[83,276]. It has also recently been demonstrated that dynamics of inhibitory connectivity may be a critical facilitating factor of synapse formation and subsequent motor learning[277].

Connections from the output neurons of the motor cortex to downstream structures were also shown be pliable. Significantly, LTP could be induced between corticospinal neurons and interneurons within the spinal cord[278]. Such LTP was also localized to the corticospinal-spinal cord pathway; connections between corticospinal cells and other neurons in the cortex remained stable. This implied that it was not just cortical circuits which could be shaped by learning but also the final output connection, hinting that the fundamental relationship between motor cortex activity and movement may be altered during learning. The possible functional impact of modifying corticospinal connections with the spinal cord was recently demonstrated in monkeys by artificially pairing activity between areas. A recurrent system was set up where activity was recorded in a corticospinal cell while a stimulating electrode was placed in the spinal cord.

Every time the targeted corticospinal cell spiked, the stimulating electrode would deliver an electrical pulse to the spinal cord. Conditioning in this way led to a substantial change in the relationship between activity in that cell and corresponding muscle activity, indicating that connection strength within the spinal cord had been altered[279]. The axon fields of corticospinal cells within the spinal cord are known to expand after injury[280] and the number of corticospinal synapses in the spinal cord can change following strength training[281], suggesting that plasticity of the corticospinal connection may also be an important target of motor learning.

Plasticity of motor cortex connections involves significant structural changes. It has been shown that motor learning is dependent on local protein synthesis, implicating restructuring within neurons[282]. Part of the contribution to motor learning likely involves altering existing connections, which is evidenced by changes in spine with during motor learning[283]. This restructuring also significantly includes the formation of new synapses as observed by ultrastructure of the motor cortex following motor learning[284]. These new synapses are also specific to the area of the motor cortex involved with the trained movement[285]. It has been further suggested that these new synapses support motor map expansion, as their presence precedes map reorganization but only occur relatively late in learning[286]. More direct evidence for synaptic changes within the motor cortex has recently been made possible by two-photon imaging. When fluorescent proteins are expressed in a sparse subset of neurons, their full architecture can be imaged through a cranial window over the course of days to weeks. By

focusing on the dendrites of these neurons, it is possible to then track the growth and elimination of dendritic spines, which are primarily sites of excitatory synapses. Using this technique, it was shown that motor learning induces substantial dynamics of dendritic spines of layer 5 neurons in the motor cortex[287,288]. Early in learning, a number of new spines grew which was remarkably proportional to the amount of practice each mouse engaged in. Following this initial growth period, there was an elimination of preexisting spines, such that the total number of spines returned to pre-training levels but with the preferred retention of newly formed spines. These dynamics in spines were further demonstrated to be task specific; retraining in the same task later did not have any effect, but training the animals in a second skilled motor task induced the process again but for a separate set of spines. These spines were later shown to be clustered, where it was suggested that spines on the same dendrite may be participating in the same circuit[289]. Furthermore, the role of sleep in retaining these new spines was investigated, where sleep deprivation impaired the formation and stabilization of learning-related spines[290]. Spine stabilization was also shown to rely on altered inhibitory connections induced by learning, such that spine formation in apical dendrites was associated with a reduction in apical dendrite inhibition by somatostatin-positive interneurons[277]. The causal role of newly formed spines in the process of motor learning has recently been evidenced, implicating that these synapses are a critical site of motor memories[220]. In addition to changes in spines on dendrites, the dendritic fields themselves can also change during learning. This has been observed as an

elaboration of both apical and basal dendrites in task-related corticospinal neurons[291], but like spine formation appears to be a transient increase[292]. These synaptic changes implicate the formation of new circuits within the motor cortex to support motor learning.

Examining plasticity in the motor cortex across motor learning

The motor cortex has been a subject of intense investigation for over a century. Substantial progress has been made in determining the role of the motor cortex in movement, the relationship between activity in the motor cortex and movement, and plasticity of the motor cortex during learning. All three of these domains remain active areas of research and contain fundamental unanswered questions. The aim of the work presented in this dissertation is to contribute to an understanding of the motor cortex in each of these respects by building on the rich history of previous studies using modern methodology. The advent of two-photon calcium imaging in behaving animals allows for the activity of large populations of neurons to be tracked throughout learning. Specific populations can also be targeted or identified using various genetic and viral strategies. This powerful technique provides a perspective on motor learning which has not been previously feasible. The following experiments utilize two-photon calcium imaging in mice during a motor learning task while subdividing neuronal populations into superficial excitatory cells, superficial inhibitory cells, and corticospinal cells. The focus is to examine plasticity of individual motor cortex neurons across learning, how they are contextualized within a neuronal population, and how those changes relate to motor control.

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Chapter 2. Emergence of reproducible spatiotemporal activity during motor learning

The motor cortex is capable of reliably driving complex movements[1,2] yet exhibits considerable plasticity during motor learning[3-10]. These observations suggest that the fundamental relationship between motor cortex activity and movement may not be fixed but is instead shaped by learning. However, to what extent and how motor learning shapes this relationship is not fully understood. Here we addressed this issue by using *in vivo* two-photon calcium imaging[11] to monitor the activity of the same population of hundreds of layer 2/3 neurons while mice learned a forelimb lever-press task over two weeks, while identifying excitatory and inhibitory neurons by transgenic labeling[12,13]. Inhibitory neuron activity was relatively stable and balanced local excitatory neuron activity on a movement-by-movement basis, while excitatory neuron activity showed higher dynamism during the initial phase of learning. The dynamics of excitatory neurons during the initial phase involved the expansion of the movement-related population which explored various activity patterns even during similar movements. This was followed by a refinement into a smaller population exhibiting reproducible spatiotemporal sequences of activity. This pattern of activity associated with the learned movement was unique to expert animals and not observed during similar movements made in the naive phase, and the relationship between neuronal activity and individual movements became more consistent with learning. These changes in population activity coincided with a transient increase in dendritic spine turnover in these neurons.

Our results indicate that a novel and reproducible activity-movement relationship develops as a result of motor learning, and we speculate that synaptic plasticity within the motor cortex underlies the emergence of reproducible spatiotemporal activity patterns for learned movements. These results underscore the profound influence of learning on the way by which the cortex produces movements.

We developed a cued lever-press task performed by mice under a microscope (Fig. 2.1a), similar to other recently reported tasks[14,15]. Briefly, a lever press beyond the set threshold during an auditory cue was rewarded with water (Methods). Mice were trained with this task daily for two weeks (n = 10). Even though mice achieved a reward in most trials, the timing of their behavior improved in later sessions (Extended Data Fig. 2.1). Furthermore, lever movements on individual trials became more stereotyped over time (Fig. 2.1b). The reproducibility of movements was evident in higher correlation of rewarded movements on individual trials within and across later sessions (Fig. 2.1c). The motor cortex is necessary for this task, as lesions prior to training prevented the emergence of movement stereotypy, and acute inactivation by pharmacology or optogenetics impaired task performance (Extended Data Fig. 2.2).

To identify how the activity of motor cortex neuronal ensembles is modified during this learning, we combined the lever-press task with chronic two-photon calcium imaging[8,16]. In this study we focused on neurons in layer 2/3, the major input layer capable of driving deeper layer neurons to produce motor cortex outputs[17,18]. Before training, we injected an adeno-associated virus

encoding the Ca^{2+} indicator GCaMP5G[19] into the right forelimb area of the motor cortex to express GCaMP5G in all neuron types. Optogenetic stimulation of this area evoked forelimb movements (Extended Data Fig. 2.3). GCaMP5G fluorescence reported spiking activity with high temporal precision (jitter of spikes and calcium events = 7.1 ± 41.4 ms, median ± SD, Extended Data Fig. 2.4). We used transgenic mice that express tdTomato in all GABAergic inhibitory neurons (*GAD2-IRES-Cre*[12] ; *Rosa-LSL-tdTomato*[13]) to identify excitatory (only expressing GCaMP5G) and inhibitory (expressing both tdTomato and GCaMP5G) neurons. Two weeks following surgery, hundreds of neurons were imaged through a chronic window. 202 ± 18 (mean ± SEM) neurons were imaged in each animal, with 20.9 ± 2.6% being inhibitory, consistent with the composition of the cortex[20].

We imaged the activity of the same population of excitatory and inhibitory neurons over the course of two weeks while mice simultaneously learned and performed the lever-press task (Fig. 2.1d, n = 7 mice). High correlations of neural activity and lever-press movements were evident both at the levels of single neurons and population average in both excitatory and inhibitory neurons (Fig. 2.1e). A large fraction of imaged neurons showed significantly more activity during lever-press movements and were thus considered movement-related (51.4 ± 5.7 % of imaged neurons, 44.0 ± 6.2 % of excitatory and 78.7 ± 5.7 % of inhibitory, were classified as movement-related in at least one session, Methods). Movement-related neurons did not show obvious spatial clustering (Extended Data Fig. 2.5).

We investigated the relationship between excitatory and inhibitory populations. We found a positive correlation between the fraction of active inhibitory and excitatory neurons on a movement-by-movement basis; during movements that activated a larger fraction of excitatory neurons, a larger fraction of inhibitory neurons were also active. This relationship of excitatory and inhibitory activity remained constant for the entire two weeks of imaging (Fig. 2.1f). Individual inhibitory neurons were particularly correlated with nearby excitatory neurons within 150 μm, consistent with their connectivity[21,22] (Fig. 2.1g). This local matching of excitatory and inhibitory activity likely provides a basis for the balance between excitatory and inhibitory inputs to individual neurons observed in the cortex (reviewed in ref.[20]). Even though the ratio of excitatory and inhibitory activity on a trial-to-trial basis was stable, the identity of movement-related neurons was dynamic. In particular, excitatory neurons on average had a higher degree of turnover than the inhibitory population, suggesting that the excitatory population is more dynamic during learning (Fig. 2.1h and Extended Data Fig. 2.6a). We therefore focused on excitatory neurons for the following analyses.

Many excitatory neurons were transiently movement-related (Fig. 2.2a). In the initial phase of learning, a large fraction of excitatory neurons developed movement-related activity, resulting in a dramatic increase of the movement-related population (Fig. 2.2b&c). Following this initial expansion, the fraction of movement-related neurons decreased gradually through the remaining course of the experiment (Fig. 2.2b&c and Extended Data Fig. 2.6b). This resulted in a

smaller and more stable population of movement-related neurons towards the end of learning (Fig. 2.2d). The expansion and refinement was not seen during spontaneous movements without training (Extended Data Fig. 2.6c). Despite these changes in the ensemble of movement-related neurons during learning, the average fraction of excitatory neurons active on each trial remained stable (Fig. 2.2e). This constant level of activity was maintained despite a changing size of movement-related populations because of the corresponding shift in the frequency of activity in individual neurons (Fig. 2.2f). Various combinations of excitatory neurons were therefore utilized within the motor cortex during the initial phase of learning, followed by a refinement of the population to form a stable network associated with the learned movement.

We next examined the timing of activity of individual neurons. As a population, the activity of movement-related excitatory neurons diverged from baseline at 105 ms before the movement onset and continued throughout the duration of movements (Extended Data Fig. 2.6d). During the first few sessions of learning, movement-related excitatory neurons showed variable timing of activity on individual trials relative to the movement onset. Conversely, movement-related neurons in later sessions showed reproducible activity timing relative to movement onset (Fig. 2.2g and Extended Data Fig. 2.6e). As a result, the temporal activity pattern became progressively more stable during learning (Fig. 2.2h). This activity sequence tiled the entire duration of movement (Fig. 2.2i&j, Extended Data Fig. 2.6f). Furthermore, the population activity shifted

towards the beginning of movements over the course of the experiment (Fig. 2.2j and Extended Data Fig. 2.6g).

The observed stabilization of motor cortex activity may result from the selection of a particular activity-movement pair out of many which are explored during initial learning. In this case, activity during the learned movement and activity during similar movements made early in learning should resemble each other. Alternatively, the 'learned' activity pattern may be unique to the expert stage after learning. To distinguish between these possibilities, we evaluated the relationship between movement and neural activity. We defined the 'learned' patterns of activity and movement as the averages of a randomly chosen 50% of trials from the expert sessions (sessions 10-14, Methods). When the remaining 50% of trials from the expert sessions were sorted according to the similarity of the movement in each trial with the learned movement pattern, a clear relationship between movement and activity became evident; the similarity of activity to the learned activity pattern increased with the similarity of movements to the learned movement (Fig. 2.3a&b). Remarkably, this relationship was much weaker when trials from naive sessions (sessions 1-3) were considered. Regardless of the similarity of movement to the learned movement pattern, the similarity of activity to the learned activity pattern was consistently low in naive sessions (Fig. 2.3a&b). In other words, the learned activity pattern was reproducibly observed only when the expert animals made the learned movement, while similar movements made in naive sessions were accompanied by very different activity patterns. Furthermore, the general relationship between

activity and movement in pairs of trials became more consistent after learning, while activity in naive animals was more variable regardless of movement similarity (Fig. 2.3c). These analyses of learning-related changes in population activity were performed using the entire movement periods. However, the results were similar when only the periods from movement onset to reward were considered (Extended Data Fig. 2.7).

The plasticity of population activity described above could simply reflect changes in other brain areas providing inputs to these neurons. However, synaptic plasticity within the motor cortex could also contribute to the changes in population activity. To test whether learning of this lever-press task induces synaptic plasticity in layer 2/3 of the motor cortex, we labeled sparse subsets of layer 2/3 neurons and chronically imaged spines on the same dendritic branches of excitatory neurons over the course of learning (n = 191 spines in 3 mice). Imaging was performed immediately prior to each training session in awake animals. We observed the formation of a number of dendritic spines during the initial sessions of training, followed by the elimination of some of the spines that were present at the beginning of the experiment. The majority (95 %, 19 / 20) of the spines that formed during training persisted for the entire two weeks. At the population level, these changes resulted in a transient 10% increase in the density of spines followed by return to the baseline (Fig. 2.4). These results are analogous to previous reports in motor cortex layer 5 neurons during different motor learning tasks[3,4]. Spines were largely stable in a separate group of animals that did not undergo training but otherwise were treated identically including

water restriction and head-fixation (Fig. 2.4b). The spine density was also stable in the hindlimb area in the motor cortex during learning (Extended Data Fig. 2.8). These results indicate that our lever-press task induces area-specific reorganizations of excitatory synapses onto layer 2/3 neurons during learning.

Previous studies suggested a relatively stable tuning and population code of motor cortex neurons in well-trained animals[23,24]. However, our understanding is quite limited as to the changes in the ensemble activity pattern during the transition from naive to expert stages. Our results suggest that the relationship between movements and activity is initially inconsistent (i.e. degenerate), and the early days of learning involve the expansion and exploration of various movement-related activity in the motor cortex. An increased variability of single neuron activity in the motor cortex has been observed during learning of visuomotor adaptation[25] and a brain-machine interface task[26]. Such trial-to-trial variability has been proposed to provide the basis for exploration of possible network states and facilitate learning[10]. Our results directly demonstrate such an exploration during initial learning at the population level. Following this period of high variability, the activity-movement degeneracy is reduced and a reproducible temporal sequence of activity emerges in a stable population of excitatory neurons (Extended Data Fig. 2.9). Such spatiotemporal activity may orchestrate the temporal dynamics of the learned movement. Reproducible temporal patterns of population activity during learned movements are hypothesized to be generated by internal connections within the motor cortex[27,28]. We note that our results do not provide a causal link between local

synaptic plasticity and changes in population activity. Nevertheless, we show that these processes occur during motor learning on similar time scales, which supports the notion that local synaptic plasticity may generate a circuit to reproduce a particular spatiotemporal activity pattern. These new circuits may be more efficient in driving movements, which could underlie the lower metabolic activity in the motor cortex observed during execution of well-practiced movements[29,30]. Our study provides a glimpse of the emergence of population activity patterns for learned movements.

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Author Contributions

AJP and TK conceived the project. Dendritic spine imaging and optogenetic silencing experiments were performed by SXC and analyzed by SXC and TK. All other experiments were performed by AJP and analyzed by AJP and TK. AJP and TK wrote the manuscript with inputs from SXC.

Methods

Animals. All procedures were in accordance with protocols approved by the UCSD Institutional Animal Care and Use Committee and guidelines of the National Institute of Health. Mice (calcium imaging: cross between *GAD2-IRES-Cre*[12] and *Rosa26-CAG-LSL-tdTomato*[13], Jackson laboratories; structural imaging: C57Bl/6, Charles River Laboratory) were group housed in disposable plastic cages with standard bedding in a room with a reversed light cycle (12h-12h). After surgery, animals were singly housed. Experiments were performed during the dark period. No randomization was used, but internal controls were included whenever possible.

Surgery. Adult mice (6 weeks or older, male and female) were anesthetized with isoflurane and injected with dexamethasone (2 mg / kg) and baytril (10 mg / kg) intramuscularly to prevent brain swelling and infection. A custom head-plate was glued to the skull and craniotomy (~2 mm diameter) was performed as described[31] over the right caudal forelimb area. Viruses (UPenn Vector Core; calcium imaging: AAV2/1-syn-GCaMP5G diluted 1:1-3 in saline, 5-6 sites; structural imaging: AAV2/1-CAG-FLEX-EGFP (1:1) and AAV2/1-CMV-PI-Cre (1:5000) diluted in saline, 3 sites) were injected in the caudal forelimb area of the motor cortex around the coordinate of 300 μm anterior and 1500 μm lateral from

bregma, according to previous microstimulation experiments[15,32-36]. We confirmed that optogenetic stimulation of this area evoked forelimb movements (Extended Data Fig. 2.3). This area also falls near the border of the abduction and adduction areas defined by ref.[2]. For control experiments targeting the hindlimb area of the motor cortex, viruses were injected around the coordinate of 1500 μm posterior and 1500 μm lateral from bregma, according to previous microstimulation experiments[32,33,35,36]. Each injection consisted of ~20 nl at a depth of ~250 μm from the pia injected over 2-4 minutes and each injection site was separated by ~500 μm. Pipettes were left in the brain for 3 minutes following injection to avoid backflow. Following virus injections, a chronic imaging window was implanted consisting of a glass plug glued onto a larger glass base. The edges between the glass plug and the skull were filled with 1.5% agarose and the window was secured using dental acrylic. Buprenorphine (0.1 mg / kg) was injected subcutaneously at the end of surgery.

Behavior. Three days after surgery, mice were water-restricted at 1 ml / day. After ~14 days of water restriction, mice were trained daily for 14 days while two-photon imaging was applied simultaneously. The lever was built using a piezoelectric flexible force transducer (LCL-113G, Omega Engineering) attached to a 1/16 mm thick brass rod. The voltage from the force transducer, which is proportional to the lever position, was continuously recorded using a data acquisition device (LabJack) and software (Ephus, MATLAB, Mathworks) working with custom software running on LabVIEW (National Instruments) which monitored threshold crossing. The behavioral setup was controlled by software

(Dispatcher, Z. Mainen and C. Brody) running on MATLAB communicating with a real-time system (RTLinux). A 6 kHz tone marked a period during which lever-press was rewarded with water (~8 µl / trial) paired with a 500 ms, 12 kHz tone, followed by an intertrial interval (variable duration, see below). Lever-press was defined as crossing of two thresholds (~1.5 mm and ~3 mm below the resting position) within 200 ms. The 3 mm threshold defined the displacement required, and the 1.5 mm threshold ensured that the mouse did not hold the lever near the lower threshold. Failure to press during the cue period triggered a loud white noise and an intertrial interval. Lever-presses during intertrial intervals were neither rewarded nor punished. The cue period was decreased during the first two sessions from 30 sec to 10 sec. The reward period was reduced during the first three sessions from 2 sec to 0.4 sec. The intertrial interval was increased over the first three sessions from 2-4 sec to 8-12 sec. Each session lasted 20-30 minutes and 100-200 trials until terminated when mice stopped performing or consumed 1 ml of water. Experiments lasted for 11-14 sessions. One mouse failed to learn the task and was thus excluded from all analyses.

Lesion. Motor cortex lesions were performed under isoflurane anesthesia. After craniotomy was performed as above, cortical tissue was aspirated using a glass Pasteur pipette connected to vacuum. Care was taken to avoid damaging the underlying white matter. After the lesion, the cavity was filled with Surgifoam (Johnson & Johnson), KWIK-CAST (World Precision Instruments) and then with a layer of dental acrylic. Mice were allowed to recover for three days after the surgery and then placed on water restriction. Behavioral training started two

weeks after lesion. The extent of lesion was determined for each mouse with *post hoc* histology.

Muscimol inactivation. Mice with imaging windows were first trained with the task for 7-14 days. On the day of inactivation, the imaging window was removed and ~70 nl of muscimol (5 µg / l µl in cortex buffer) was injected over 2-3 min in the center of the craniotomy at the depth of 300 µm, under isoflurane anesthesia. The craniotomy was then sealed and mice were allowed to recover in their home cage on a heating pad for 60 min before behavioral experiments. For control injections, muscimol was injected into the barrel cortex, using the coordinate of 1.4 mm posterior and 3.1 mm lateral from bregma.

Optogenetic inactivation. Surgery was performed on PV-Cre mice as above to inject AAV2/1-CAG-Flex-ChR2-tdTomato around the forelimb area of the motor cortex at 5 sites. 80 nl was injected at each site at each depth of 400 µm and 800 µm. Behavioral training started three weeks following surgery. After seven sessions of daily training, the cortical area was inactivated in 20 % of trials by activating PV neurons[37] by blue light from an LED (~40 mW, 470 nm, Doric Lenses) delivered directly onto the center of the craniotomy covered with a chronic glass window. Blue light was delivered starting from 1-2 sec before the cue period until the end of the cue period (i.e. reward delivery or time out). Blue light delivery was performed in seven successive sessions. In the last two sessions, the glass window was covered with curable silicone (KWIK-CAST, World Precision Instruments) and these served as control sessions.

Optogenetic microstimulation. Surgery was performed on C57Bl/6 wildtype mice as above to inject AAV2/1-CAG-ChR2-Venus around the forelimb area of the motor cortex at 9 sites. 20 nl was injected at each site at the depth of 300 μm. Three weeks following surgery, mice were head-fixed. Blue light from an LED (~40 mW, 470 nm, Doric Lenses) was delivered directly onto the center of the craniotomy covered with a chronic glass window for 1 sec / trial with the intertrial interval of 8 - 12 sec. Stimulation was performed without anesthesia. The effect of the light stimulus on forelimb movements was quantified by manually identifying movie frames in which the forelimb movements were observed on videos cropped so that the optical stimulation was not visible. Pre-light periods were defined as movie frames lasting from 2 to 1 second before light onset. Scoring was done by two individuals independently and trials which differed between the scorers were excluded (1/79 light trials, 3/79 pre-light trials).

Imaging. Imaging was conducted with a commercial two-photon microscope (B-scope, Thorlabs) running Scanimage using a 16x objective (NIKON) with excitation at 925 nm (Ti-Sa laser, Newport). Imaging was always conducted in awake animals. For calcium imaging, images (512 x 512 pixels covering 472 x 508 μm) were recorded at approximately 28 Hz in continuous segments about 2 minutes long each with inter-segment intervals of 12 seconds. The trials that overlapped with the intervals were discarded. Signals for the start of each trial were also recorded, which was used to align images and behavior data. Slow drifts in imaging field were manually corrected using reference images. For structural imaging, stacks of image planes (512 x 512 pixels covering 94 x 104 μm)

were acquired at approximately 28 Hz, 20 frames / plane, 80 – 120 planes / animal with a z-axis step size of 1.0 μm between planes.

Movement analysis. Movement bouts were identified in the lever displacement traces (voltage recordings from the force transducer) that were downsampled from 10 kHz to 1 kHz and then filtered (4-pole 10 Hz low-pass Butterworth). The velocity of the lever was then determined by smoothing the difference of consecutive points with a moving average window of 5 ms. The envelope of the velocity was then extracted using a Hilbert transform, and movement bouts were defined by the envelope crossing a threshold (4.9 mm / sec). Each movement bout was extended by 75 ms, bouts separated by less than 500 ms were considered continuous, and then the start and end times were fine-tuned as follows. The start time was defined by finding when the lever position crossed a threshold exceeding the resting period before the movement, and the end time was defined by finding when the lever position went below a threshold defined by the resting period following the movement. Thresholds were the resting position plus the 99th percentile of noise distribution defined as the difference between the Butterworth smoothed trace and the original trace. These processes were chosen empirically based on visual inspection. For trial-based analyses, the trials in which animals were moving the lever at the onset of cue were excluded.

Image analysis. For calcium imaging data, lateral motion was corrected using full-frame cross-correlation image alignment (Turboreg[38] plugin for ImageJ). Motion within each frame was negligible due to the fast frame rate. Regions of

interest (ROIs) were manually drawn using a custom MATLAB program by visually inspecting movies from all sessions and selecting neurons that showed at least one fluorescence transient in at least one session. Therefore, our analysis excludes neurons that do not show any fluorescence transients during our imaging periods. ROIs were aligned across sessions using a semi-automated method and classified as excitatory or inhibitory based on tdTomato expression. ROIs which presented with a nucleus filled by GCaMP5G at any point during the experiment, indicating possibly abnormal physiology[39], were excluded from all analyses. Other than these nucleus-filled neurons, GCaMP-expressing neurons have been shown to display normal physiological properties including input resistance, resting membrane potential, input-output relationship, synaptic input maps, and normal synaptic plasticity[8,39].

For structural imaging data, lateral motion for each image plane (20 frames) was corrected using full-frame cross-correlation image alignment (Turboreg[38] plugin for ImageJ), with the average of the 5 most consistent consecutive frames as the reference image. After this alignment, all 20 frames within a plane were averaged. Different image planes were then aligned using recursive alignment of stacks of images (Stackreg, plugin for ImageJ).

Fluorescence analysis. Pixels within each ROI were averaged to create a fluorescence time series. Background fluorescence fluctuations were subtracted from each ROI to remove neuropil contamination as follows. A ring-shaped "background ROI" was created from the border of each neuronal ROI to a width of 6 pixels. From this background ROI, pixels containing transients that did not

contaminate the neuronal ROI were excluded. These excluded pixels were identified as those that contained time points at which pixel values exceeded the neuronal ROI by 2 times the standard deviation of the difference between each background ROI pixel time series and the neuronal ROI time series. The remaining pixels were averaged to create a background fluorescence trace, and the ΔF of the background fluorescence trace was subtracted from the neuronal ROI fluorescence trace to create the final background-subtracted fluorescence trace for each neuronal ROI.

The time-varying baseline (F_0) of a fluorescence trace was estimated by smoothing inactive portions of the trace using the iterative procedure detailed below.

Inactive portions of the trace were initially estimated as when the raw trace loess-smoothed with a 1 second window was below a threshold. These inactive portions were further shortened by 5 seconds on each end to exclude tails of active portions. The threshold for activity was estimated by first creating a preliminary F_0 approximation, which was a 1 minute moving average of the original fluorescence trace. This preliminary F_0 was subtracted from the raw trace to yield a preliminary ΔF. The noise of the fluorescence was calculated as the standard deviation of the difference between the preliminary ΔF and the smoothed preliminary ΔF (loess, 1 sec). An offset of the preliminary F_0 was then estimated as the mode of the smoothed preliminary ΔF. The threshold for detecting active portions was then set as preliminary F_0 + the offset + two times the fluorescence noise. The remaining inactive portions of the trace were then

concatenated and subjected to a second round of activity extraction using the same procedure, but by defining inactive portions where values fell within ± two times the fluorescence noise.

Inactive portions were concatenated and smoothed (loess, 1 sec). The resulting smoothed trace was then broken up according to their original time points and values were linearly interpolated across gaps (i.e. active portions), resulting in an F_0 estimation that was independent of activity and slow drifts. This F_0 estimation was fine-tuned for an offset as follows. The F_0 was subtracted from the raw trace, yielding a new ΔF. The fluorescence noise was once again estimated by the standard deviation of the difference between ΔF and smoothed ΔF (loess, 1 sec). The offset was then estimated by the mode of Gaussian-fit of the distribution of values of inactive portions of the ΔF. Inactive portions were defined as when the ΔF values were within ± two times the noise values, and were shortened by 5 seconds on either end. This offset was added to the F_0 estimation, yielding the final F_0.

Activity analysis. Activity event traces were created from normalized background-subtracted fluorescence traces. For excitatory neurons, events were defined if the first derivative (velocity) of the smoothed fluorescence trace (loess, 1 sec) crossed 5 times the standard deviation of the inactive velocity trace (inactive velocity trace was derived from periods when the fluorescence was within 3 times the standard deviation of the fluorescence trace). This velocity criterion detected sharp rises in the fluorescence trace. For these detected events, the start and end times were defined using the following

iterative process. The peak time of the event was first roughly estimated as the time when the velocity drops below zero for the first time after it crossed the threshold as above. The peak time (i.e. the end time of the event) was then defined as the time of the highest $\Delta F/F_0$ value within 5 frames before and after the initial estimate. We next defined the 'baseline' $\Delta F/F_0$ value for the event as the $\Delta F/F_0$ value at the first time point when velocity was above zero before the peak time. (This 'baseline' $\Delta F/F_0$ for each event is similar to the baseline of the fluorescence trace (zero) except in cases when the event occurs during a decay of another event. See the last event in Extended Data Fig. 2.4b for such an example.) The start time of the event was then defined as the last time point before the peak time when the $\Delta F/F_0$ value is within noise level from the 'baseline' $\Delta F/F_0$ ('noise' being 3 times the standard deviation of the difference between the raw $\Delta F/F_0$ trace and the loess-smoothed $\Delta F/F_0$ trace.) An activity event trace was then constructed which was zero except for frames with detected events, and each event was assigned an amplitude equal to the difference between the peak $\Delta F/F_0$ and the 'baseline' $\Delta F/F_0$ for that event. This eliminated the decay of the calcium signal[19], but the use of velocity preserved events which occurred on top of the decays from other events. For inhibitory neurons, activity event traces were generated with the following procedure. The fluorescence noise was defined as the mean of the absolute difference between the raw trace and a 1 second moving window loess smoothed fluorescence trace. A low threshold of 1 time the noise and a high threshold of 3 times the noise were then set. Events were required to cross the high threshold.

The start of an event was defined as the time when the fluorescence trace crossed the low threshold going up to capture the start of activity, and the end was defined as the time when the fluorescence trace crossed the high threshold going down. An activity event trace was then constructed which was zero at all frames except during detected events which were assigned values of the original $\Delta F/F_0$ for those frames.

Classification of movement-related neurons. We observed higher levels of activity during movement periods. In individual neurons, the average percentages of image frames that contained activity during movement periods vs. non-movement periods were 0.68 ± 0.03 % vs. 0.23 ± 0.02 % in excitatory neurons and 14.45 ± 0.49 % vs. 6.95 ± 0.27 % in inhibitory neurons (mean ± SEM). Neurons whose activity was significantly higher during movement periods were classified as movement-related using the following procedure. The amount of activity during movement was calculated for each neuron as the mean value of the activity event trace during movement epochs (defined as described above, and extending individual epochs by 5 image frames before and after each movement). The movement trace was then shuffled (10,000 times) such that complete movement epochs were kept intact but their position in the trace and relation to each other was randomized. A measure of activity during these shuffled movement epochs was calculated in each shuffle as above. The neuron was classified as movement-related if the real value was higher than the 0.5 percentile value of the shuffled values. Classification using only rewarded movements instead of all detected movements gave nearly identical results.

For analyzing the longitudinal dynamics of the fractions of movement-related neurons over sessions (Fig. 2.2b), the fraction of movement-related neurons in each session in each animal was normalized to the highest and lowest values of the animal. The correlation was significantly positive for the data points in sessions 1-3 ($r = 0.60$, $p < 0.01$) and significantly negative for sessions 4-14 ($r = -0.36$, $p < 0.01$).

Population activity correlation. The stability of the population across days was assessed by correlation of population vectors (Fig. 2.2d). Each square represents the correlation coefficient of the excitatory neuron population activity vectors in a pair of sessions. The population activity vectors are the averages of binarized population activity during rewarded movements, collapsed across time of each movement. (For example, if neurons a, b and c are active in 20 %, 70 % and 30 % of rewarded movements, respectively, then the population activity vector of the session is (0.2, 0.7, 0.3).)

Activity onset timing analysis. We performed two analyses to define the timing of activity of movement-related excitatory neurons relative to movement onset. To define when the population activity diverged from baseline, the activity of each neuron was first averaged across movements. Then the population activity vector in each image frame was compared to the baseline activity (all non-movement periods from all sessions) by bootstrap (10,000 repetitions), yielding a p value for each image frame. We identified image frames in which p-values were below 0.01 in at least five successive frames, and defined the first frame of those as the time of divergence. The time of first population activity divergence

from baseline was 105 ms before movement onset (Extended Data Fig. 2.6d). Similarly, to identify the timing of activity modulation of individual neurons, the activity of a neuron in each image frame across movements was compared to its baseline activity (420 – 315 ms before movement onset) by bootstrap (10,000 repetitions). We defined the activity onset timing as the first image frame in which p-values were below 0.01 in at least five successive frames. 9.2 % of movement-related excitatory neurons showed significant activity prior to movement onset (Extended Data Fig. 2.6f).

Analysis of movement-activity correlation. The relationship between movements and activity (Fig. 2.3) was analyzed using 3 seconds (or 500ms, Extended Data Fig 7) starting from the onset of each rewarded movement, which was approximately the duration of rewarded movements for all animals over all sessions (2.62 ± 0.02 seconds, mean ± SEM). The learned movement and activity patterns were created by averaging the lever traces and the activity of excitatory neurons, respectively, of a randomly chosen half of the trials of sessions 10-14, considered the expert stage. The trials used to generate the learned patterns were excluded from correlation analysis. The movement correlation for each trial was the correlation coefficient of the lever trace of the rewarded movement in that trial with the learned movement pattern. The activity correlation for each trial was the correlation coefficient between the concatenated activity time series of all excitatory neurons in the trial and the concatenated learned activity pattern. For results shown in Fig. 2.3b, the

random choice of trials to define the learned patterns was repeated 1000 times and the results were averaged.

Dendritic spine dynamics. Dendritic spines were manually scored over the entire 14 training sessions using a custom-written IGOR program (J. Boyd and K. Haas). Spine analysis was done in 3-dimensions and the criteria were as previous described[31]. Analysis was done blind to the session number of each image, which was randomized. We assumed that rapid 'flickering' of spines (elimination and immediate reformation, or formation and immediate elimination) is rare and corrected our blind scoring accordingly. While this corrected for mistakes in scoring, we may be slightly underestimating spine dynamics. Specifically, if a spine was scored as absent in one session (session X) and present in the immediately preceding (session X-1) and following (session X+1) sessions, then it was called present on session X. Furthermore, if a spine was scored as present in one session (session X) and absent in the immediately preceding (session X-1) and following (session X+1) sessions, then it was called absent on session X. No more than one correction was applied on any given spine. If a spine score contained these gaps after one correction, that spine was excluded from following analyses. These exclusions were rare (4 / 191). 8 % of spines were corrected (16 / 191). The results closely matched those from independent scoring of the same data without shuffling dates (data not shown).

Simultaneous two-photon guided cell-attached recordings and calcium imaging. Mice which had previously been used for calcium imaging were allowed full access to water prior to electrophysiology. On the day of the

experiment, mice were anesthetized with isoflurane and the glass window was removed and replaced with a glass half-window which was secured with superglue. The animals were then head-fixed in the imaging rig and allowed to recover from anesthesia. Loose patch recordings were performed with glass pipettes (~5-7 MΩ) filled with 100μM Alexa Fluor 488 in saline. Excitatory neurons (negative for tdTomato) expressing GCaMP5G without fluorescence in the nucleus were targeted for recording. Signals were amplified 500x by an Axon CNS amplifier (Molecular Devices), filtered at 2 kHz, recorded (Ephus) at 10 kHz, and synchronized to the start of image acquisition. In 4 out of 6 neurons, imaging was done at the same zoom as the population imaging experiments (field of view 472 x 508 μm), and at three times higher zoom for the other 2 neurons. The results were similar at both zooms.

Statistics. Non-parametric tests were used when possible to avoid assumptions about data distributions. Sample sizes were determined based on the statistical significance of our main findings, which is highly significant. Multiple comparisons were corrected for by Bonferroni corrections. Sample sizes (n) are as follows where applicable. Mice per session: 6, 6, 7, 7, 7, 7, 7, 7, 7, 7, 7, 6, 6, 5. Imaged, rewarded trials without movement at cue / total trials per session: 98/313, 173/526, 458/968, 488/1071, 438/966, 519/1081, 424/946, 453/1082, 320/784, 457/981, 481/889, 417/959, 412/853, 280/702. Movement-related / all imaged excitatory neurons: 79/874, 118/874, 202/1122, 210/1122, 201/1122, 213/1122, 181/1122, 191/1122, 159/1122, 171/1122, 158/1122, 136/995, 149/995, 89/843. Movement-related / all imaged inhibitory neurons: 103/262, 117/262, 155/297,

167/297, 154/297, 133/297, 133/297, 124/297, 106/297, 125/297, 119/297, 119/260,

114/260, 68/183.

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Figure 2.1 Lever-press task and chronic calcium imaging of excitatory and inhibitory populations. **a,** Task schematic. **b,** Lever movement traces in rewarded trials from one mouse. Grey: 10 individual trials; black: average of all trials; red dotted line: movement onset. **c,** Top, median pairwise correlation coefficients of rewarded movements on individual trials over 3 seconds, averaged across animals. Bottom, pairwise movement correlation on individual trials within and across sessions corresponding to the black and gray arrows indicated on the top, respectively. Individual movements became more similar within ($r = 0.35$, $p < 0.001$) and across ($r = 0.37$, $p < 0.001$) sessions. **d,** Top and middle, GCaMP5G expression in layer 2/3 neurons imaged two weeks apart. Insets, zoom of outlined areas. Bottom, merge of tdTomato expressed in all inhibitory neurons (red) and GCaMP5G (green). **e,** Top, activity of all simultaneously imaged movement-related 38 excitatory (green) and 42 inhibitory (red) neurons from one animal. Each row represents a neuron. Middle, $\Delta F/F_0$ traces from one neuron each and average $\Delta F/F_0$ of all imaged neurons of each type (152 excitatory and 77 inhibitory). Bottom, task-related events; yellow shading, movement epochs. **f,** Fractions of active inhibitory neurons and excitatory neurons during rewarded movements are correlated on a movement-by-movement basis ($r = 0.63$-67, $p < 0.001$). This relationship is stable throughout learning ($p = 0.92$, one-way ANOVA comparison of median excitatory-inhibitory ratios). **g,** Pairwise correlation coefficients between inhibitory and excitatory neuron activity decrease with distance ($p < 0.001$, comparison between pairs within 150 μm and all other pairs, Wilcoxon rank sum test). **h,** Individual movement-related inhibitory neurons are classified on more sessions, showing that excitatory neurons are on average more dynamic than inhibitory neurons ($p < 0.001$, Wilcoxon rank sum test). All error bars are SEM.

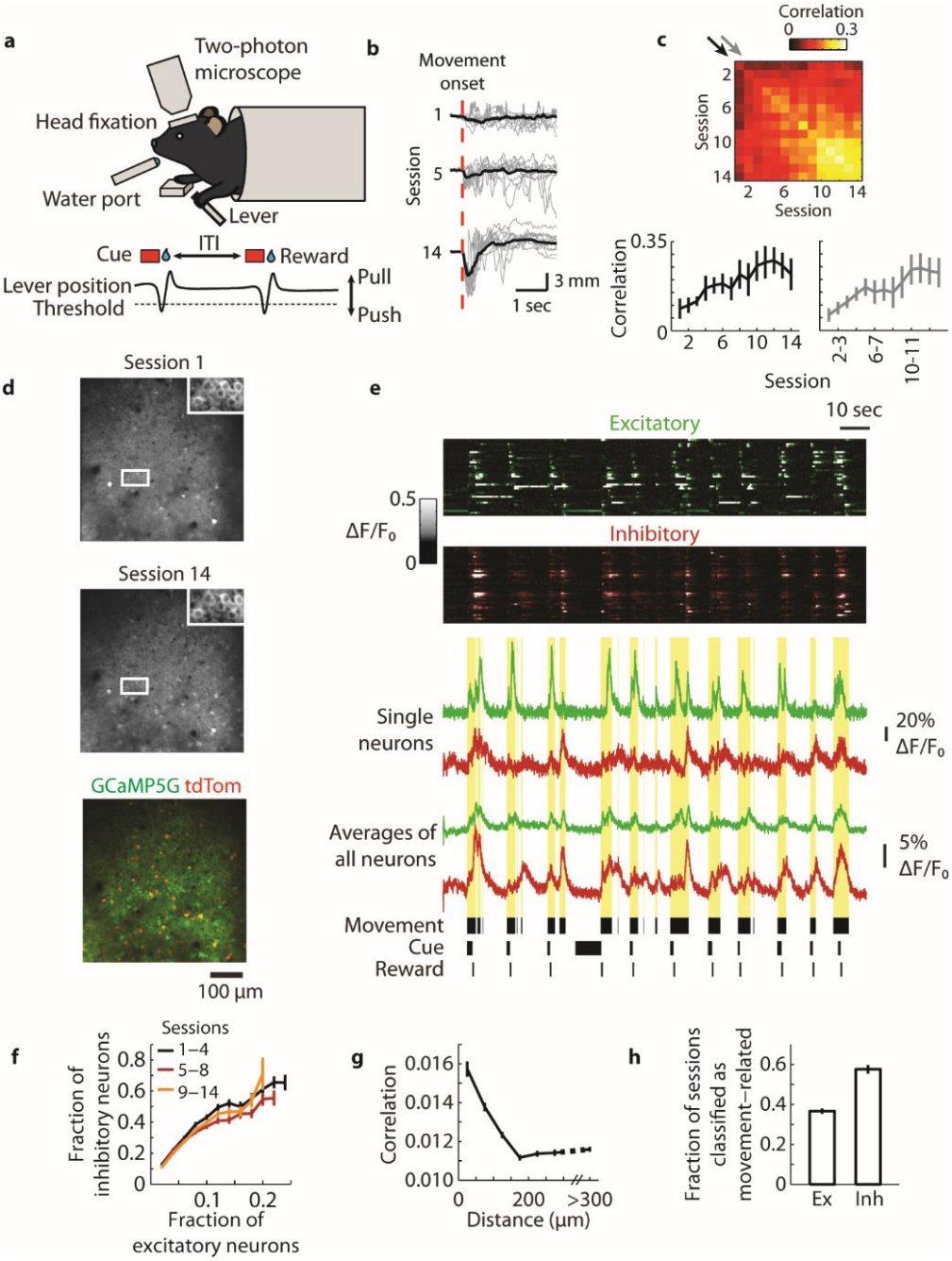

a
Two-photon microscope
Head fixation
Water port
Lever
Cue
ITI
Reward
Lever position
Threshold
Pull
Push
b
Movement onset
Session
1
5
14
3 mm
1 sec
c
Correlation
0
0.3
Session
2
6
10
14
Correlation
0.35
0
2-3
6-7
10-11
d
Session 1
Session 14
GCaMP5G tdTom
100 µm
e
10 sec
Excitatory
Inhibitory
0.5
ΔF/F0
0
Single neurons
20% ΔF/F0
Averages of all neurons
5% ΔF/F0
Movement
Cue
Reward
f
Sessions
1–4
5–8
9–14
Fraction of inhibitory neurons
0.8
0.6
0.4
0.2
0
0.1
0.2
Fraction of excitatory neurons
g
Correlation
0.016
0.014
0.012
0.010
0
200
>300
Distance (µm)
h
Fraction of sessions classified as movement–related
0.6
0.4
0.2
0
Ex
Inh

Figure 2.2 Dynamics of spatiotemporal activity of excitatory neurons during learning. **a,** Dynamics of three excitatory neurons. Top rows, images of neurons confirming reliable identification. Bottom rows, black, mean $\Delta F/F_0$; gray, SEM; arrow, movement onset. **b,** Mean fraction of excitatory neurons classified as movement-related in each session (increase over sessions 1-3, $p < 0.01$; decrease over sessions 4-14, $p < 0.01$, Methods). **c,** Dynamic population of excitatory neurons from one mouse. Green, movement-related excitatory neurons; gray; non-classified excitatory neurons. **d,** Correlation of population activity of all excitatory neurons during rewarded movements across sessions (Methods). The population of movement-related excitatory neurons became more stable in later sessions ($p < 0.001$, session 1-4 pairs vs. session 10-14 pairs, Wilcoxon rank sum test). **e,** Average fraction of excitatory neurons that are active in each individual movement out of all excitatory neurons remains stable throughout learning ($r = 0.00$, $p = 0.98$). **f,** Cumulative distribution of fraction of trials in which each movement-related excitatory neuron is active. In sessions 3-4, individual movement-related neurons are active in fewer trials compared to sessions 1-2 or 11-14 ($p < 0.001$, Kolmogorov-Smirnov test). **g,** Standard deviation of the timing of activity onsets for movement-related excitatory neurons decreases over sessions, ($r = -0.24$, $p < 0.001$). Neurons that were active in less than 5 trials of a given session were excluded from this analysis. **h,** Pairwise trial-to-trial correlation of temporal population activity vectors increases with learning ($r = 0.43$, $p < 0.001$). Temporal population activity vector was a concatenation of the activity traces of all movement-related neurons and thus maintained temporal information within each movement. **i,** Activity onsets of excitatory neurons from one animal that are movement-related and active in at least 10% of trials on the sessions indicated. Arrow, movement onsets; colors, individual neurons sorted according to their preferred timing. Note that same colors across sessions are not necessarily the same neurons. **j,** Maximum-normalized average activity from all movement-related neurons from all animals in session 2 (left, 106 neurons) and session 14 (right, 84 neurons) aligned to movement onset (arrow). Activity timing is refined over time, shown by narrower peaks and lower background in session 14, and shifts towards movement onset. All error bars are SEM.

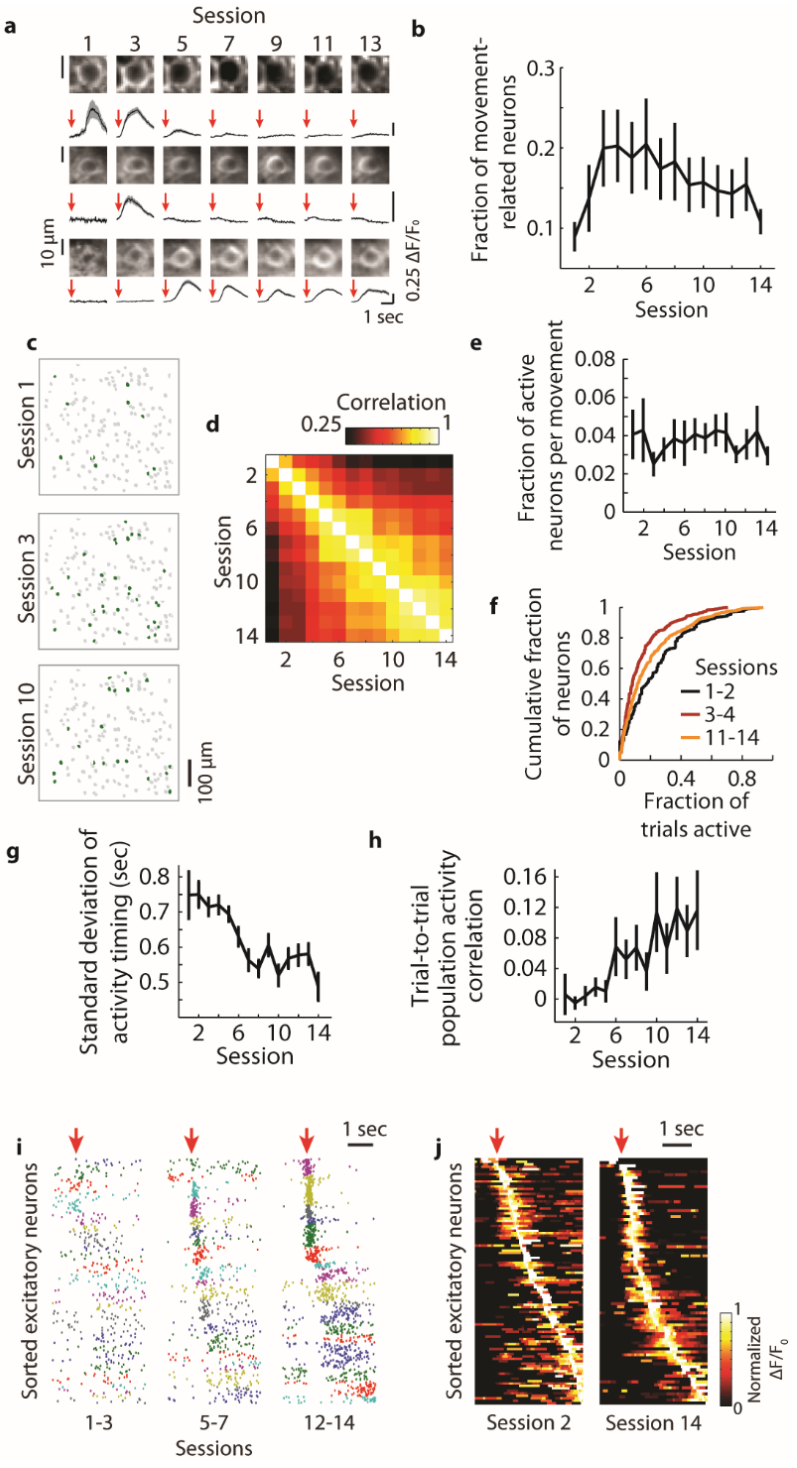
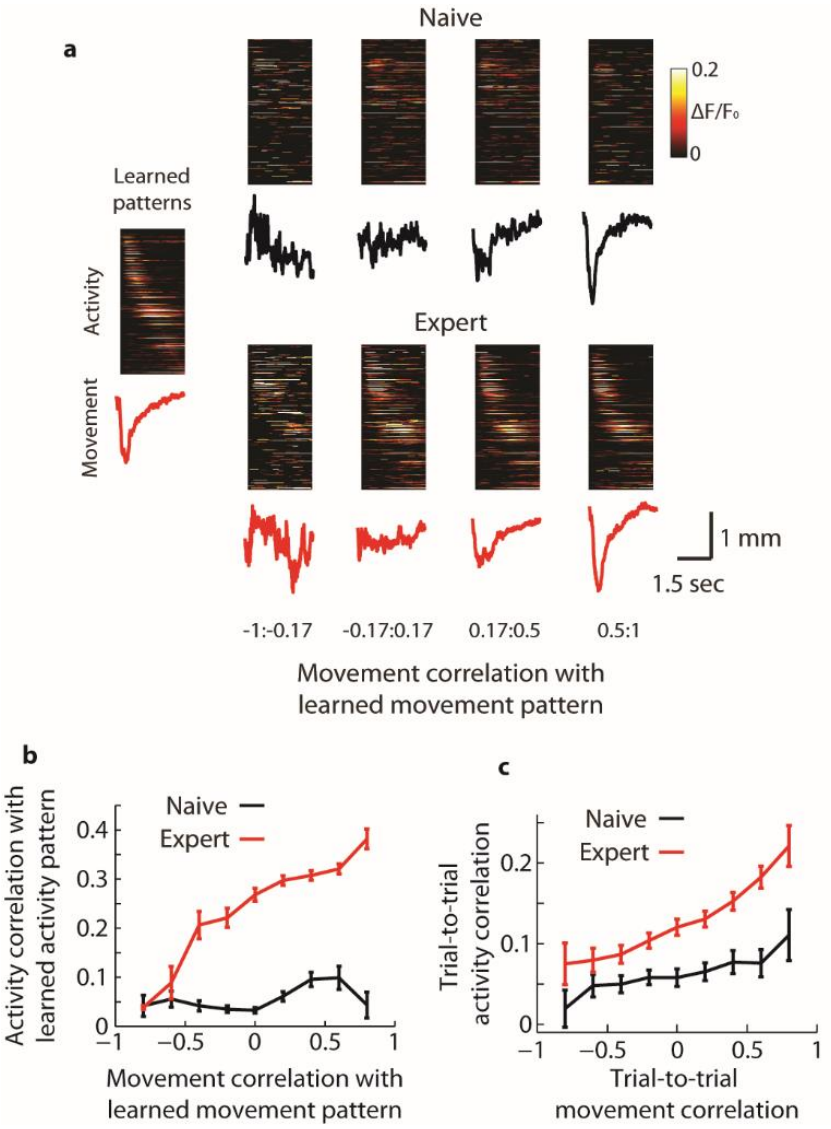

a
Session
1 3 5 7 9 11 13
10 µm
0.25 ΔF/F₀
1 sec
b
Fraction of movement-related neurons
0.3
0.2
0.1
2 6 10 14
Session
c
Session 1
Session 3
Session 10
100 µm
d
Correlation
0.25 1
Session
2 6 10 14
e
Fraction of active neurons per movement
0.08
0.06
0.04
0.02
0
f
Cumulative fraction of neurons
Sessions
1-2
3-4
11-14
0 0.4 0.8
Fraction of trials active
g
Standard deviation of activity timing (sec)
0.8 0.7 0.6 0.5
h
Trial-to-trial population activity correlation
0.16 0.12 0.08 0.04 0
i
Sorted excitatory neurons
1 sec
1-3 5-7 12-14
Sessions
j
Sorted excitatory neurons
1 sec
Session 2
Session 14
Normalized ΔF/F₀

Figure 2.3 Learning-related emergence of reproducible spatiotemporal activity. a, Heat maps, mean activity of movement-related excitatory neurons classified during expert sessions of all animals aligned to movement onset (left edge). Traces, mean lever movements. Trials are binned according to the correlations of the movements on those trials with the learned movement pattern (Methods). Left, learned patterns; top, naive sessions (sessions 1-3); bottom, expert sessions (sessions 10-14). **b,** Correlation of trial activity with the learned activity pattern increases with the correlation of trial movement with the learned movement pattern in expert sessions. Movements similar to the learned movement pattern but made in naive sessions display activity very different from the learned activity pattern ($p = 0.83$, $= 0.35$ and < 0.001 in the bins 1, 2 and 3-9, respectively, Wilcoxon rank sum test). **c,** Pairwise trial-to-trial correlation of temporal population activity vectors (defined as in Fig. 2.2h) plotted as a function of movement correlation on those trials. A stronger relationship between population activity and movement emerges during learning ($p = 0.38$, $= 0.18$, $= 0.04$, $= 0.002$, < 0.001, and $= 0.02$ in bins 1, 2, 3, 4, 5-8 and 9, respectively, Wilcoxon rank sum test). All error bars are SEM.


a
Naive
0.2
ΔF/F₀
0
Learned patterns
Activity
Movement
Expert
1 mm
1.5 sec
-1:-0.17
-0.17:0.17
0.17:0.5
0.5:1
Movement correlation with learned movement pattern
b
Activity correlation with learned activity pattern
Naive
Expert
0.4
0.3
0.2
0.1
0
−1
−0.5
0
0.5
1
Movement correlation with learned movement pattern
c
Trial-to-trial activity correlation
Naive
Expert
0.2
0.1
0
−1
−0.5
0
0.5
1
Trial-to-trial movement correlation

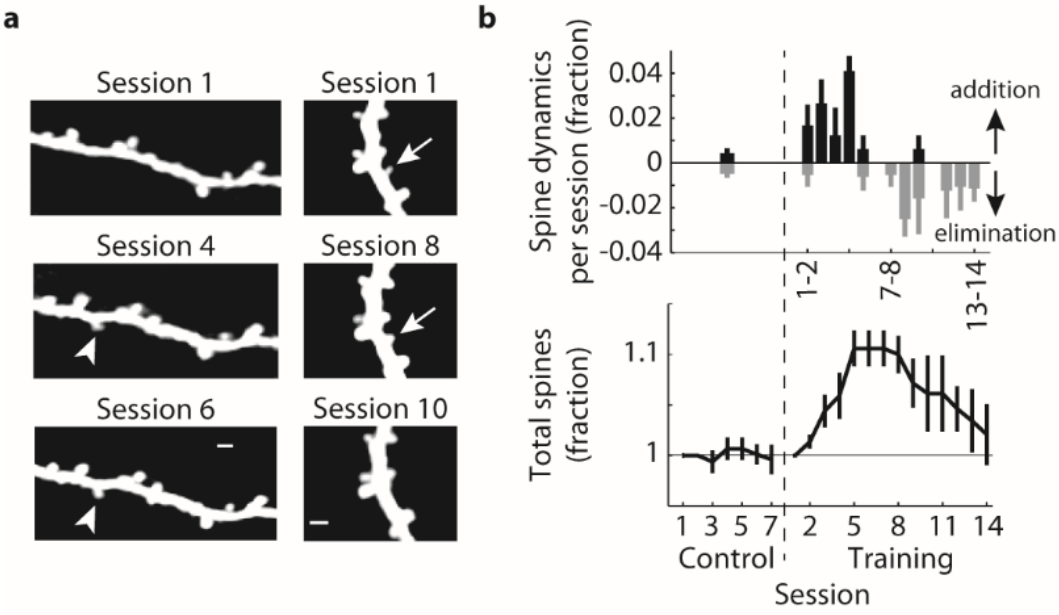
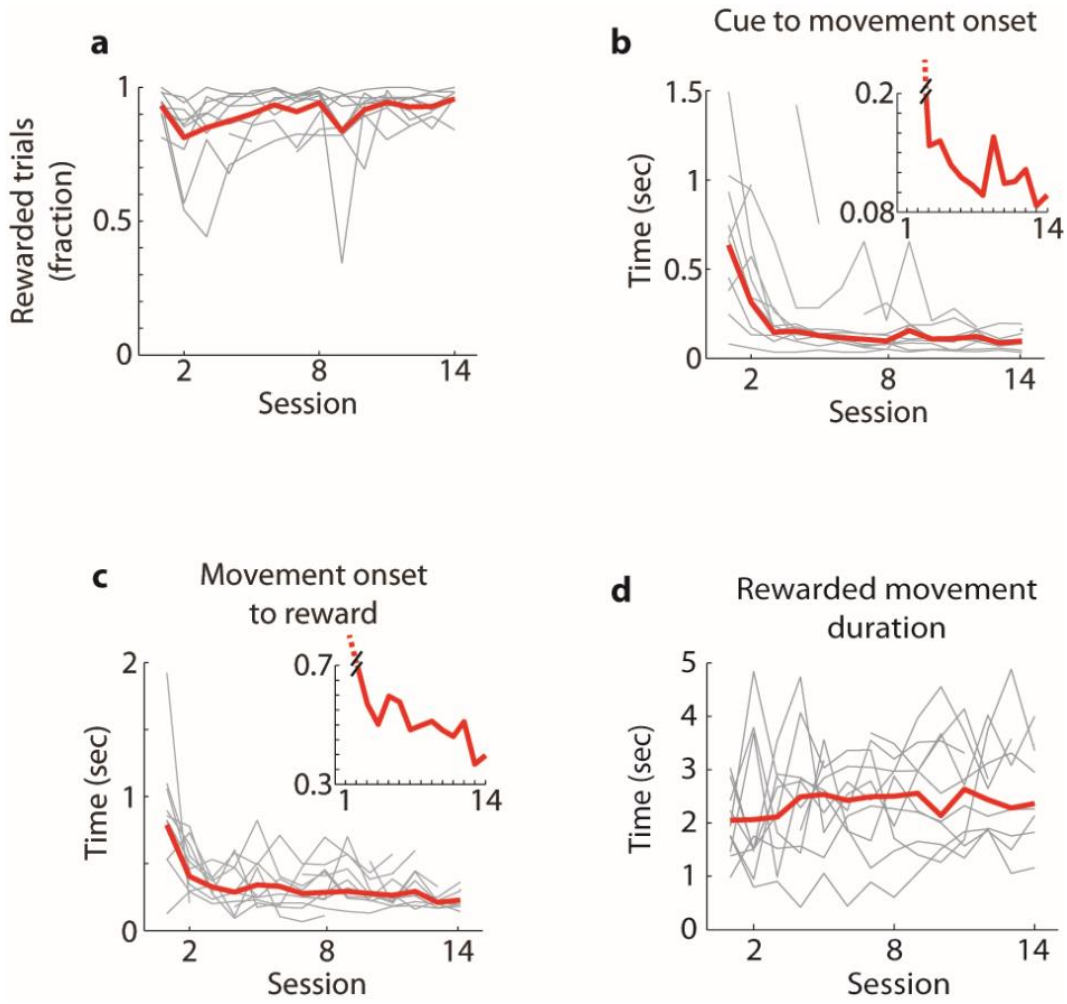
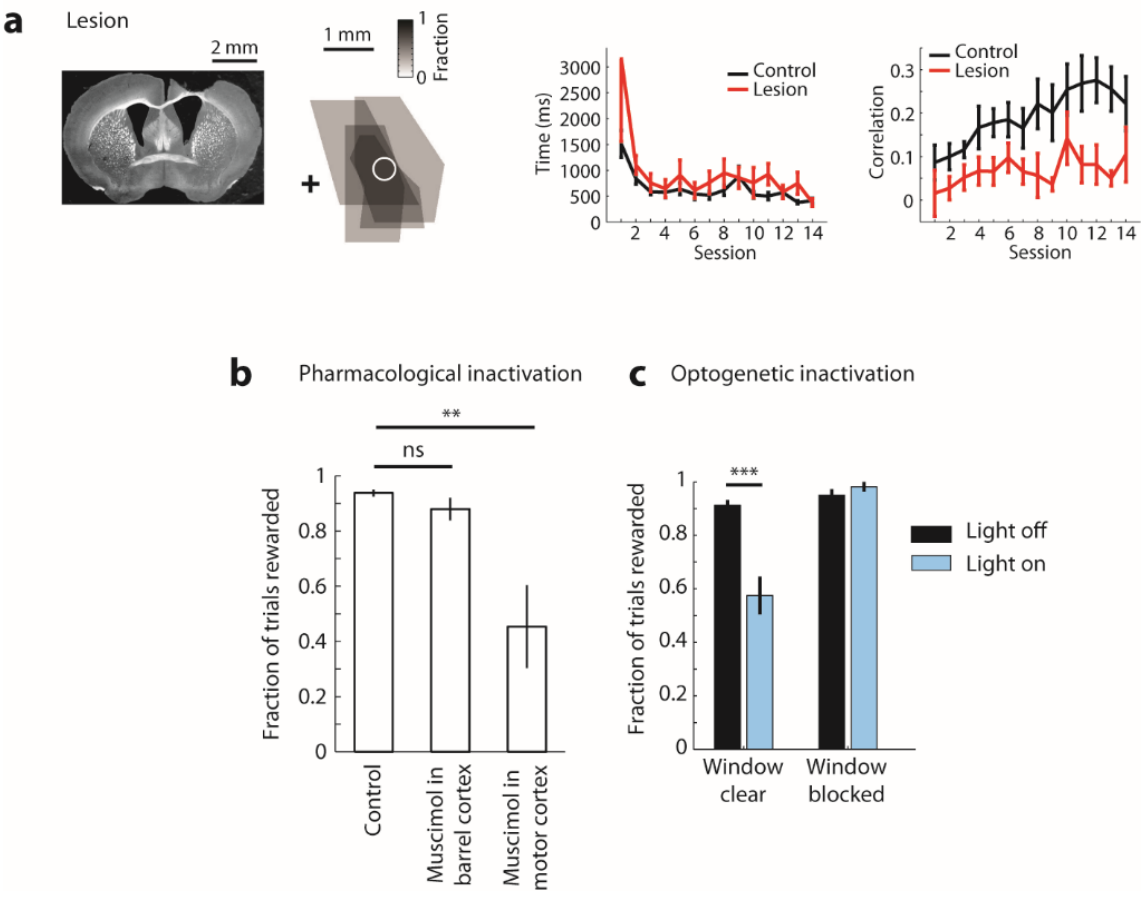


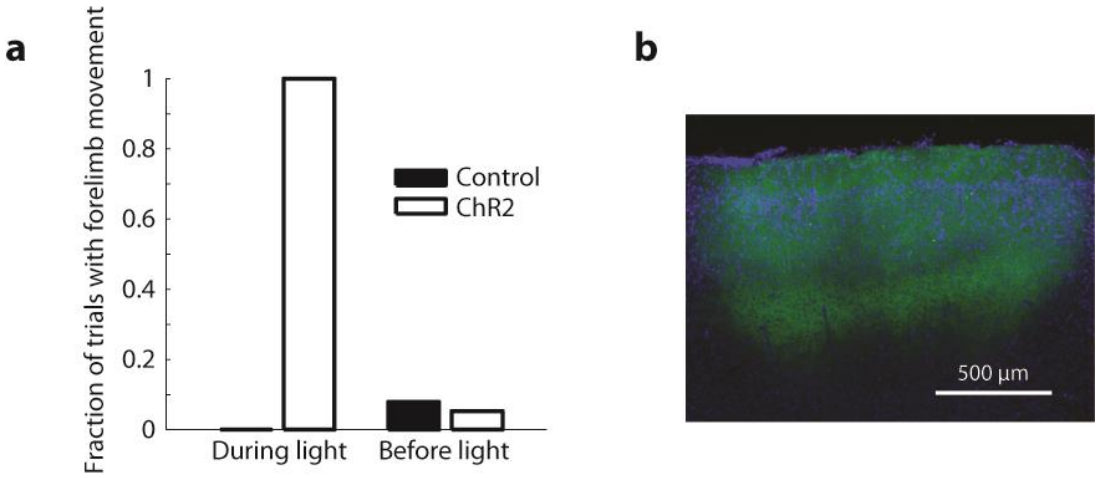
Figure 2.4 Learning-related plasticity of dendritic spines. a, Example layer 2/3 excitatory neuron dendrites imaged in awake mice throughout learning. Arrowheads, added spine; arrows, eliminated spine; scale bars, 2 μm. **b,** Summary of spine dynamics in trained and control animals. Top, spine additions (black) and eliminations (grey) in each session. For control animals, data from all sessions are combined. Bottom, total spine number across sessions normalized to session 1 in each condition. Spine density transiently increases during learning ($p < 0.001$, Control vs. Training sessions 4-7, Wilcoxon rank sum test). All error bars are SEM.



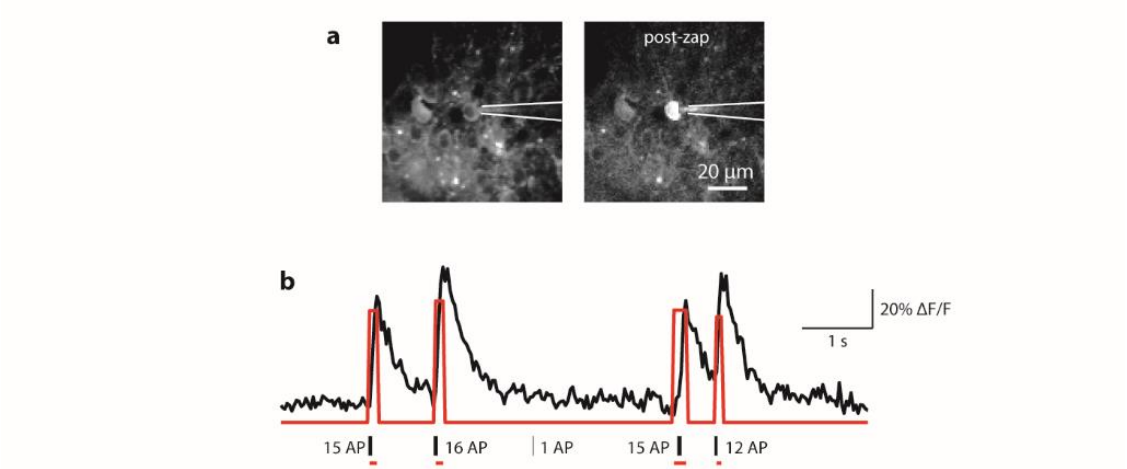
Extended Data Figure 2.1 Behavior. The fraction of rewarded trials is consistently high but the timing of behavior improves during learning. **a**, Fraction of trials which are rewarded. **b**, Time from cue onset to movement onset decreases ($p < 0.001$, one-way ANOVA), inset; zoom. **c**, Time from movement onset to reward decreases ($p < 0.001$, one-way ANOVA), inset; zoom. **d,** the duration of each rewarded movement is stable throughout learning ($p = 0.94$, one-way ANOVA). Grey: individual mice; red: mean of all animals (**a**) or median of all trials (**b**-**d**).

Extended Data Figure 2.2 Motor cortex is required for the lever-press task. **a,** Aspiration lesion of motor cortex impairs learning. Mice were allowed to recover for 14 days after lesion before training. Left, Histological image showing lesion in the right motor cortex and quantification of lesion extents in four mice shown as a density map of the fraction of animals in which the area was lesioned. Anterior is to the top, lateral to the right. + denotes bregma. The white circle indicates the imaged area. Middle, average time from movement onset to reward throughout learning. This time is longer in mice with motor cortex lesion ($p < 0.01$, two-way ANOVA), indicating that the mice with lesion are less efficient in their movements. Right, Correlation of lever movements in all pairs of trials within each session throughout learning. This correlation is lower in the mice with lesion ($p < 0.001$, two-way ANOVA), indicating that the mice with lesion do not develop reproducible movements. **b,** Injections of muscimol, a GABA receptor agonist, into the imaged area acutely impairs performance (control vs. muscimol in motor cortex, ** $p < 0.01$, Wilcoxon rank sum test). Muscimol injections in the barrel cortex had no significant effect (control vs. muscimol in barrel cortex, $p = 0.35$, Wilcoxon rank sum test). Control, n = 18 sessions in 6 mice; barrel cortex, n = 6 sessions in 6 mice; motor cortex, n = 6 sessions in 6 mice. **c,** The imaged cortical area was acutely inactivated by stimulation of ChR2 in parvalbumin-expressing inhibitory neurons by blue light in interleaved 20% of trials (n = 10 sessions in 2 animals). This optogenetic inactivation of the imaged area impaired performance on a trial-by-trial basis (***, $p < 0.001$, Wilcoxon rank sum test). Blue light had no effect on behavior when the window was covered with opaque silicone (n = 4 sessions in 2 animals). All error bars are SEM.


a
Lesion
2 mm
1 mm
1
0
Fraction
+
Time (ms)
3000
2500
2000
1500
1000
500
0
Control
Lesion
2 4 6 8 10 12 14
Session
Correlation
0.3
0.2
0.1
0
Control
Lesion
2 4 6 8 10 12 14
Session
b
Pharmacological inactivation
**
ns
Fraction of trials rewarded
1
0.8
0.6
0.4
0.2
0
Control
Muscimol in barrel cortex
Muscimol in motor cortex
c
Optogenetic inactivation
***
Fraction of trials rewarded
1
0.8
0.6
0.4
0.2
0
Window clear
Window blocked
Light off
Light on



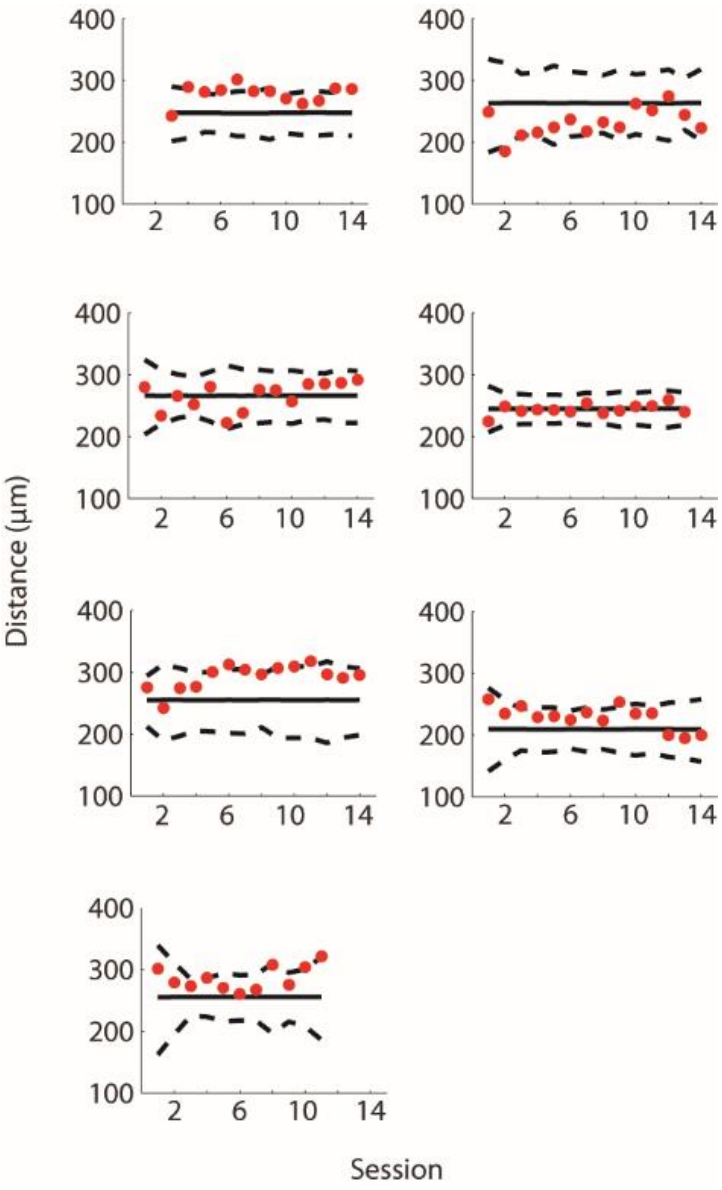
Extended Data Figure 2.3 Optogenetic stimulation of the imaged area evokes forelimb movements in awake mice. a, Optogenetic excitation of the imaged area triggers forelimb movements in mice expressing ChR2 but not in control mice not expressing ChR2 ($p < 0.001$, chi-square test). ChR2 expression does not alter spontaneous movement frequency in the absence of stimulation ($p = 0.64$, chi-square test). 'During light', 1-sec light stimulation; 'before light', 2 to 1 sec before light onset, n = 40 'during light' trials and 38 'before light' trials in 2 ChR2 mice, 38 'during light' trials and 38 'before light' trials in 2 control mice. **b,** Histological section showing the expression of ChR2 in the motor cortex. Green, ChR2-YFP; blue, DAPI.



c

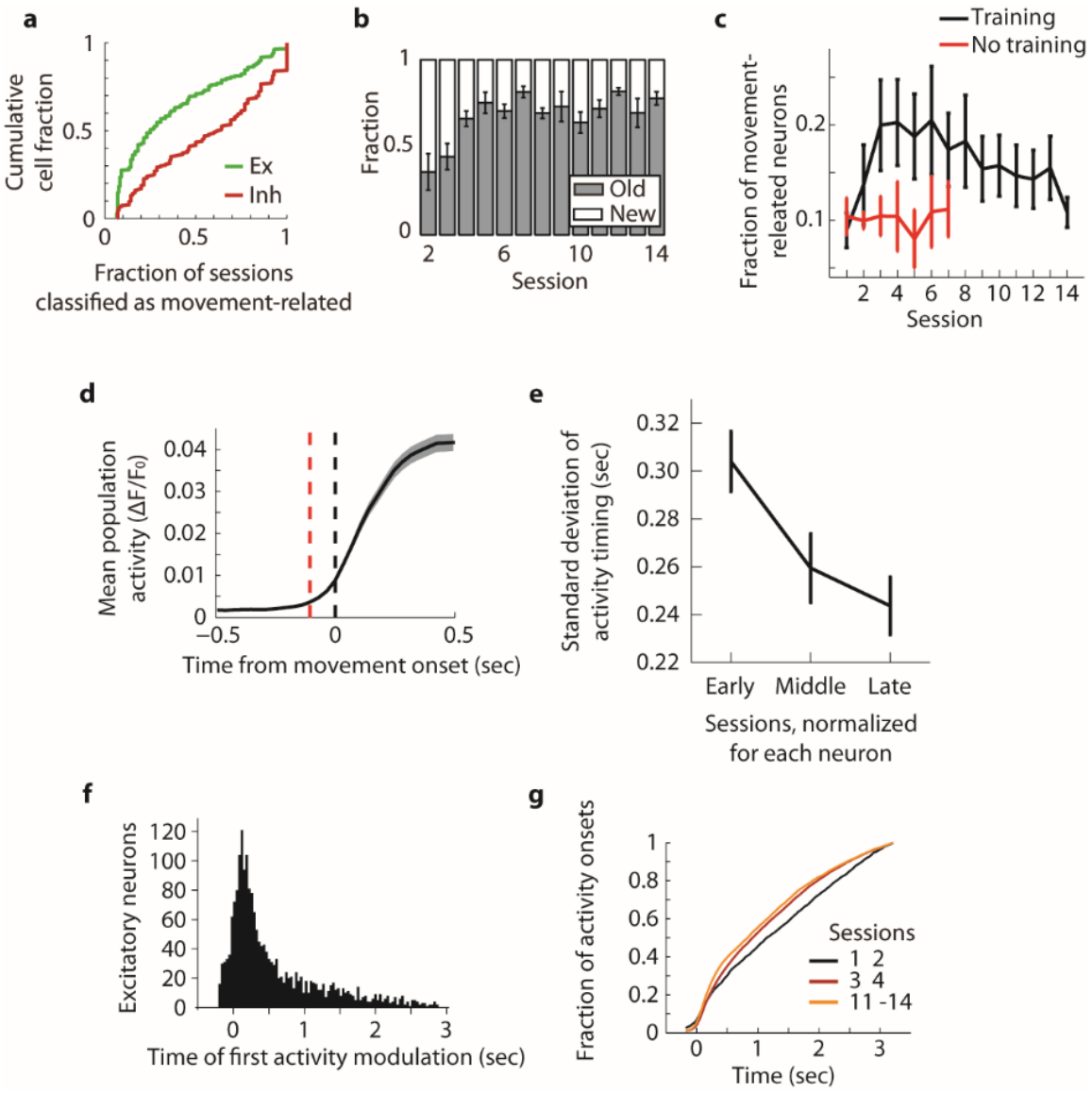
Neuron	1	2	3	4	5	6	Combined
Median offset (ms)	19.0	10	21.8	17.2	-39.1	-8.5	7.1
SD (ms)	62.9	17.4	44.5	30.9	48.9	34.8	41.4
# of events	12	16	17	24	9	30	108

Extended Data Figure 2.4 Simultaneous cell-attached recordings and two-photon calcium imaging in awake mice. **a,** Left, *in vivo* two-photon image of motor cortex neurons expressing GCaMP5G. The neuron in the center is targeted with a patch electrode. Right, after the recording session, voltage step was applied to the electrode to activate the recorded neuron. The increased GCaMP5G fluorescence in the middle neuron confirms that the neuron was indeed targeted. **b,** Example GCaMP5G fluorescence trace (top, black: fluorescence trace; red: detected calcium events) and simultaneously recorded action potentials (black vertical ticks, the numbers indicate the number of action potentials contained in each burst). Horizontal red lines at bottom indicate the duration of detected calcium events. Note the precise temporal relationship between action potentials and calcium events. **c,** Table summarizing data from 6 neurons in 2 mice. Positive offsets indicate the lag of the onset of detected calcium events relative to the first spike in the burst. The offset (7.1 ± 41.4 ms) is on the order of the temporal resolution of our imaging (~35 ms / image frame).

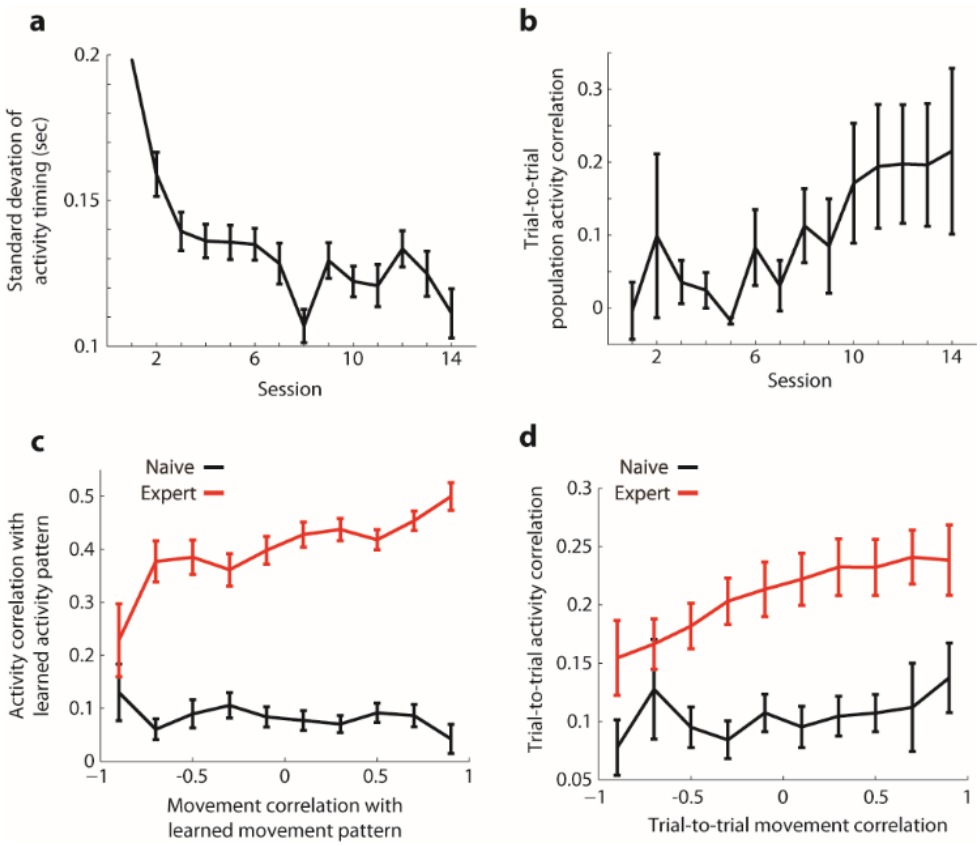


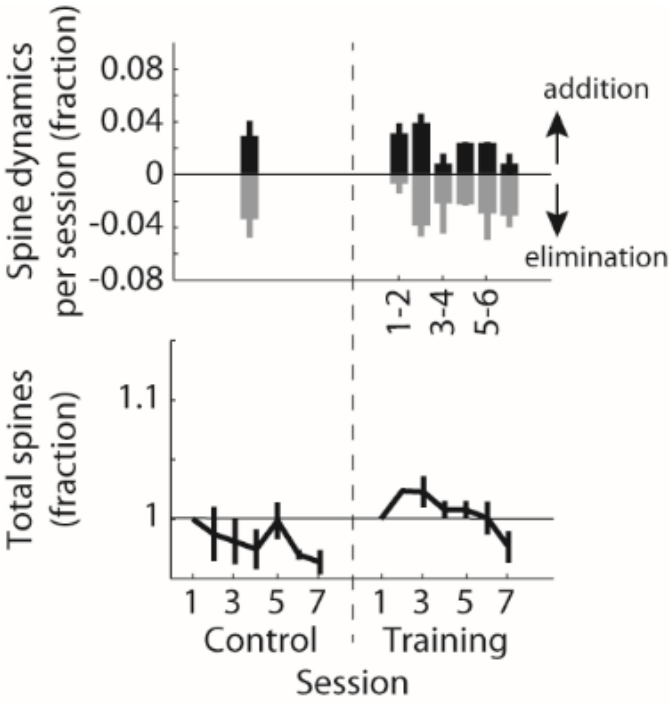
Extended Data Figure 2.5 Lack of spatial clustering of movement-related excitatory neurons. Each plot represents one animal. Red dots show the mean pairwise distance between movement-related excitatory neurons. Solid and dotted black lines show the mean and 95% confidence intervals, respectively, obtained from shuffling the identities of movement-related neurons among all excitatory neurons 10,000 times. Dots below the lower dotted line would indicate significant clustering of cells, while dots above the upper dotted line would indicate the significant dispersion of cells ($p < 0.05$).

Extended Data Figure 2.6 Additional analysis of population activity. a, Cumulative distribution of fraction of sessions classified as movement-related for inhibitory (red) and excitatory (green) neurons, showing the relative invariance of inhibitory neurons and dynamism of excitatory neurons ($p < 0.001$, Kolmogorov-Smirnov test). **b,** Movement-related excitatory neuron populations in each session compared to the previous session. Grey: fraction of neurons classified in the previous session; white: not classified in the previous session. A large number of newly movement-related neurons were added in the first few sessions ($p < 0.001$, comparison between sessions 2-4 vs. 10-14, Wilcoxon rank sum test). **c,** Fraction of excitatory neurons classified as movement-related in each session. Black, training (n = 7 mice, this is the data shown in Fig. 2.2b); red, no training (n = 6 mice). The expansion of movement-related neurons is specific to animals that underwent training ($p = 0.74$, sessions 1-2 combined; $p < 0.001$, sessions 3-7 combined; Wilcoxon rank sum test). **d,** Average population activity aligned to movement onset (black dotted line). Average activity (calcium event trace) of each movement-related excitatory neuron was averaged. The population activity diverged from baseline 105 ms before movement onset (red dotted line, Methods). **e,** Standard deviation of activity timing of individual movement-related excitatory neurons across sessions. Focusing on neurons that are classified as movement-related in 3 or more sessions, the standard deviation of activity onset timing relative to movement onset is plotted across sessions. Sessions were binned into one thirds of the total number of sessions each neuron was classified. Activity timing became more stable on the neuron-by-neuron basis ($r = -0.14$, $p < 0.001$). **f,** Histogram of the time from movement onset that the activity of each movement-related neuron significantly diverged from baseline. 9.2% of movement-related excitatory neurons show significant pre-movement activity, a composition similar to a previous study[14]. 82.7% of activity of movement-related neurons occurred during the periods between 105 ms before movement onset and movement offset (Methods). **g,** The cumulative fraction plot of the timing of all activity onsets of movement-related excitatory neurons during rewarded movements. Each group of sessions is shown as a line, with different colors representing different sessions. The distribution of activity onset timing during later sessions shifts towards the movement onset ($p < 0.001$, Kolmogorov-Smirnov test for all three comparisons). All error bars are SEM.

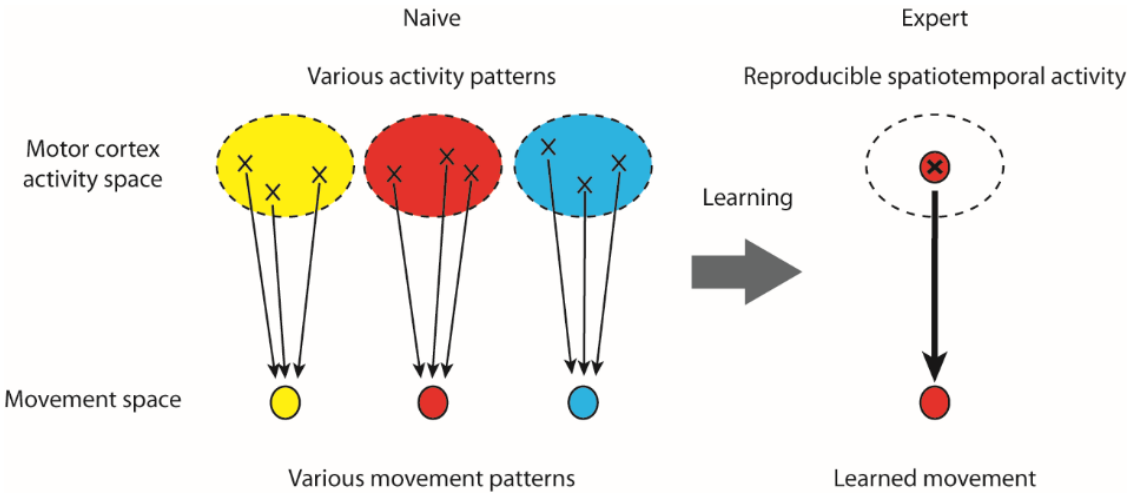

a
Cumulative cell fraction
1
0.5
0
0
0.5
1
Ex
Inh
Fraction of sessions classified as movement-related
b
Fraction
1
0.5
0
Old
New
2
6
10
14
Session
c
Training
No training
Fraction of movement-releated neurons
0.2
0.1
2 4 6 8 10 12 14
Session
d
Mean population activity (ΔF/F0)
0.04
0.03
0.02
0.01
0
−0.5
0
0.5
Time from movement onset (sec)
e
Standard deviation of activity timing (sec)
0.32
0.30
0.28
0.26
0.24
0.22
Early Middle Late
Sessions, normalized for each neuron
f
Excitatory neurons
120
100
80
60
40
20
0
0 1 2 3
Time of first activity modulation (sec)
g
Fraction of activity onsets
1
0.8
0.6
0.4
0.2
0
Sessions
1 2
3 4
11 -14
0 1 2 3
Time (sec)

Extended Data Figure 2.7 Activity analysis focusing on the first 500 ms of each movement. For the activity analyses in the main figures that used the duration of 3 seconds after movement onset, we repeated the same analyses focusing on the first 500 ms of each movement (median time from movement onset to reward = 506 ms) This early activity shows progression throughout learning, similar to when activity over 3 seconds was considered. **a,** Standard deviation of the timing of activity onsets for movement-related excitatory neurons over sessions, indicating a gradual refinement of activity timing ($r = -0.18$, $p < 0.001$). Neurons that were active in less than 5 trials of a given session were excluded from this analysis. The first bin contains only one data point and thus does not have an error bar. This analysis is equivalent to Figure 2g. **b,** Pairwise trial-to-trial correlation of temporal population activity vectors increases with learning ($r = 0.38$, $p < 0.001$). Temporal population activity vector was defined as a concatenation of the activity traces of all movement-related neurons and thus maintained temporal information within each movement. This analysis is equivalent to Figure 2h. **c,** Correlation of spatiotemporal activity with the learned activity pattern is a function of the correlation of movement with the learned movement pattern in expert sessions. Movements similar to the learned movement pattern but made in naive sessions display activity very different from the learned activity pattern ($p = 0.28$ and < 0.001 in the bins 1 and 2-10, respectively, Wilcoxon rank sum test). This analysis is equivalent to Figure 3b. **d,** Pairwise trial-to-trial correlation of temporal population activity vectors plotted as a function of movement correlation on those trials. A strong relationship between population activity and movement emerges during learning ($p = 0.08$, $= 0.08$, $= 0.004$, < 0.001, $= 0.002$, < 0.001, $= 0.001$, $= 0.002$, < 0.001, and $= 0.046$ for each bin, Wilcoxon rank sum test). This analysis is equivalent to Figure 3c. All error bars are SEM.


a
Standard devation of activity timing (sec)
0.2
0.15
0.1
2
6
10
14
Session
b
Trial-to-trial population activity correlation
0.3
0.2
0.1
0
2
6
10
14
Session
c
Activity correlation with learned activity pattern
Naive
Expert
0.5
0.4
0.3
0.2
0.1
0
−1
-0.5
0
0.5
1
Movement correlation with learned movement pattern
d
Trial-to-trial activity correlation
Naive
Expert
0.3
0.25
0.2
0.15
0.1
0.05
−1
-0.5
0
0.5
1
Trial-to-trial movement correlation



Extended Data Figure 2.8 Dynamics of dendritic spines in the hindlimb area during learning of the lever-press task. Summary of dendritic spine dynamics in the hindlimb area during control period (seven days before training) and subsequent seven days of training. Mice were water restricted in both conditions. Top, spine additions (black) and eliminations (grey) in each session. For control sessions, data from all sessions are combined. Bottom, total spine number across sessions. Values are normalized to the total spine number in session 1 in each condition. Unlike the forelimb area, the density of dendritic spines in the hindlimb area is relatively stable during learning ($p = 0.07$, comparisons between Control vs. Training sessions 4-7, Wilcoxon rank sum test). All error bars are SEM.



Extended Data Figure 2.9 Schematic of learning-related changes in the relationship of motor cortex activity and movement. Top, abstract space of activity patterns. Bottom, abstract space of movements. Circles in the movement space represent observed movements, and ovals in the activity space represent possible activity patterns that can lead to corresponding movements. X's and arrows represent example individual trials of activity-movement pairs. In naive animals, each trial involves variable activity and movement patterns as illustrated by scattered X's and multiple movements. In this stage, the relationship between activity and movement is inconsistent (i.e. degenerate), such that same movement is derived from different activity patterns in different trials. During learning, this degeneracy is reduced and a reproducible spatiotemporal activity pattern emerges in the motor cortex that reliably generates the learned movement. This learned activity pattern (thick X) is rarely, if at all, observed in naive stages.

Chapter 2 is a reprint of the material as it appears in Peters AJ, Chen SX, Komiyama T. Emergence of reproducible spatiotemporal activity during motor learning. Nature. 2014; 510(7504):264-267. The dissertation author was the primary investigator and author of this paper.

Chapter 3. Reorganization of corticospinal output associated with movement during motor learning

Summary

The motor cortex can control movements through direct connections to motor circuitry within the spinal cord. Motor skill learning is accompanied by significant changes within the motor cortex, but it is unknown whether these changes are ultimately funneled through a stable corticospinal output or if the corticospinal output itself is a locus of plasticity. We investigated the consistency of motor cortex output by recording the activity of corticospinal neurons through *in vivo* two-photon calcium imaging in mice across learning of a lever-press task. Neurons active during movement were inconsistent across days, where the population first increased and then settled into a subset of newly active cells. Most importantly, similar movements early and late in learning were accompanied by different patterns of corticospinal activity. These results indicate that the output of the motor cortex targeting the spinal cord changes with learning, suggesting that global cortical plasticity takes precedence over stability.

Introduction

The motor cortex is defined by its ability to drive movement when stimulated[1]. This characteristic feature is presumed to be mediated by a direct projection from a subset of motor cortex neurons to motor components within the spinal cord[2,3]. These corticospinal neurons are located within layer 5b of the motor cortex, but are spatially intermingled with non-corticospinal neurons[4]. The

activity of neurons within the motor cortex has been closely linked to movement both specifically in corticospinal neurons[5] and in the general population[6,7], suggesting its role in guiding ongoing behavior. In particular, the motor cortex has been implicated in motor skill learning[8]. Behaviorally, this function is evidenced by the requirement of an intact motor cortex to learn new movements[9] and a deficit in dexterous and skilled movements following acute motor cortex inactivation[10], motor cortical lesions[11], and corticospinal tract transection[12]. Moreover, motor skill learning induces plasticity of the motor cortex at multiple levels, including stimulation-evoked movement maps[13], activity of neurons during learned behavior[14], and dendritic spine growth and turnover[15]. The organization of the motor cortex according to ethological behaviors further supports the notion that it plays a central role in storing circuits that facilitate learned movements[16,17].

Learning-related synaptic plasticity within the motor cortex has been nearly ubiquitously demonstrated. Inputs to the motor cortex are capable of long-term potentiation, including from the thalamus[18] and horizontal connections within the cortex[19]. Learning-dependent dendritic spine growth has also been observed in both superficial[20] and deep[15] layer motor cortex neurons, including in corticospinal neurons[21]. These forms of plasticity are also dependent upon and interact with plasticity in local inhibitory interneurons[22] and downstream structures like the striatum[23]. Given this wide-scale potential for reorganization within the motor cortex, a fundamental question arises as to whether circuits within the cortex operate through a functionally stable output to the spinal cord, or

whether the downstream impact of corticospinal neurons itself changes with motor learning. These two possibilities represent separate schemas of motor cortex plasticity: malleable circuitry within the cortex could overly and ultimately be funneled through a consistent output channel, or otherwise the basic relationship between the corticospinal output of the motor cortex and movement could be flexible.

Different lines of evidence lend credence to each possibility. Individual layer 5 neurons within the motor cortex can be associated with specific components of movement, and changes in activity during learning can directly reflect changes in corresponding movement components[24]. Corticospinal cells specifically have also been suggested to consistently relate to movement across learning[25]. On the other hand, dynamics in the fraction of behavior-related layer 5 cells has been observed during motor learning[26], and artificial feedback can alter muscle activity associated with corticospinal activity[27]. Directly addressing this issue therefore requires specifically monitoring the activity of corticospinal populations across learning in conjunction with a continuously measuring movement. We approached this using targeted *in vivo* two-photon calcium imaging in a lever press task previously used to examine plasticity within layer 2/3 of the motor cortex[20]. By utilizing Cre-dependent expression of calcium indicators and imaging the apical dendrites of deep neurons, we were able to track the activity of corticospinal neurons every day for two weeks while animals learned and performed the task. We found that a subset of imaged neurons were active selectively during movement, but surprisingly that a larger number of neurons

were selectively active during quiescence. The behavioral correlation of each neuron moreover was dynamic across time, where different neurons were active during movement after learning and neurons active during movement early in learning could become selectively active during quiescence after learning. We found that the temporal activity of neurons active during movement was also initially variable but stabilized with learning. Finally, we demonstrated that these changes reflected an unstable relationship between corticospinal activity and movement kinematics. These results indicate that plasticity within the motor cortex during learning is not restricted to inter-cortical circuitry, but extends to the functional output of the motor cortex.

Results

Two-photon calcium imaging of corticospinal neurons during motor learning

We utilized a Cre/FLEX system to selectively express the calcium indicator GCaMP6f[28] in corticospinal cells. This was achieved by injecting a retrograde adeno-associated virus encoding Cre recombinase into the C7/C8 segments of the spinal cord[29] and injecting an adeno-associated virus encoding Cre-dependent GCaMP6f into the right caudal forelimb area of the motor cortex (Figure 3.1A). The caudal cervical segments of the spinal cord were targeted because they contain motor neurons innervating muscles for both limb and paw control[30], and previous studies have shown that corticospinal cells projecting to these segments exhibit plasticity during forelimb motor learning tasks[21]. Fluorescent cells in layer 5b of the motor cortex were observed two weeks after surgery, and these cells projected via the pyramidal tract to the spinal cord

(Figure 3.1B). Fluorescently-labelled axons sent collaterals to the intermediate and ventral lamina of the cervical spinal cord, consistent with targeting motor circuitry within the spinal cord[31]. Axons within the corticospinal tract typically did not extend beyond the thoracic spinal cord (3 out of 4 mice), indicating that labelled cells were specific to forelimb control[32] (Figure S3.1A). Many collaterals were observed in regions outside of the spinal cord, consistent with reports of these cells projecting to multiple areas[33,34] (Figure S3.1B).

GCaMP6f-expressing corticospinal cells were visible under a two-photon microscope, but somata were too deep to allow for direct imaging. Instead, we focused on the apical dendrites passing through the superficial layers (Figure 3.2A). The location of these apical dendrites was stable across days and the same dendrites could be reliably located each day (Figure 3.2B). Activity within these dendrites was observed as bright discrete points in a very low-noise background, allowing for automated region-of-interest (ROI) creation. ROIs that exhibited very similar activity were assumed to be branches from the same soma, and their activity was combined by weighted average. Calcium events were then detected within baseline-normalized traces through thresholding to eliminate fluctuations not related to spiking[20].

In two mice with sparsely labeled populations and with the aid of a module to shorten laser pulse time (DeepSee, Maitai), we were able to track dendrites to their respective somata and confirmed that calcium fluorescence in branches from a single soma were highly correlated (Figure S3.2), in agreement with previous reports[35]. We were also able to simultaneously image certain

somata with their apical dendrites in these mice, and found a high degree of overlap between calcium events in both structures, albeit with disparity between event amplitudes (Figure S3.2). Back-propagating action potentials from cortical pyramidal somata into apical dendrites are known to induce calcium influx through voltage-gated calcium channels and yield corresponding calcium indicator fluorescence events[36,37]. It is notable that in deep cortical cells the coupling between calcium events in the apical dendrite and action potentials is not perfect[38], but because regenerative calcium events in dendrites complement action potentials in the soma[39] and we observed high fidelity between somatic and dendritic calcium events, we suggest that our apical dendrite signals can serve as a proxy for somatic spiking.

We performed apical dendrite imaging while mice were trained in a cued lever-press task previously used to examine plasticity in superficial motor cortex[20,22]. Mice were water restricted for two weeks prior to training to induce motivation for water rewards. They were then trained in the task in approximately one half hour session each day for two weeks. During training, mice were head-fixed under a microscope and rested their right paw on a stationary block and their left paw on a lever attached to a force transducer (Figure 3.3A). The displacement of the lever was continuously recorded, allowing for a measurement of movement kinematics. The task structure consisted of a variable inter-trial interval followed by an auditory cue, during which a press of the lever past a threshold produced a brief tone and a water reward. Mice learned this

task over the course of two weeks and developed an increasingly stereotyped movement to achieve reward (Figure 3.3B).

Corticospinal activity correlates with movement or quiescence

We previously found that population activity within layer 2/3 of the motor cortex is highly correlated with bouts of movement[20], and began by investigating this feature in corticospinal neurons (n = 8 mice). Prior studies have demonstrated that neurons in the deep layers of motor cortex can be differentially tuned to either the direction or magnitude of exerted force[40]. In order to determine parameters of movement relevant to activity, we quantified the distribution of the average position, velocity, and speed during activity for all ROIs. We found that there did not appear to be ROIs which were active during specific velocities corresponding to preferential activation during pushing or pulling (Figure 3.4A). The lack of velocity tuning could potentially be explained by the relatively low temporal precision of our activity measurements with respect to movement dynamics. In order to define a more general measure of movement, the envelope of the lever velocity was used to quantify speed. The distribution of activity relative to speed was highly divergent from chance and included ROIs that preferred both low and high speeds (Figure 3.4A). It has been suggested that different components of the corticospinal system carry information about tonic and phasic aspects of movement[41]. It is unlikely that the low-speed ROIs corresponded to tonic force however, as there was no positional preference in these ROIs (Figure 3.4A). We therefore proceeded to classify ROIs by preferential activity during movement or quiescent epochs, yielding a substantial population

of movement-related ROIs and surprisingly a larger population of quiescent-related ROIs (Figure 3.4B). This is in strong contrast with layer 2/3, where the majority of ROIs have activity preferentially during movement (Figure S3.3A). We validated that binary classification was similar to classification by graded speed preferences, suggesting two disparate populations instead of a continuum (Figure S3.4). The process of classification extracts ROIs which are differentially active given the behavioral state of the animal and are therefore likely involved in movement, and these ROIs were used for all following analyses.

It is possible that these movement- and quiescent-related classes of corticospinal activity are distinguishable during any behavior, but it may likewise be the case that they only emerge under certain conditions. General forelimb use in rodents does not depend on the motor cortex[12], although we have previously demonstrated that increased movement stereotypy in the current task does require an intact motor cortex[20]. We therefore sought to determine whether behavior that was not directed towards the task produced a similar dichotomy of activity classes. To ensure that mice in this condition were not attempting to perform the task, we carried out a separate set of experiments in which mice were subject to the same preparations and conditions as described except task contingency was removed (n = 8 mice). In this "no-task" condition, the water reward was not dependent on lever press but was instead delivered on every trial after a variable cue tone presentation. Mice in this condition moved the lever spontaneously but not out of task necessity. Compared to task mice, no-task mice moved the lever for a comparable duration both within each

movement bout and throughout the session, with primary differences being an increased tendency to pull as opposed to push and a lower amplitude (Figure S3.5A). A similar divergence of activity relative to movement was seen in these mice as in task mice (Figure S3.5B). No-task mice also exhibited an equivalent fraction of movement-related ROIs. Interestingly however, the fraction of quiescent-related ROIs was greatly reduced from task condition mice and equal to the fraction of movement-related ROIs. This result suggests that the overrepresentation of quiescent-related ROIs is unique to task-directed behavior.

Movement-related populations are dynamic and stabilize across learning

The fraction of ROIs which were classified as quiescence-related was consistently higher than those classified as movement-related across learning. The fraction of quiescent-related ROIs increased within the first few sessions and then remained stable, while the fraction of movement-related ROIs increased during the first week and decreased to initial levels during the second week (Figure 3.5A). This increase and decrease in fraction of movement-related ROIs is qualitatively similar to what was previously observed in layer 2/3, although with a slower rise and larger subsequent drop[20]. Also as seen in layer 2/3, the transient increase in fraction of movement-related ROIs was specific to learning and was not observed in the no-task condition (Figure S3.5B).

The change in fraction of movement-related ROIs suggested an explorative process for utilizing different neurons during movement across learning. We addressed this possibility by quantifying ROIs that shared classification across days. The population of movement-related ROIs underwent

a period of turnover and expansion in the first week. During this stage, a new group of ROIs became movement-related that were not classified as such in the first few days. A consistent decline followed these initial changes, in which the movement-related ROIs of each day were a subset of those from the previous day (Figure 3.5A). Over the course of learning, this resulted in an increasingly stable population of movement-related ROIs (Figure 3.5C). The quiescent-related population in contrast appeared to steadily drift, where adjacent days were more similar than separated days (Figure 3.5A). With the exception of the first two days, the stability of the quiescent population did not change over learning (Figure 3.5C).

We then examined cross-over between the two classes to determine whether and to what extent individual ROIs reversed their relationship to behavior. This showed a strong unidirectional bias, where movement-related ROIs in the first week became quiescent-related within the second week, but quiescent-related ROIs did not become movement-related at any point (Figure 3.5A). Mice in the no-task condition on the other hand did not exhibit this restricted pattern of class-switching (Figure S3.5D). The tendency for movement-related ROIs to become quiescent-related could result from different types of changes. First, activity during quiescence may be consistent across days but initially high activity during movement could decrease, effectively flipping the balance between quiescent and movement activity. In this case, there may be a steady "baseline" activity while the movement-related activity is selectively dampened. Otherwise, activity during quiescence might itself increase. This

would imply that activity during quiescence may not merely be "baseline" activity, or that the role of the neuron in the circuit had fundamentally changed. We therefore quantified the activity of ROIs which switched from movement- to quiescence-related during movement and quiescence epochs. We found that while activity during movement of these cells was decreased, this was paired with an increase in activity during quiescence, implicating the latter scenario (Figure S3.6). Notably, the total activity of these ROIs across the entire session did not change, suggesting a possible homeostatic process.

Temporal activity is variable across neurons but becomes more consistent with learning

Movement- and quiescent-related classification indicates a global segregation of activity relative to behavior without regard to temporal dynamics. In order to examine temporal features of activity, we extracted portions of activity surrounding rewarded movements and aligned to movement onset and offset. The average activity of movement-related ROIs correspondingly demonstrated sustained activity selectively during movement, while the average activity of quiescent-related ROIs exhibited sustained activity during quiescence (Figure 3.6A). The activity for each class of ROI was not evenly distributed however, where movement-related activity was biased towards movement onset while quiescent-related activity was biased towards movement offset (Figure 3.6A). The sustained average activity of movement-related ROIs was the result of tiling from ROIs with variable preferred timings, while quiescent-related ROIs had broader temporal activity but more highly distributed towards

movement offset (Figure 3.6B). We previously observed a similar arrangement of movement-related activity during movement in layer 2/3, although activity in layer 2/3 shifted towards movement onset across learning. Corticospinal movement-related activity on the other hand did not shift over learning, and instead the distribution of activity onsets relative to movement onset stayed consistent across days (Figure 3.6C, D).

Given the temporal variability of activity across ROIs, we investigated the consistency of activity within ROIs across learning. Movement-related ROIs in this way could produce a unique but reliable pattern of activity whenever they are positively classified. Alternatively, the activity of movement-related ROIs might change over time even when they are positively classified on multiple days. We therefore correlated movement-aligned temporal activity across days using only ROIs that were classified as movement-related between each pair of days. Movement-related activity stabilized across learning, indicating that individual movement-related ROIs did not use fixed activity patterns but instead were malleable across days (Figure 3.7).

The relationship between activity and movement across learning is novel but equally redundant

A fundamental issue in understanding the function of plasticity in the motor cortex is the stability of the relationship between activity and movement. Because mice in this task gradually alter their movements over the course of learning, it is possible that corresponding changes in activity are explained by variability in movement. We addressed this issue by taking advantage of our

lever trajectory recordings, which provided a measurement of movement kinematics across learning. In particular, we focused on how similarity of movement was related to similarity of activity on a trial-by-trial basis both across in within days.

Mice in this task gradually alter and stereotype their movements leading to reward. These changes in movement could take place overlying a fixed association with corticospinal activity, in which similar movements early and late in learning are accompanied by similar activity. This would suggest a stable regime of motor cortical influence over movement. If similar movements early and late in learning are instead accompanied by dissimilar activity, the relationship between corticospinal activity and movement would appear to be inconsistent. In this case, movements would not be deterministic of activity. We distinguished between these two possibilities by comparing the activity and movements of trials across days. We did this by making templates of "learned" activity and movement by averaging across subsets of trials late in learning, and compared these templates on a trial-by-trial basis to early and late days. We found that movements more similar to the template movement were paired with activity similar to the template activity late in learning; however, this relationship was not present early in learning (Figure 3.8A). This indicates that the relationship between activity and movement is itself plastic, resulting in novel activity being associated with learned movements. We previously observed the same shift in layer 2/3[20], indicating that this principle is common throughout the motor cortex.

A separate feature of the relationship between activity and movement is degeneracy within a stage of learning. Movements could be associated with differing degrees of variable activity, where similar movements may be reliably paired with a certain activity pattern or could otherwise be paired with a wide range of activity patterns. Effectively, this indicates how many different activity patterns are used to produce the same movement. We quantified this degeneracy by comparing activity and movements in a pairwise fashion across trials within early and late stages of learning. We found that this measurement did not change across learning, such that similar movements were accompanied by activity patterns with equal variability early and late in learning (Figure 3.8B). Interestingly, this is in contrast to a reduction in degeneracy across learning previously observed in layer 2/3[20].

Discussion

The motor cortex is thought to play a fundamental role in motor learning and is capable of extensive plasticity. It has been unclear however whether activity within the motor cortex operates through a stable output to the spinal cord, or whether the function output of the motor cortex is itself plastic. We addressed this issue by imaging the activity of corticospinal neurons across learning. We found that the population of corticospinal neurons was diversely correlated with movement, such that certain neurons were active selectively during movement while a larger fraction of neurons was selectively active during quiescence. The activity of corticospinal neurons moreover was not consistent across days, where different cells were active during movement or quiescence

through learning and the temporal activity within cells was variable. These changes ultimately lead to stable spatiotemporal activity associated with movement. Importantly, differences in activity were not attributable to differences in movement, but instead the association between movements and activity shifted with learning.

Heterogeneous correlation of corticospinal activity and movement

The fact that corticospinal neurons can be either correlated or anti-correlated with movement corroborates previous findings dating back to the earliest recordings of motor cortex activity[41]. Moreover, it has been suggested that quiescence-related neurons are found exclusively in intermediate and deep layers of the cortex[6], reinforcing our observed differences between previously recorded layer 2/3 activity[20] and corticospinal cells. We report here though a larger fraction of quiescence-related neurons than typically found in the motor cortex[6,26]. While the reason for this discrepancy is unclear, one possibility is that this balance is specific to corticospinal neurons and not the general deep layer population, or that the movement in our task was particularly effective in eliciting quiescent-related activity. As we demonstrated through the no-task condition, not all movements equally expose these classes of activity.

The diversity of corticospinal activity is not unexpected given the heterogeneity within the corticospinal neuron population[42,43]. The mechanism behind biasing a neuron's activity towards movement or quiescence however could either be a slowly-adjustable intrinsic property of each neuron or a rapidly flexible circuit property. Previous research has supported both of these

alternatives. Towards an intrinsic property, baseline firing rate was originally found to be related to axonal conduction velocity, where low-baseline neurons had faster conduction velocities than high-baseline neurons[41]. Correspondingly, low-baseline neurons were active during movement, while high-baseline neurons could be enhanced or suppressed during movement. It was suggested that these two fast and slow components drive corresponding phasic and tonic aspects of movement[44]. These morphological differences suggest that the activity of corticospinal neurons is at least party linked to inherent features of each neuron. More recently, an increase or decrease of activity during movement of corticospinal neurons was linked to bidirectional responses to neuromodulators, again suggesting a cell property origin of activity differences[45]. Conversely, another study which observed a high fraction of movement-suppressed corticospinal neurons found that these neurons were movement-activated during other types of movements on the same day, suggesting a degree of online control[46]. It is therefore likely that aspects of both these possibilities are in play. These may be reflected in our data respectively through different activity profiles across the classes of neurons but the requirement of directed movement to draw out a difference in activity.

Motor cortex output is flexibly associated with movement

A main finding from the current work is that corticospinal activity changes with learning to create a novel relationship between activity and movement. We previously found this same phenomenon in layer 2/3[20], although extending this result to corticospinal neurons indicates that the motor cortex can fundamentally

alter its functional output. This does not strictly require a change in how downstream motor circuitry is influenced by motor cortical input however. It is possible for example that coupling between corticospinal and spinal cord activity is degenerate, where one subset of possible activity patterns for a given movement are observed before learning and another subset after learning. It is consequently well documented that movement is not perfectly paired with activity of related neurons, and instead the relationship between motor cortex activity and muscles is highly context dependent both generally[47] and specifically in corticospinal neurons[48]. A number of lines of evidence suggest however that there is plasticity downstream of the motor cortex which is important for learning. Corticospinal neurons send collaterals to many other areas besides the spinal cord[34], including the MdV nucleus of the brainstem which is required for learning motor skills[49]. Corticospinal plasticity within the spinal cord is also observed both spontaneously after injury[50] and in an activity-dependent manner[51,52]. Motor learning is therefore likely to be supported by nervous system-wide reorganization.

If corticospinal output is indeed inconsistently predictive of downstream movement, there are also interesting implications for how motor activity is integrated throughout the brain. Collaterals from corticospinal neurons have been thought to carry an efference copy of movement to other areas[53]. These efference copies are proposed to be used to shape ongoing movement and interact with sensory functions, and are hypothesized to act through a forward model where efference copies reliably relay information about movement[54,55]. If

the relationship between corticospinal activity and movement changes across learning, this suggests that corresponding efference copies are not reliable but must instead be actively updated.

Activity changes across learning are different between layer 2/3 and corticospinal neurons

In comparing the current results with experiments focusing on layer 2/3[20], we find certain distinct differences complementary to previous findings[25]. First, we observe more quiescent-related corticospinal neurons than layer 2/3 neurons. This might point to contrasting functions in driving movements between the two populations. The major pathway of the motor cortex consists of external inputs activating recurrent circuitry within superficial layers which subsequently influence deep layer neurons[56,57]. It is therefore possible that while corticospinal cells more directly drive movement and therefore must be selectively excited or suppressed for a given movement, layer 2/3 neurons are sparsely activated as necessary to sculpt deep layer activity.

Second, we found that activity in layer 2/3 shifted towards movement onset across learning while corticospinal activity remained consistently distributed throughout movement. This again has interesting potential implications for the functional role of each population. For example, it has been proposed that temporally complex deep layer activity associated with movement could be initiated by an initial temporally uniform input[58], in this case by layer 2/3. This shift in activity could also reflect different interactions with other motor regions, namely the basal ganglia and cerebellum. The basal ganglia and

cerebellum recipient zones of the thalamus project to the motor cortex in lamina-specific patterns, which may in turn exploit laminar activity for initial movement selection and ongoing movement execution respectively[59].

Finally, we found that degeneracy between activity and movement reduced across learning in layer 2/3 but remained stable in corticospinal populations. This may reflect a higher capacity for degeneracy from layer 2/3 to drive corticospinal cells than corticospinal cells to drive spinal circuits. For example, layer 2/3 circuits might be constructed to activate deep layer neurons from initially broad connectivity to fewer but more reliable connections. Conversely, the variance in corticospinal activity capable of producing given spinal activity may exist within a functional limit. In this case, downstream connectivity might change and therefore shift associations in activity, but the space for variance could be held constant. These results together support future endeavors into examining lamina-specific function of the motor cortex along with learning-related plasticity in downstream motor circuits.

Methods

Animals. All procedures were in accordance with protocols approved by the UCSD Institutional Animal Care and Use Committee and guidelines of the National Institutes of Health. All mice were male and acquired from Charles River Laboratory (C57Bl/6 wild type). All surgeries and experiments were carried out in adult mice (6 weeks or older). All animals were group housed before surgery and singly housed afterwards in disposable plastic cages with standard bedding, nestlets, and a running wheel and kept in a room on a reversed light cycle (12

hour). All experiments were performed at approximately the same time each day during the dark period.

Surgery. Surgeries consisted of two parts, the first being a spinal cord injection and the second being cortical injection and cranial window preparation. Mice were anesthetized with isoflurane and fixed on a bite bar with a nose clamp over a heating pad. The back was shaved from below the shoulder blades to the top of the neck and prepared with iodine and alcohol. A midline incision was made in the skin from below the shoulder blades to the middle of the neck. Fat tissue was removed as necessary to expose the trapezius muscles. The trapezius muscles were then cut along the midline at the shoulder blades to expose the spine. The spinous process on the T2 vertebra was identified and separated from attached musculature. The spine was then fixed using custom metal wedges held by a stereotaxic frame. This was accomplished by lifting the spine from the T2 spinous process while placing the wedges under the trapezius muscles to support the spine from underneath. Fat tissue over the spine was then removed and muscles directly overlying the spine were cut. Laminectomies were performed in the range of the C7 to C5 vertebrae, exposing the C6 to C8 segments of the spinal cord. A viral solution of AAV2/9-CaMKII-Cre (University of Pennsylvania Vector Core Facility) was injected into two sites on the left side of the spinal cord, each injection being 200 nl and placed 400 µm from the midline, 700 µm from the surface, and separated by 600 µm rostrocaudally. After injecting, the wedges fixing the spine were removed, the trapezius was sutured with 5-0 vicryl sutures, and the skin was sutured with 5-0

silk sutures. Cortical injections and cranial windows were then prepared as previously described[20]. Skin overlying the skull was removed, the skull was scraped clean, and a custom headplate was glued to the skull and fixed with dental cement. A craniotomy was then performed over the right caudal forelimb area of the motor cortex as stereotaxically defined[60]. A viral solution of 1:4 diluted AAV2/1-Syn-FLEX-GCaMP6f (University of Pennsylvania Vector Core Facility) was injected into the cortex in five sites in a plus shape, each injection being 40 nl and placed 700 μm from the surface, separated by 500 μm, and centered at 1500 μm lateral and 300 μm anterior from bregma. A glass window consisting of a base and concentrically attached smaller plug was held against the skull and brain respectively, the gap between plug and skull was filled with 1.5% agarose, and the base was fixed in place with dental cement. Baytril (10 mg/kg) and buprenorphine (0.1 mg/kg) was injected subcutaneously at the end of surgery. Animals did not display motor detriments following surgery, and were often observed running on their wheels within one day of surgery.

Behavior. Animals engaged in a lever press task as previously described[20]. Mice were water restricted at 1-2 ml per day beginning three days after surgery for two weeks prior to training. Mice were then trained in the lever press task during two-photon imaging for one session per day lasting approximately one half hour. Mice rested their body and feet in a tube, and placed their right paw on a stable block and their left paw on a movable lever. The lever consisted of a handle glued to a piezoelectric flexible force transducer (LCL-113G, Omega Engineering). Voltage from the force transducer, which was linearly proportional

to the lever displacement, was continuously monitored using a data acquisition device (LabJack) and software (LabVIEW, National Instruments). Presses of the lever were defined as displacement through two thresholds within a short time (~1.5mm to ~3mm below resting position within 200 ms). Task structure consisted of a variable inter-trial interval followed by a cue period during which lever presses triggered water reward. Cue periods and rewards were paired with separate tones, and a failure to press the lever within the cue period resulted in a short burst of white noise. The cue period was reduced during the first two sessions from 30 s to 10 s, and the inter-trial interval was increased during the first three sessions from 2-4 s, to 5-7 s, to 8-12 s to encourage discrete cued movements.

Mice in the no-task condition underwent the same preparations, training, and task structure as defined above, except that water rewards were not contingent on lever press and instead were delivered on every trial after 0.5-2 seconds of the cue tone.

Two-photon imaging. Two-photon imaging was conducted through a 16x, 0.8 NA objective (Nikon) mounted to a commercial two-photon microscope (B-scope, Thorlabs) and using a 925 nm laser (Ti:Sapphire laser, Newport). Images were acquired with Scanimage 4.1 (Vidrio Technologies) at a rate of ~28 Hz, covering ~340 μm^2 with 512x512 square pixels. Frame triggers, lever voltage, and the start times of trials were recorded with Ephus (Vidrio Technologies), allowing for alignment between behavior and imaging. Drifts in the imaging field within a session were manually monitored and corrected. Images were motion corrected

offline by maximizing 2D cross correlation between raw images and an average reference image. For simultaneous dendrite and soma imaging, a z-stack was first collected consisting of 51 slices spanning 400 μm to identify dendrite branching and corresponding somas, with each slice being an average of 100 motion corrected frames. Multiple z-planes were then imaged simultaneously using a piezo mounted to the objective (Physik Instruments). Eight z-planes separated by 50 μm were used to allow sufficient time for the piezo to travel the full range of 400 μm, however only the first and last z-planes were used for analysis.

Automated region of interest generation. Many hundreds of dendrites were imaged within each field which could be sparsely active and invisible when inactive, motivating the use of automated region of interest generation. This was done by first registering maximum projections from all sessions together through affine alignment to account for slight differences in fields across days. All subsequent steps were performed on movies in which each frame consisted of an average of 50 raw images to increase the signal to noise ratio. Each frame was registered according to the affine transformation matrices calculated during session alignment, ensuring that each pixel across the experiment represented the same location in the brain. Any pixels along the edges that were not imaged within and across all sessions were not used any further. Active portions of the field were then defined on a frame-by-frame basis across the entire experiment by subtracting the average image within each session from all frames of that session, and thresholding the average-subtracted frames by one

standard deviation of all average-subtracted pixel values. Dendrites were often closely neighboring and point-spread resulted in overlapping thresholded regions across dendrites, so that time-invariant thresholding was not informative. Instead, we took advantage of the temporal diversity of activity across dendrites to define co-varying thresholded pixels. Our approach was similar in intent to methods using independent components analysis (ICA), however we found ICA to be insensitive and not easily and accurately segmentable, while our eventual method was highly sensitive and cleanly segmented as verified by visual inspection. We began by finding the centroids of all discrete active spots within each frame and summing all active centroids across the entire experiment. Active spots corresponding to a single dendrite could expand and retract depending on the fluorescence amplitude due to point spread, but because the point spread function is symmetrical the centroid remained relatively constant. Because of this, even very closely neighboring dendrites had separate clusters of active centroids. Clusters of active centroids were then reduced to a single local maximum, representing the centers of all objects which were ever active during the experiment. The shapes of dendrites corresponding to those centroids were then determined by finding all frames when a centroid was active, and a border was drawn over connected pixels that were over threshold on at least half of all centroid-active frames. These borders defined regions of interest (ROI) which were then refined. Any ROIs which occupied less than 10 pixels were dilated by 1 pixel. ROIs which overlapped by greater than 50% were usually the same dendrite detected twice or two dendrites which had both

common and unique ROIs, so the larger of the two ROIs was deleted. Any further overlap between ROIs was dilated by 1 pixel to allow a buffer zone and removed from ROIs. ROIs were then dilated by 1 pixel and resulting overlap was removed, ensuring a buffer zone between immediately neighboring ROIs. Any remaining ROIs occupying less than 5 pixels or not encircling an originally defined active centroid were deleted. Finally, ROIs were affine aligned to each session through the inverse of the transformation matrices used to align sessions. Any ROIs not fully imaged in every session were deleted.

Fluorescence analysis. Traces for each ROI were created by averaging enclosed pixels and subtracting background fluorescence. Background subtraction was critical for extracting the activity of single dendrites, as the signal from neighboring dendrites often invaded each ROI. Within each raw imaging frame, background signal was estimated by removing fluorescence values within each ROI and 2D interpolating the missing values from the surrounding fluorescence (inpaint_nans MATLAB function, J. D'Errico, MATLAB File Exchange). Two traces were then produced for each ROI, one averaging the raw imaged pixels and one averaging the background-estimated pixels. The changes in fluorescence (ΔF as defined below) of the background trace was then subtracted from the raw trace, producing a final background-subtracted trace. This process errs on the side of over-estimating background signal because the point spread function decreases superlinearly from the source but interpolation was linear, and because some signal originating from within the ROI was

included. The amplitude of calcium events are therefore somewhat reduced, but contaminating signals are effectively eliminated.

The background-subtracted fluorescence trace for each ROI was then normalized to units of $\Delta F/F_0$, where F_0 represents a continuously-defined baseline. Baseline estimation was performed as previously described[20]. Briefly, a recursive process identified and removed portions of the trace which were active. The resulting "inactive" trace was then loess smoothed and interpolated across active periods, producing a time-varying baseline. The normalized trace was calculated by subtracting the baseline trace from the raw trace and dividing the difference by the baseline trace.

Apical dendrites often have multiple branches within the superficial layers, which led us to combine the traces of ROIs with similar that which likely originated from the same soma. Activity similarity between ROIs was calculated by the dot product of baseline-normalized traces loess smoothed within each session and L2 normalized across all sessions. Any ROI pairs with a normalized dot product over 0.8 were considered sibling branches, and groupings of branches were determined by multi-way comparisons. Final traces for each neuron were derived by weighted averaging of all sibling branch traces according to their across-session L2 norm to take advantage of the highest signal to noise ratios. Each "ROI" in further analysis can therefore correspond either to a single ROI or combined sibling branch ROIs.

Baseline-normalized fluorescence traces within ROIs were subject to calcium event detection in order to remove signals within the trace caused by

calcium indicator dynamics instead of neuronal activity. Two thresholds were defined, one being 3 times the noise to find active portions and the other being 1 times the noise to define baseline. Noise was estimated as the standard deviation of negative fluorescence values mirrored about zero to simulate the noise distribution. Active portions of the trace were identified by a 30-frame loess smoothed trace crossing the active threshold and extended to begin when the baseline threshold was last crossed by the unsmoothed trace. Falling periods of the smoothed trace during active portions were set to inactive. All remaining active portions were considered calcium events and set to the difference between the maximum and minimum values within each event, with all other points were set to zero.

Movement analysis. Voltage from the piezoelectric lever was continuously recorded during each session and parsed into movement and quiescence epochs as previously described[20]. Briefly, movement was first identified by velocity threshold. Movement epochs were then refined by combining nearby epochs, eliminating small epochs, and refining the start and end times of movement epochs according to when the lever position respectively left or entered a baseline defined by adjacent quiescent epochs. Visual inspection confirmed accurate demarcation of behavior.

ROI classification. ROIs were classified as movement-related or quiescent-related similarly as previously described[20]. The fraction of movement frames which contained activity in each ROI was first calculated. This value was compared to a shuffled distribution, where movement and quiescent epochs

were kept intact but shuffled relative to each other 10,000 times. Activity during shuffled movement epochs was compared to activity during actual movement epochs. Actual values that were above the 97.5 percentile of the shuffled distribution were classified as movement-related while actual values that were below the 2.5 percentile of the shuffled distribution were classified as quiescent-related.

Tissue preparation. Mice were anesthetized and transcardially perfused with ice-cold 0.1 M PBS (pH 7.4), followed by a perfusion with ice-cold 4% paraformaldehyde (PFA) solution. Isolated brains and spinal cord were postfixed over night at 4°C in 4% PFA and cryoprotected in 30% sucrose solution for at least 24 h at 4°C.

Microtome-cut (Thermo Scientific Microm HM 430) 60 µm free-floating brain (coronal) and brainstem (sagittal) sections were collected in PBS and stored at 4°C. Cryostat-cut (Leica CM 1900) 20 µm spinal cord sections were collected on microscopy slides (Fisherbrand Superfrost Plus) and stored at -80°C.

Immunofluorescence. Antibodies were diluted in staining buffer consisting of 0.1% (wt/vol) bovine serum albumin (BSA, OmniPur) and 0.3% (vol/vol) TritonX-100 (Alfa Aesar) in PBS. Primary antibodies were incubated over night at 4°C followed by a 3 times wash step in PBS. Secondary antibodies were incubated for 1h at RT followed by a 3 times wash step in PBS. Tissue was mounted using CC/Mount (Sigma).

Primary antibodies used were:

- guinea pig anti NeuN (1:1000, Synaptic Systems, No. 266004)

- ch anti GFP (1:400, Aves, No. 1020)
- goat anti guinea pig Alexa 594 (1:1000, Invitrogen, A11076)
- goat anti chicken (1:1000, Invitrogen, A11039)

Histology imaging. Images of brain and spinal cord sections were taken with Zeiss Imager.M2 with the Apotome.2 attachment controlled with the AxioVision 4.8 software. Taken images were stitched with Microsoft Image Composite Editor Version 1.4.4.0 and color levels were post-processed using Adobe Photoshop CS6.

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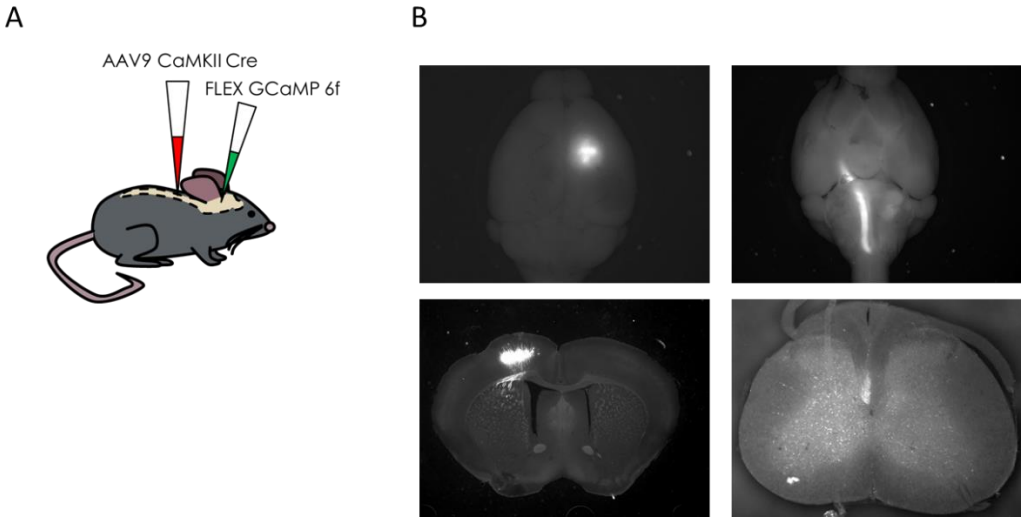

Figure 3.1 Corticospinal labeling. a. Schematic of injections. **b**. GCaMP6f-expressing cells are located in deep layers of cortex and send axons through the pyramidal tract to the spinal cord. Top left: dorsal view of the brain. Top right: Ventral view of the brain. Bottom left: Coronal brain slice including the motor cortex. Bottom right: coronal slice of the cervical spinal cord.

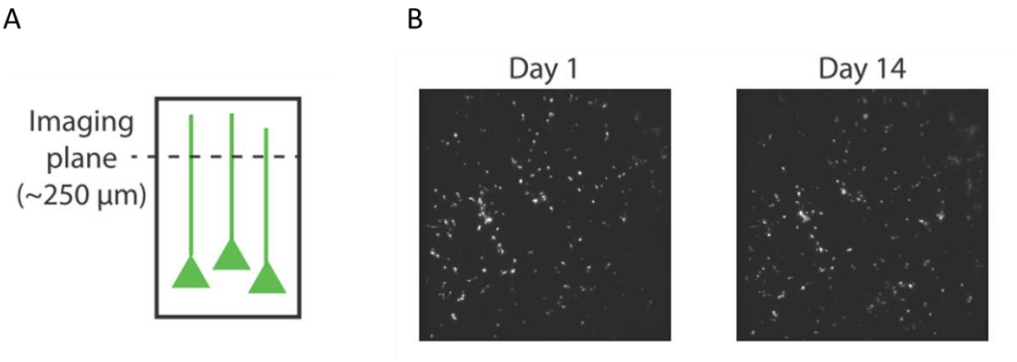

Figure 3.2 Imaging apical dendrites. a. Schematic of imaging plane. **b**. Example imaged fields across days.

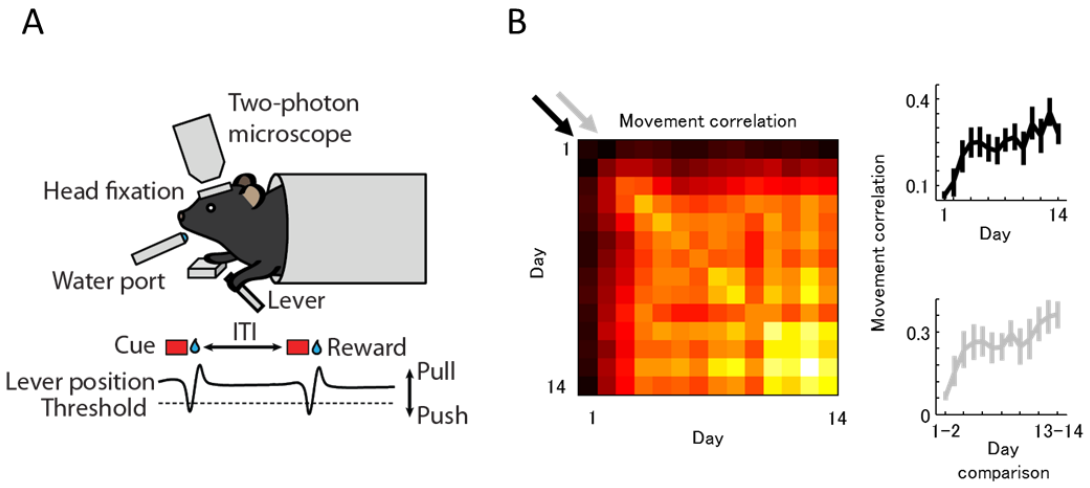

Figure 3.3 Lever press task. a. Schematic of task. **b**. Rewarded movement stereotypy increases across days. Left: median correlations between rewarded movements of all pairs of days. Top right: rewarded movement correlation within days corresponding to the diagonal of the left plot indicated by the black arrow. Bottom right: rewarded movement correlation across adjacent days corresponding to the diagonal of the left plot indicated by the gray arrow.

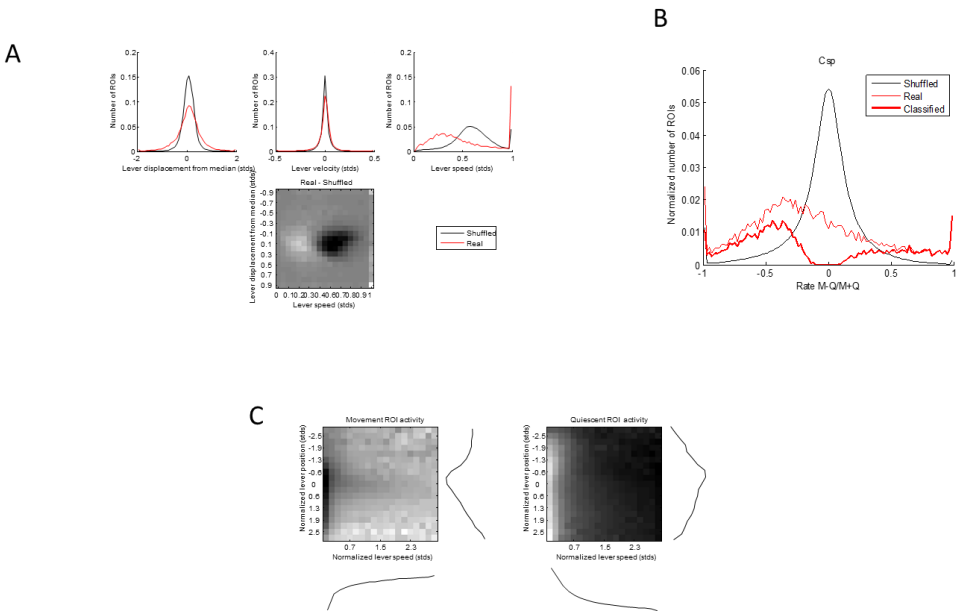

Figure 3.4 ROIs are bidirectionally modulated relative to movement. a. Top: distribution of ROIs relative to displacement from median (left), velocity (center), and speed (right). Bottom: distribution relative to lever displacement and speed independently. Note that ROIs which are shifted towards lower speeds do not have a shifted displacement distribution, suggesting no role in tonic force. Red lines indicate real distributions, black lines indicate shuffled distributions. Values are obtained through the average lever parameter during activity of each ROI across all mice and days. Shuffled distribution is determined by shuffling movement and quiescent epochs within a lever trace 100 times. **b**. Distribution of ROIs relative to movement and quiescent epochs. The red line indicates real distributions, the thick red line indicates subset of ROIs which are classified as significantly modulated by movement or quiescence, the black line indicates the shuffled distribution. Values are obtained by the average fraction of active frames during movement and quiescence for each ROI across all mice and days. **c**. Activity of classified ROIs relative to lever displacement and speed independently. 2-dimensional plots represent binning by parameters independently, adjacent 1-dimensional plots represent binning by each parameters separately. Left: Movement-related ROIs. Right: Quiescent-related ROIs.

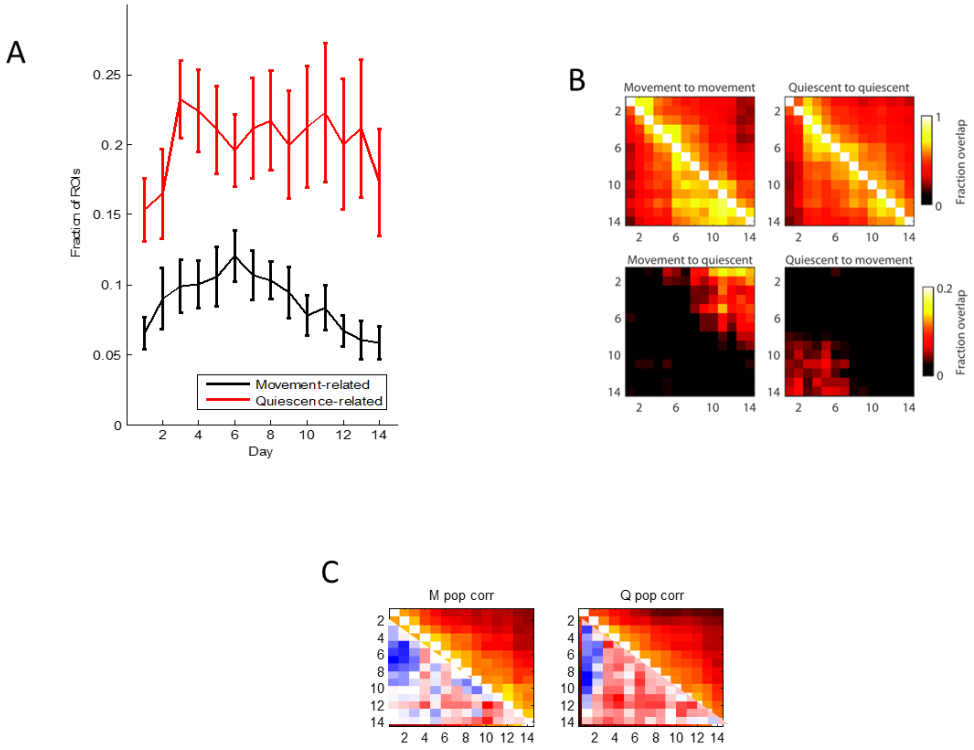

Figure 3.5 Activity relative to movement changes over learning. a. Fraction of classified ROIs across days. The fraction of movement-related ROIs increases in the first week and decreases in the second week, while the fraction of quiescence-related ROIs increases initially and then remains stable. The black line indicates movement-related ROIs, the red line indicates quiescence-related ROIs. **b**. Switch of classification across days. Values corresponding to the X- and Y-axes are the number of overlapping classified ROIs on day X and Y divided by the number of classified ROIs on day Y. **c**. Stabilization of classified population across days. Values in the upper triangular portion represent correlation between binary classification vectors, values in the lower triangular portion represent difference in correlation from mean values shuffled along the diagonals to highlight changes. Left: movement-related ROIs. Right: quiescent-related ROIs.

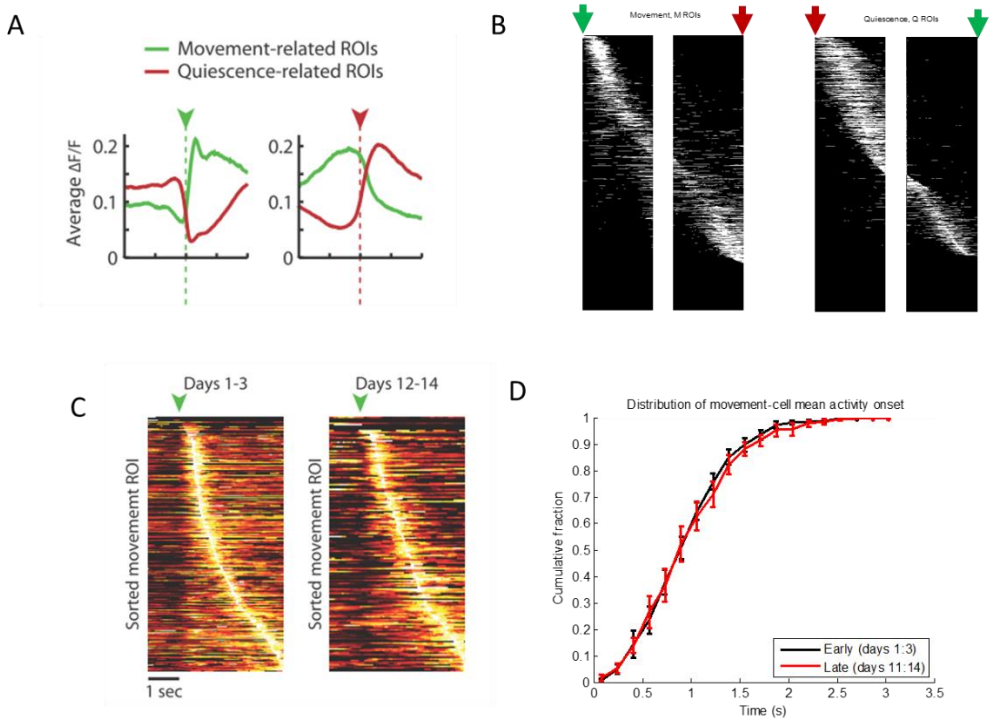

Figure 3.6 Activity occurs consistently throughout behavior. a. Average temporal activity of all classified ROIs relative to movement. Left: activity aligned to movement onset, denoted by the green dotted line. Right: activity aligned to movement offset, denoted by the red dotted line. Values are from all classified ROIs across all mice and days. **b**. Windows of preferred timings are spaced across movement for movement ROIs, but are broader and less evenly distributed for quiescent ROIs during quiescence. Values represent frames which contain significantly more activity than expected from shuffling activity within each trial independently. Left: Movement-related ROI activity timing during movement. Right: Quiescent-related ROI activity timing during quiescence. Green arrows indicate movement onset and red arrows indicate movement offset. **c**. Trial-averaged activity of movement-related ROIs across days. Green arrows indicate movement onset. **d**. Distribution of activity onsets of movement-related ROIS relative to movement onset across days.

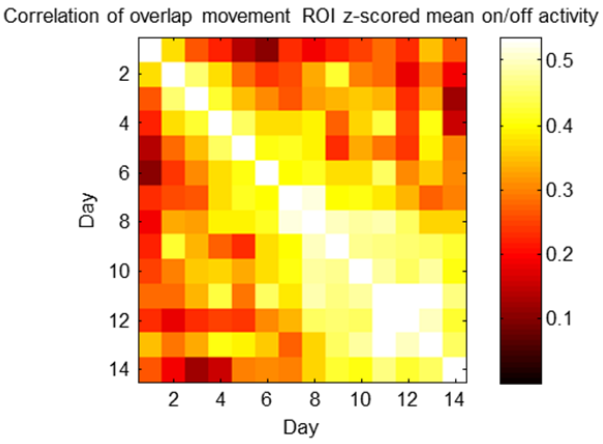

Figure 3.7 Temporal activity of movement-related ROIs stabilizes.
Temporal activity of movement-related ROIs stabilizes. Values are obtained by correlating concatenated movement onset- and offset-aligned activity. Only ROIs which are classified as movement-related on each pair of days are compared.

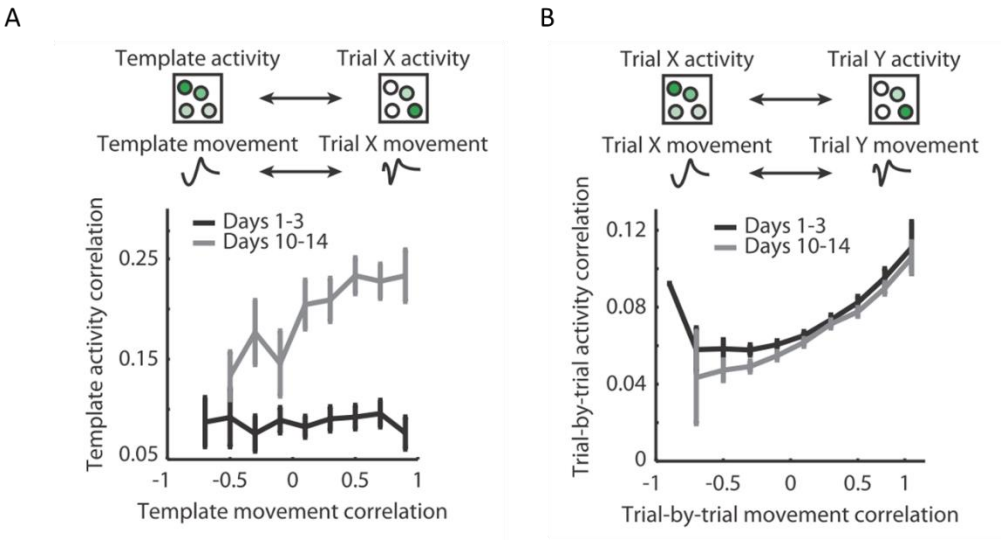

Figure 3.8 The relationship between movement and activity across learning is novel but similarly degenerate. a. Template activity and movement correlation. **b**. Pairwise activity and movement correlation

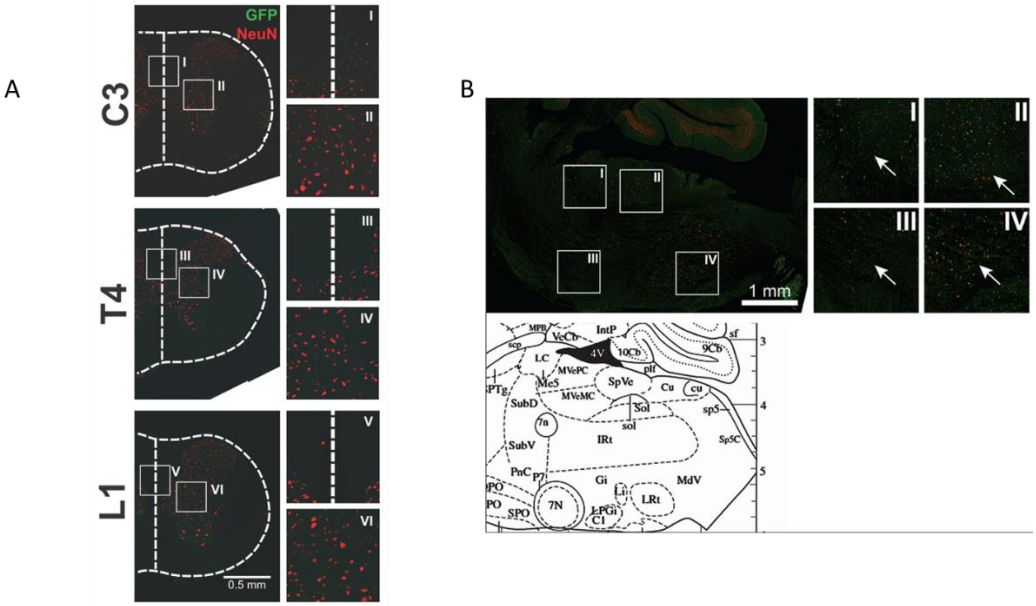

Figure S3.1 Characterization of corticospinal targets. a. GCaMP6f-expressing axons are observed in the cervical but not lumbar segments of the spinal cord. Fluorescent axon terminals are observed in the intermediate and ventral lamina. **b**. GCaMP6f-expressing axonal terminations are found outside of the spinal cord, including the cortex, striatum, and brainstem.

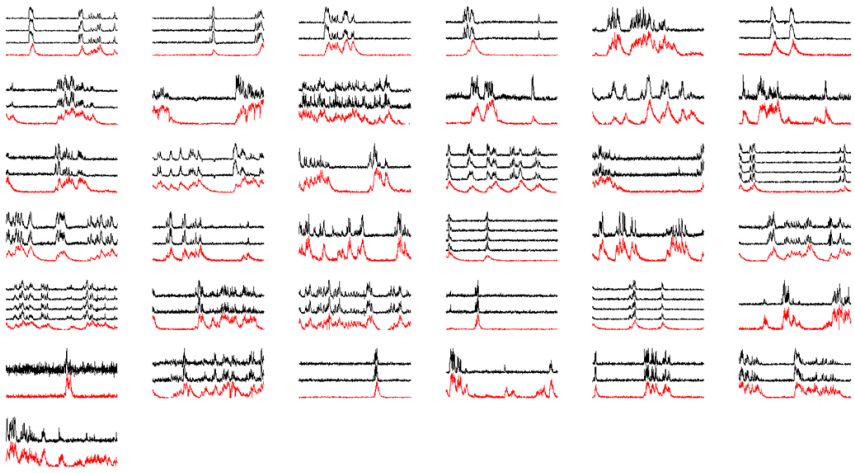

Figure S3.2 Simultaneous soma and apical dendrite imaging. Fluorescence traces from all simultaneously imaged somata and apical dendrites. Red lines indicate somata, black lines represent each branch of the apical dendrite.

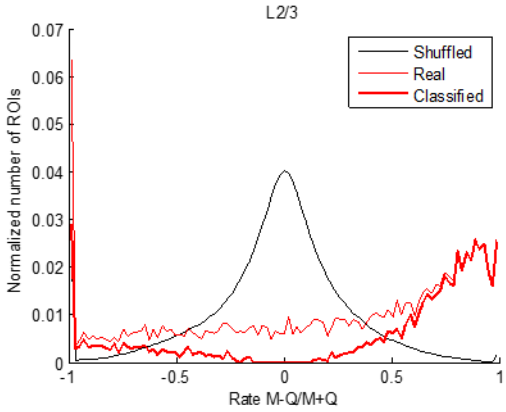

Figure S3.3 Comparisons with layer 2/3. Distribution of layer 2/3 ROIs relative to movement and quiescent epochs, as in Figure 4B. The red line indicates real distributions, the thick red line indicates subset of ROIs which are classified as significantly modulated by movement or quiescence, the black line indicates the shuffled distribution. Values are obtained by the average fraction of active frames during movement and quiescence for each ROI across all mice and days. Data was from previous study.

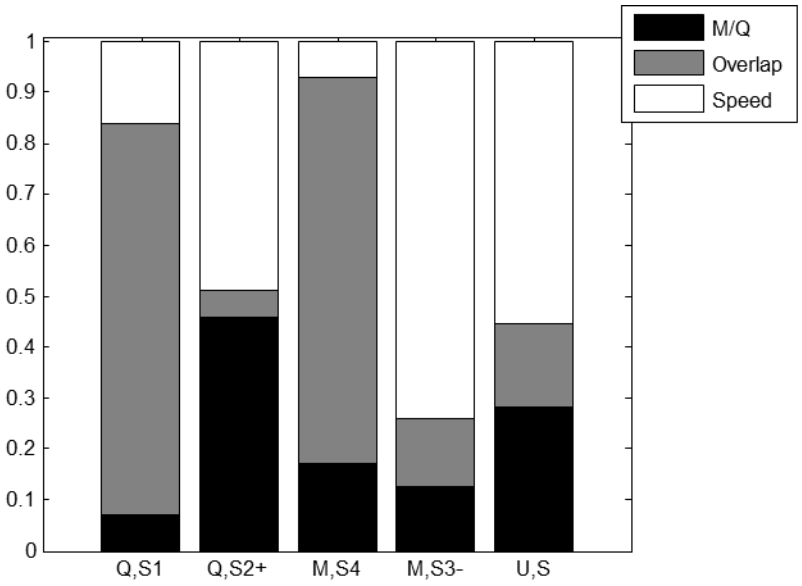

Figure S3.4 Classification by speed and movement or quiescent epochs is equivalent. Overlap of binary movement- or quiescent-related classification with graded speed classification. Speed was grouped into 4 bins respectively constituting 0-0.25, 0.25-0.5, 0.5-0.75, and 0.75-Inf standard deviations. Most quiescent-related ROIs were also significant within the first speed bin, and most movement-related ROIs were significant within the last bin.

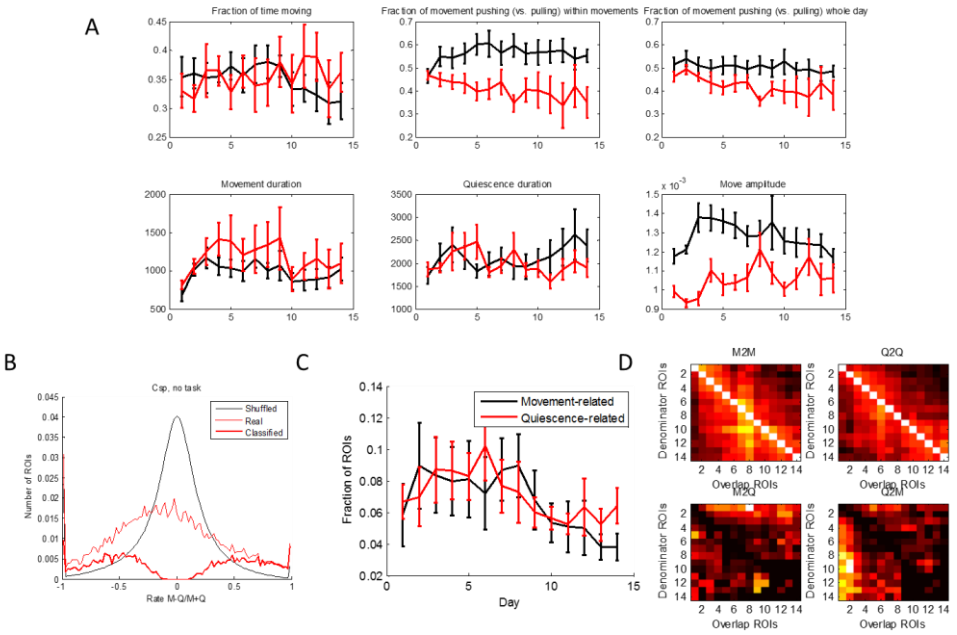

Figure S3.5 Mice not engaged in a task show different activity dynamics. **a**. Parameters of movement across days. Task mice indicated by black lines and no-task mice indicated by red lines. **b**. Distribution of ROIs relative to movement and quiescent epochs in no-task mice, as in Figure 4B. The red line indicates real distributions, the thick red line indicates subset of ROIs which are classified as significantly modulated by movement or quiescence, the black line indicates the shuffled distribution. Values are obtained by the average fraction of active frames during movement and quiescence for each ROI across all mice and days. **c**. Switch of classification across days, as in Figure 5B. Values corresponding to the X- and Y-axes are the number of overlapping classified ROIs on day X and Y divided by the number of classified ROIs on day Y.

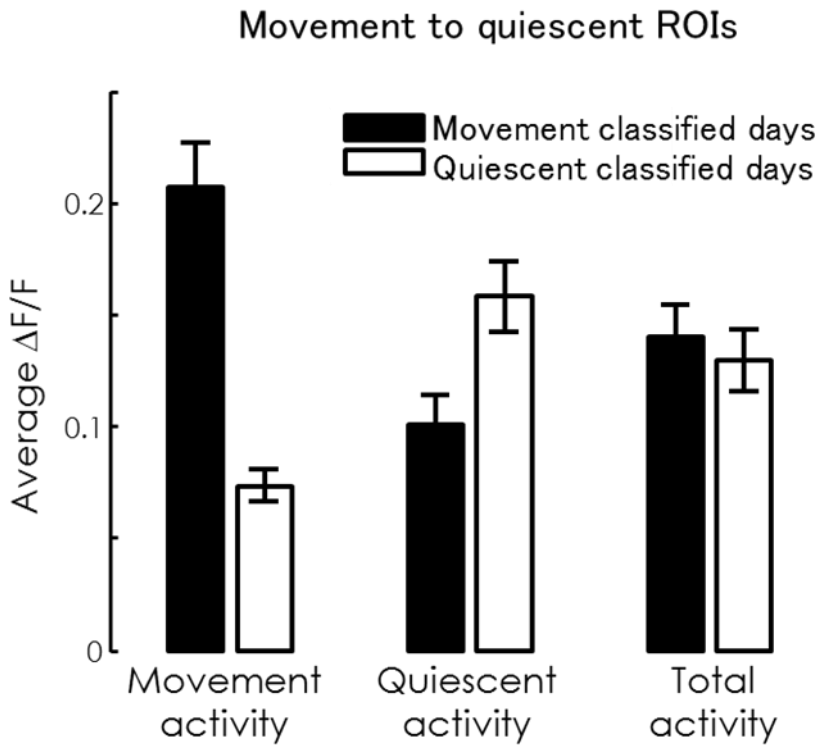

Figure S3.6 ROIs which switch from movement- to quiescence-related redistribute activity. Average activity of ROIs which are movement-related in the first week and quiescent-related at any point in the second week. Activity is separated by movement and quiescent epochs.

Chapter 3 is currently being prepared for submission for publication of the material. Peters AJ, Lee J, Komiyama T. Reorganization of corticospinal output associated with movement during motor learning. The dissertation author was the primary investigator and author of this material.