**ABSTRACT**

The motor cortex is responsible for the planning, controlling and execution of movement. Damage to this region or any associated cortex results in impaired motor skills. Investigating the functions of the different regions that make up the motor cortex and how they relate to each other is crucial for better understanding the origins of movement. In this thesis, we set out to resolve a controversy within the study of movement. In the motor cortex, two regions known as the primary motor cortex (M1) and the premotor cortex (PMC) are synaptically connected. However, limited and conflicting research has resulted in a lack of consensus on whether the PMC influences movement independently or is mediated through M1. We used new statistical methods of causal analysis to look for evidence of a motor cortical hierarchy in mice. Individual recordings of neurons in rostral forelimb area (RFA), which can be considered the mouse equivalent of PMC, and caudal forelimb area (CFA), which is similar to M1 region, were analyzed using jitter-corrected cross correlogram (JCCG) analysis which showed weak evidence of a connectivity that could be indicative of a hierarchical relationship. Further statistical analysis, including transfer entropy, showed slightly more significant information flow from RFA to CFA which is consistent with the hypothesized directionality of the hierarchical relationship. From these results, we propose that if there is a functional hierarchy between RFA and CFA in mice, it is very slight. These results can be used for further understanding the question of hierarchy between the PMC and M1.

**INTRODUCTION**

*Broader Significance*

Movement informs how an animal interacts with its environment. Furthermore, the ability to perform complex, coordinated movements is often evolutionarily advantageous (Welniarz et al., 2017). In rodents, for instance, adaptive behaviors stemming from learned movements such as climbing and burrowing are required for finding food and nesting, illustrating the evolutionary necessity of complex movement (Latham and Maston, 2004). Studies have implicated many brain regions, such as the cerebellum, basal ganglia and motor cortex, in controlling the motor system. In vertebrates, both the cerebellum and basal ganglia are highly conserved; however, the motor cortex is more developed in mammals than amphibians, reptiles, or birds **(**Butler and Hodes, 2005; Shmuelof and Krakauer, 2011). Currently, the motor cortex is being researched as a potential control center for movement in mammals.

The motor cortex is a region of neocortex that serves an important role in the planning, coordination, and execution of movement. Stimulation of motor cortex neurons causes muscle contractions, meaning that the motor cortex is capable of driving movement (Leyton and Sherrington, 1917; Penfield and Boldrey, 1937; Van Acker III et al., 2013) . Additionally, in lesion studies with both nonhuman primates and rodents, the animals were unable to perform complex movements, including reaching and grasping. Previously learned behaviors such as running on a treadmill were still possible (Piecharka et al., 2005; Alaverdashvili and Whishaw, 2008). However, many of the nonhuman primates eventually showed full recovery after lesion (Darling et al., 2011). This implies that there may be some compensatory pathway that is redundant with some of the functions of the motor cortex. However, in a study that accounted for potential compensatory pathways by looking at short latencies after inactivation, it was observed that significant motor control begins at the motor cortex (Miri et al., 2017). Therefore, regardless of compensatory pathways, the motor cortex is both capable of driving and coordinating movement.

There are still many questions left to be answered about the functional organization of the motor cortex. In particular, it is debated exactly how the discrete regions encompassed in the motor cortex coordinate in the planning and execution of movement. The lack of a clear picture of organization of this cortical region comes from many limitations. Some of them include limited studies, behavioral paradigms that do not capture natural movement, and a host of technical limitations that arrive from attempting to record large areas of neuronal activity in real time. One prevailing organizational theory states that there is a hierarchy amongst distinct functional regions within the motor cortex that dictate movement control. In this thesis, we take a new approach to tackling this potential motor cortical hierarchy. By using multi-region recording, we can simultaneously record multiple areas of the brain to get a real time picture of information flow at cellular resolution and perform novel methods of directional analysis based on synaptic connectivity between regions. This approach allows for more specific and direct analysis of causality, creating a unique perspective on the central question of motor function hierarchy that has never been considered before.

*A Brief History of Motor Cortex Organization*

In attempting to reconcile the observations made both in older, cellular-based cortical movement studies and newer, population-based ones, two conflicting models of the motor cortical organization emerged. One states that there are cortical clusters that are distinct in the behavior they provoke. The other concludes that there is a processing hierarchy within the motor cortex that integrates movement bilaterally. The conversation regarding these two theories of movement dominates discourse about cortical influence on movement.

Since its discovery by Fristch and Hitzig in 1870, the motor cortex has been researched as a control center for movement. Fritsch and Hitzig observed that specific cortical regions of the brain, when stimulated, can cause a dog to twitch involuntarily. Ferrier would go on to replicate this research in a wide variety of animals and find that stimulation could also lead to movement involving multiple coordinated muscles (Ferrier, 1873). These findings informed many further studies including Sherrington’s, which proved that stimulating different regions of the brain would result in particular coordinated movements. Additionally, they showed that function was recovered after lesion, implying compensatory pathways. (Leyton and Sherrington, 1917). Based on these findings, Sherrington created a map of a monkey brain that was largely based on movements, not muscles (Sherrington, 1939; Graziano, 2008). This map was largely overshadowed, however, by the picture Penfield would publish of the human brain: the homunculus (Penfield and Boldrey, 1937). Despite the picture’s overly simplistic suggestion that the motor cortex has a strict, somatotopic organization, this map would go on to inform views of the motor cortex for decades to come and is still fairly influential today.

Novel technological advances in the second half of the twentieth century allowed for more complex, integrative mapping of the motor cortex. For the first time, after ~100 years of motor cortical research, scientists were able to go beyond surface stimulation of the cortical regions because of the invention of microelectrodes which allowed for researchers to give short pulses of lower levels of stimulation directly onto targeted regions of the motor cortex (Graziano 2008). This reduced the issue of overlapping current spread that informed many of the existing maps of the motor cortex, including Penfield’s. Furthermore, the development of spike-triggered averaging techniques, when combined with microstimulation, was integral for many important observations made about the organization of the motor cortex. In 1985, Cheney and Fetz performed a series of experiments that proved that neurons were capable of influencing more than one muscle by using these new techniques in monkeys (Cheney and Fetz, 1985).

One model emerged from further studies that showed the motor cortex as being organized by specific motor behaviors. One study injected a retrograde virus into hand muscles, revealing that not only do many neurons project to the same, singular muscle, but one neuron innervates multiple muscles (Rathelot and Strick, 2006). This shows that some motor neurons exhibit divergence indicative of being able to control a network of muscles. In addition, it was found that the motor map can be changed with behavioral experience. When monkeys were trained to combine the use of two arm joints, the motor cortical representation of these joints overlapped more in the motor cortex (Nudo et al., 1996**)**. This leads to a model of movement that focuses on behaviors that are mapped across the motor cortex.

More recent theories have focused on the firing patterns across a population of neurons for determining motor output (Pandarinath et al., 2018; Warriner et al., 2020). For example, the premotor cortex alters population activity in the primary motor cortex in response to unpredictable stimuli (Perich et al., 2017). Since premotor cortex stimulation alone is not involved in movement, it implies a shaping role. This means that different areas of the cortex are active in motor planning, even if they play no direct role in the execution. This provides some evidence for a hierarchical model of motor cortical control.

*Hierarchy: A Controversial Method for Organizing Cortical Regions*

A potential hierarchical organization of the motor system has undergone many revisions in the past century, influenced primarily by the available technology at the time, and remains in constant debate. However, in order to have a hierarchical representation, it is necessary to first establish distinct functional cortical fields. While today, the functionally-defined regions of motor cortex are predominantly the premotor cortex (PMC), the primary motor cortex (M1), the supplementary motor area (SMA), which is often considered part of the PMC, and sometimes cingulate motor areas, this was not always the case. In 1905, Campbell, based on the appearance of cells and fiber tracts, proposed two cytoarchitectural regions of the motor cortex: the “precentral” cortex (a posterior region) and the “intermediate precentral” cortex (an anterior region). Additionally, due to the cellular makeup of the two areas, the “precentral” cortex was stated as more important to movement (Campbell, 1905). Brodmann observed the same regions but referred to them as “area 4” and “area 6” respectively, which is the terminology still used today (Brodmann, 1909). While we now know that it is dubious to determine the function of the brain from its appearance, Campbell’s suggestion of a hierarchical division would inspire many future studies.

Further investigation into the theory of distinct fields within the motor cortex resulted in the discovery of different motor maps. Vogt and Vogt divided the motor cortex of monkeys into three cytoarchitectural regions known as areas 4, 6aɑ, and 6aβ, similar to Brodmann’s definitions (1926). At low current levels of stimulation, both areas 4 and 6aɑ evoked simple twitches while 6aβ had no effect on movement. On the other hand, high current levels evoked complex movements when applied to 6aɑ and 6aβ but no movement from area 4 (Vogt and Vogt, 1926). According to these results, area 6, as a combination of 6aɑ and 6aβ, is capable of two vastly different pathways for movement. For complex movement, area 6 directly projects to deeper areas such as the spinal cord. For simple movements, area 6 is mediated laterally by area 4. This theory would later be confirmed by a series of lesion experiments done in monkeys (Bucy, 1933). It still remained ambiguous, however, whether this was indicative of a hierarchical series or instead the two areas operate in parallel whilst processing different aspects of motor behavior.

Further studies expanded upon the potential of a hierarchical cortical relationship. The term PMC, as it is understood today, became popularized by a series of experiments done by Fulton on monkeys (1934). Lesions to only the PMC resulted in the disorganization of voluntary movement whereas lesions to the M1 alone caused temporary paralysis. Lesions to both areas resulted in permanent paralysis (Fulton, 1934). Fulton concluded that the PMC controlled complex motor movements while the M1 evoked simple movements, a similar conclusion to that of Vogt and Vogt. Fulton speculated that while there may be a natural motor hierarchy, there is still some level of lateral connection between the two regions because they both had separate projection tracts to the spinal cord.

Many scientists were more reluctant to recognize a motor cortical hierarchy including the premotor cortex because it seemed to overly simplify a complicated system. Penfield and Welch proposed an alternative model: a second motor area on the medial part of the hemisphere that they would name the SMA. This conclusion was reached from observations of somatotopic maps of monkeys as well as stimulation studies of the medial maps evoking movement that combined both sides of the body whereas the lateral map tended to only evoke one side (Penfield and Welch, 1951). These findings suspended further research into the PMC as a distinct cortical field for thirty years as well as established the SMA as a separate motor area. However, despite these disagreements on the location and function of separate cortical regions, it was becoming undeniable that the motor cortex consisted of a mosaic of cortical fields that generated complex movements by mediating motor output and high-order cognitive processing. The conversation was no longer if separate regions with their own functionality existed, but instead focused on how to define these regions and how they related to each other.

The PMC and SMA were considered to be operating at a more specialized level than the M1. This is consistent with high-order processing that can be interpreted as operating on a higher cortical level. Roland and his colleagues recorded cerebral blood flow in human subjects as they performed a variety of tasks, they came to the conclusion that the PMC participated in learning new movements and modifying previous ones, the SMA participated in coordination of complex movement, while the M1 was involved in the basic execution of motor tasks (Roland and Larsen, 1976; Roland et al., 1980; Roland et al., 1980). These results, however, do not prove the existence of hierarchy for these differences may arise from heterogeneity in neuronal populations as well as behavior specialization of cortical regions in the brain (Graziano, 2008).

Similar high-order processing has been found in both the ventral and dorsal PMC. The neurons in the ventral PMC are capable of responding to tactile, visual, and auditory signals and of remembering these signals (Fogassi et al., 1996; Rizzolatti et al., 1981; Graziano et al., 1999; Graziano et al., 1997). Based on these results, it was speculated that the ventral PMC was responsible for defending the body. This theory was later confirmed when stimulation of this area resulted in defensive behaviors. To contrast, the stimulation of the dorsal PMC results in reach-to-grasp movements that require both preparation and planning (Graziano et al., 2002). Therefore both the ventral and dorsal PMC are capable of high-order processing and therefore may participate in a cortical hierarchy with the M1.

*Mouse: An Emerging Model for Cortical Motor Control*

Recently, the mouse has emerged as a new motor system model for physiological and anatomical studies of the motor cortex. This is opposed to historical research where monkeys were most often the animal of choice due to their similarity to humans, ability to perform dexterous and complicated movement, and that their behaviors are often both predictable and identifiable (i.e. climbing, reaching, leaping, and defensive maneuvers) (Warriner et al., 2020). This leads to the mouse often being overlooked as a model for movement despite the fact that their motor cortex actually closely resembles those of cats, rats, and monkeys (Tennant et al., 2011).

However, there are many modern papers that use mice for studying cortical control of movement because of the many benefits this model organism provides (Warriner et al., 2020). One advantage is the ability to directly relate the organization of neural systems to their function due to the relatively smaller size of a mouse compared to a monkey. It is also comparatively easier to optically record neural activity with calcium indicators during free behavior (Ghosh et al., 2011; Cai et al., 2016) as well as when head-fixed (Dombeck et al., 2009). Altogether, this allows for an opportunity to examine the complete process of cortical movement control, instead of fragmented sections of the cortex, that can potentially be instrumental in achieving a fuller understanding of cortical inputment in movement than what currently exists. Furthermore, using a new model system also provides an opportunity to make more generalizable statements about the function of the motor cortex as well as how evolutionary differences may manifest in movement control.

*Relevant Regions of the Mouse Motor Cortex*

In mice, the forelimb area is important for voluntary movement and is subsequently the largest area of movement representation, making it the ideal place to study movement control by the motor cortex (Tennant et al., 2011). When stimulated, this forelimb area elicited movements of the elbow, wrist, and digit (Tennant et al., 2011;Li and Waters 1991; Pronichev and Lenkov 1998). Within the forelimb area, mice have two distinct spatial maps called the rostral forelimb area (RFA) and caudal forelimb area (CFA) that were identified both by intracortical microstimulation (Neafsey et al., 1986; [Rouiller et al., 1993](https://www.frontiersin.org/articles/10.3389/fncir.2013.00055/full#B31); Tennant et al., 2011) and Channelrhodopsin-2 (ChR2) photostimulation mapping (Hira et al., 2013). Both the relatively smaller RFA and larger CFA contain corticospinal neurons. However, RFA mainly consists of elbow and wrist representations and in CFA digit representation is dominant (Tennant et al., 2011). These representation differences suggest a model where RFA and CFA may overlap in function whilst serving distinct roles in cortical movement control. While research on the mouse motor cortex is relatively new, there is evidence that RFA and CFA regions have their own motor hierarchy, serving as the rodent equivalent of the primate’s PMC and M1 respectively.

There is a distinct structural connectivity between RFA and CFA that is indicative of a rodent motor hierarchy, similar to the potential primate motor hierarchy. Anterorade and retrograde tracers in rats revealed found asymmetrical projections between the two areas. Corticospinal neurons of CFA project to layers 5 (L5) and 6 (L6) in RFA whereas neurons in L5 and L6 of RFA project to all layers of CFA (Rouiller et al., 1993). Experiments using mice and ChR2 photostimulation mapping paired with in vivo electrical showed similar asymmetry. L5 neurons of RFA received functional projections from layer 2/3 and/or layer 5a of CFA but not layer 5b. Conversely, L5 neurons of CFA received input from layer 5b of RFA (Hira et al., 2013). Typically, higher sensory areas project to lower areas from L5 whereas the reverse projections originate predominantly from upper layers (Maunsell and Van Essen, 1983; Coogan and Burkhalter, 1990). Therefore, these results are strong evidence for a model that places RFA as a higher motor area, such as the premotor cortex.

Furthermore, RFA and CFA have divergent corticocortical connections. In comparison to CFA, RFA receives a smaller portion of projections from the primary somatosensory cortex (S1) compared to the secondary somatosensory area (S2). The S2 input to RFA largely consists of non-forelimb body representations while the S1 input to CFA is primarily forelimb. (Rouiller et al., 1993; Mohammed and Jain, 2016). This is consistent with RFA being a higher motor area that is potentially capable of integrating forelimb movements with the rest of movement.

Moreover, RFA shows more evidence of higher-order processing because of its strong connections to other cortical areas. Tracing experiments revealed that RFA has dense reciprocal connections with the insular cortex, similar to the SMA in primates (Rouiller et al., 1993). The insular cortex, as well as RFA, has been implicated in value-based action selection ( Sul et al., 2011; Parkes and Balleine, 2013). Further research showed substantial inputs from the orbitofrontal cortex to the RFA (Mohammed and Jain, 2016). Together, this information shows that RFA takes on a higher level of control by participating in voluntary and choice-based movements.

Additionally, thalamocortical and corticostriatal connections from RFA and CFA mirror PMC and M1. In primates, the ventrolateral nucleus (VL) projects to the M1 (McFarland and Haber, 2002). Similarly, in rodents, thalamic inputs to CFA also originate from the VL. RFA inputs come from the ventromedial (VM) and mediodorsal (MD) nuclei (Rouiller et al., 1993; Mohammed and Jain, 2013), the latter of which is interconnected with the dorsolateral and orbital prefrontal cortices in primates and eventually relays to thalamus and then to PMC (McFarland and Haber, 2002). Finally, RFA bilaterally projects to the dorsal striatum, similarly to PMC. CFA and M1 project to the ipsilateral striatum (Rouiller et al.,1993). RFA and CFA in rodents can therefore be considered equivalent to PMC and M1 in primates, with RFA operating as a higher motor cortical region.

*Cortical Control of Forelimb Movement in Rodents*

These anatomical observations led to further research on the functionality of RFA and CFA. In-vivo calcium imaging revealed that during different movements (reaching, grasping, forelimb elevation, etc), specific clusters of neurons within the forelimb area, CFA, are preferentially active (Dombeck et al., 2009). Further experiments have revealed a map of activation patterns amongst the forelimb area, including RFA and CFA. Primarily, RFA shows increased activity before grasping and CFA after grasping. Consequently, RFA inactivation increased errors in a pellot-grasping task, but reaching behaviors were only disrupted when CFA was inactivated (Wang et al., 2017). Additionally, CFA inactivation eliminates fine digit multiplication in a joystick task. However, both RFA and CFA were necessary for correct choice in movement and execution (Morandell and Huber, 2017). These findings suggest that the contributions of RFA and CFA to control of movement are overlapping, but very much distinct in both phase of movement and complexity of task. However, limited research on the synaptic interactions between RFA and CFA neurons obfuscates the complete picture of cortical control of movement in rodents.

Current research focuses on a potential functional hierarchy between RFA and CFA regions. For example, it was found that RFA stimulation modulates CFA output with little to no effect on latency. These powerful cortical interactions underlie RFA’s essential role in contrical control of forelimb movement including motor recovery following lesion (Deffeyes et al., 2015). Pharmacological inactivation of CFA abolished the forelimb-RFA response suggesting that sensory inputs are transmitted via corticocortical pathway from CFA to RFA. This pathway is closely related to the generation of complex forelimb movement (Kunori and Takashima, 2016). This too suggests a hierarchical model with RFA operating at a higher-order. However, an animal’s environment was shown to affect RFA activation implying that RFA does not solely determine CFA output, but is instead recruited when it is required (Saiki et al., 2014). This points towards a model where both RFA and CFA are capable of high-order processing, depending on the behavioral situation. However, a recent study in mice showed that motor cortical neurons fire in a behaviorally specific manner, which could account for some discrepancies seen amongst different behavioral paradigms (Miri et. al., 2017).

Further studies are needed to decode the exact functional relationship between RFA and CFA themselves and their role in the preparation and execution of forelimb movement in rodents. In this thesis, we directly look at the functional relationship between neurons in RFA and CFA using techniques that have, to our knowledge, never before been applied to this region. While the motor cortex in mice is not the exact same as the one in primates, and movement capabilities are not the same in mice and primates, there is still use in asking the same questions about motor systems. Particularly, there is much that can be learned regarding a potential existence of a motor cortical hierarchy in mice.

*Real Time Neural Recordings*

Neural recordings allow for a picture of information flow in the cortex; however, previous methods of neural recordings are incapable of capturing the real-time coordination of large regions of neural activity which is required for analyzing sensory, motor, and cognitive operations **(**Lewis et al., 2015) . Extracellular recordings, which have excellent temporal and spatial precision, can only record dozens of neurons at a time. Calcium imaging, which is capable of recording large amounts of neurons, lacks temporal precision. In order to progress in our understanding of cortical brain activity, it was necessary to engineer multiple multi-area arrays, capable of both precision and volume in two regions, simultaneously .

By using newly developed silicon probes called Neuropixels, we are able to get a clearer picture of neuronal activity during movement. Neuropixels were designed with the goal of simplifying and ameliorating neural recordings. This means that one animal can therefore be recorded over multiple days and experience very little damage in the process. Furthermore, each probe has 384 channels that are capable of identifying and recording distinct neurons within a 10mm region whilst maintaining temporal and spatial resolution. In effect, Neuropixels combine the best qualities of previous techniques and are therefore extremely suitable for this project where we not only need to record multiple regions of the cortex at the same time, but also need exact temporal precision inorder to assess direct interactions that would result from a hierarchical relationship. We used two Neuropixel probes, one recording the rostral forelimb movement representation and the other the caudal forelimb movement representation, which combined can record over 700 well-defined neurons from multiple brain regions in real-time (Jun et al., 2017).

*Real Time Motor Output Recordings*

Understanding cortical control of movement requires recording motor output with good temporal precision as the mouse behaves. In studies without precise muscle recordings, they must rely on established quality-defined movements, such as reaching. This creates ambiguity in whether the neural recordings actually represent the behavior being performed as well as how exactly coordination is being controlled on a movement-by-movement basis (Veuthey et al., 2020). Therefore, it can be difficult to draw conclusions about information flow since the result of the information processing, the movement itself, is not temporally precise.

Electromyographic (EMG) recordings allow for a measurement of motor output that is temporally specific and can capture real time movements. Created for use in larger mammals, EMG electrodes were made smaller and adapted for mice (Pearson et al, 2005; Akay et al, 2006).

Importantly, they do not disrupt the natural movement of mice, allowing for good recordings from multiple muscles simultaneously whilst performing a variety of natural behaviors (Pearson et al., 2005). By combining real time recordings of muscle activity with neural recordings that also have strong temporal resolution, we can see more directly how cortical motor input affects the execution of movement.

*Motor Behavioral Paradigms for Mice*

Since research about the cortical control of movement in mice is relatively new, it follows that behavioral paradigms are also somewhat limited. Paradigms ideally would have to capture complex, diverse, and natural behavioral sequences in a temporally segregated manner. This allows for neural recordings that show control of planning, executing, and adaptation of movement whilst also being a robust model for a variety of behaviors (Warriner et al., 2020). Moreover, the paradigm cannot be disrupted by the neural and EMG recordings or unnaturally affect these recordings. If a paradigm were to fail in any of these requirements, it would be difficult to make strong claims about motor cortical influence on behaviors in mice. Specifically for this project, a robust paradigm is necessary to track information flow during movement.

To address these requirements, we used a complex version of a simple voluntary movement behavioral paradigm in head-fixed mice. The mouse has to reach out and grasp a water droplet from a spout. Similar paradigms have shown to be both tolerable and successful in rodents (Guo et al., 2014a; Schwarz et al., 2010). Importantly, the reach-to-grasp-to-mouth behavior is ethologically relevant and simple to learn, often in just three to five sessions (Galiñanes et al, 2018). Furthermore, cortical involvement in movement has been shown to change as the behavior is practiced and eventually learned **(**Kawai et al, 2015). To mimic natural practices, the mouse would have to adapt to their surroundings as opposed to just repeating a learned behavior. To accomplish this, we used four separate water sprouts, one of which was used on each trial. The mouse received an indication of which spout would give the reward is a light-emitting diode (LED) light. Our paradigm therefore requires sensory adaptation, the mouse adapts to the location of the light, which is a crucial characteristic of natural behavior that many previous paradigms fail to account for (Mathis et al, 2017; Morandell and Huber, 2017; Saiki et al, 2014; Warriner et al, 2020).

There are many additional advantages that come from using water as a reward instead of the traditional food pellets. Voluntary reach and grasp paradigms often use pellets as a reward. However, this limits the number of trials that can be done as the mouse gets satiated and no longer wants the reward. Water, which can be delivered in smaller portions, can have significantly more trials allowing for greater volumes of neural information (Bachmanov et al., 2002). To further aid in engagement, we included a second sprout at each of the four locations that sucks the water out of the first spout after a set amount of time. This encourages quick reach movements a feature that is new to movement paradigms with water-based rewards.

Finally, the paradigm we used does not disrupt neural or muscular recordings. Head-fixing the mice creates stability for neural recordings which is important for spatial and temporal precision. Additionally, as opposed to food, water does not need to be manually given. Manual distribution is impractical for both sophisticated motor paradigms as well as many recording techniques such as two-photon microscopy (Ellens et al., 2016, Guo et al., 2015). Moreover, since water is rapidly consumed, it will not produce masticatory vibrations that could affect electrophysiological and imaging recordings (Galiñanes et al, 2018). Recent studies have shown that using water rewards for forelimb-based tasks can be used in combination with optogenetics, electrophysiology, and two-photon imaging (Miri et al., 2017; Morandell and Huber, 2017; Estebanez et al., 2017; Hasegawa et al., 2017). In our paradigm, it is important that we are able to achieve both good neural and EMG recordings, which will be permitted by the head-fixing and the reward system, respectively. This will allow for clear representation of movement initiation which is when the planning of movement occurs.

*Analyzing Significance and Direction of Information Flow Between Cortical Regions*

Using statistically robust methods to measure correlation in synaptic activity amongst neurons has proven to be useful for finding underlying mechanisms (Smith and Kohn, 2008). This correlation is said to arise from exhibitory and inhibitory input that is shared between neurons (Moore et al., 1970; [Lytton and Sejnowski, 1991](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2656500/#B44); [Morita et al., 2008](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2656500/#B51)). However, in the past, it has been difficult to distinguish between common synaptic input, and direct connection between cells. Additionally, common techniques such as cross-correlation do not detect direction of information flow. Finally, analysis techniques often required heavy computation that would take weeks, or potentially years, for computers to run. For this project, we used new statistical analysis techniques that have recently shown some promising results.

Cross correlogram (CCG) calculations are able to answer not only if there is evidence of direct connection between cortical neuron pairs, but also in which direction the information flow is most often occuring. CCG determines hierarchy by looking at the functionality of neurons. Specifically, it compares relative spiking activity between individual pairs of neurons. If one neuron in one area is consistently spiking at a predictable time lag after another area spikes, this implies a level of temporal synchronicity consistent with common functionality. Furthermore, it informs on the direction that information is being transferred between the two regions. Siegle et al., used a form of CCG called jittered cross correlogram (JCCG) to find a cortex hierarchy for visual control in mice. A JCCG includes a secondary calculation that removes irrelevant slow-scale connectivity. When compared with the expected anatomical hierarchy as well as pre-existing hierarchical statistical analysis, JCCG was found to be a good measure of hierarchical relationships between cortical regions (Siegle et al., 2019).

Another method used in this thesis is transfer entropy (TE). TE measures the information about the future state of a variable provided by another variable in the past given the information provided by the past state of the original variable (Schreiber, 2000). TE has been crucial in analyzing sets of spike times datasets in extracting the flow of information between brain regions. As opposed to previous analysis, TE does not require a linear assumption meaning it can account for many different pathways for information flow. Researchers were able to find a strong correlation between the coupling strength of neurons and higher TE values. TE was also found to hold stable for networks of all sizes: the more connected the networks, the higher the number of connections. (Walker et al, 2018). Garofalo used various methods in a neuronal network model made up of 60 synaptically connected neurons, and then in cultures of neurons to find synaptic strength. Their results suggest that TE was the best method for both the excitatory and inhibitory models (Garofalo et al. 2009). Overall, TE contributes a different perspective than our previous hierarchical analysis. While JCCG gives information about the functional connection between specific neuron pairs, transfer entropy tells more about the overall flow of information between cortical regions. Using both methods allows for a clearer picture of causality in the motor cortex.

*The Current Study*

We took an entirely novel approach to studying a potential motor cortical hierarchy between RFA and CFA regions. Using Neuropixels, we were able to record hundreds of neurons simultaneously as well as sync these recordings to movement using EMG data. Additionally, we used novel statistical methods that inform on both individual pairs of neurons, JCCG, and overall information flow, TE. Altogether, this creates an entirely unique approach to the question of motor cortical hierarchy.

We found evidence of significant within region connectivity amongst RFA and CFA neuron pairs using both JCCG analysis that was later confirmed with TE analysis. Additionally, for between region pairs, JCCG showed evidence of significant connection. The calculated directionality of this functional connection was slightly biased in the expected direction, RFA to CFA. This result was also confirmed with TE analysis. Therefore, our results do not strongly indicate motor cortical hierarchy. This does mean, however, that if there is a hierarchical relation between RFA and CFA, it is either very weak or very slight in directional bias. Finally, when comparing JCCG and TE analysis results, we observed a lack of correlation on an individual mouse basis whilst achieving the same overall results and conclusions.

In summary, this work helps shed some light on the relationship between motor cortical regions in the coordination of movement. We did not find evidence of a hierarchical relationship. Furthermore, we have shown that if there is one, it is very slight and would require higher power recordings and statistical analysis. Additionally, we have provided a model of analysis that can be used on many other cortical relationships. By using these techniques in other cortical regions, one can look for other causal relationships indicative of hierarchy.

**METHODS**

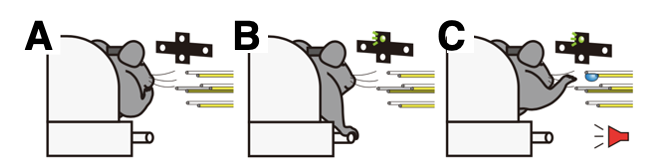
*Animal Husbandry*

6 adult male VGAT-ChR2-EYFP line 8 mice (B6.Cg-Tg(Slc32a1-COP4\*H134R/EYFP) were used. Mice were individually housed under a 12 hour light-dark cycle in a temperature and humidity-controlled room. At the time of data acquisition, all animals were 10-12 weeks old and weighed approximately 23-28 g. NIH guidelines were followed in all experiments and Northwestern Institutional Animal Care and Use Committee approved all experiments.

*Behavioral Paradigm*

We used a voluntary movement paradigm that included both reaching and grasping behavior (**Figure 1**). A mouse was head-fixed with their right paw on a touch sensor. In front of them were 4 ports each with 2 pipes. One pipe delivered the reward, water, while the other pipe took the reward away by suction after 1 second. When the mouse puts their paws on a sensor, an LED light turns on that correlates to one of the four ports (**Figure 1B**). After a certain amount of hold time, up to 2.5 seconds, there will be a reward cue in the form of a sound. After hearing the sound, the reward will be given from a random port and the mice will have to reach out and grasp the water droplet (**Figure 1C**). If the mouse was too slow at grasping the water, the LED light would turn off and the water would be taken away by the suction pipe. This reaction time was made continuously shorter, up to 1 second. This effectively trained the mice to obtain the reward quickly. The mice were each trained in this behavioral paradigm for four days before data acquisition, for an hour a day. Each mouse had multiple recording sessions but only had one per day.

**Figure 1**: *Steps of Mouse Behavioral Paradigm. (A) Mouse is head-fixed facing 4 ports, each with two pipes, and an LED light board. (B) Mouse holds onto the touch sensor which turns on a LED light that corresponds to the port that the water droplet will come out of. (C) Water droplet emerges from the indicated port and is accompanied by a sound. The mouse has 1 second to reach and grab the water droplet before it is sucked back in the second pipe at that port.*



*Electromyography recordings*

Electromyography (EMG) recordings were done according to established procedures (Miri et al, 2017) . Six muscles were recorded chronically with six different electrodes, each with two braided steel wires (793200, A-M Systems) knotted together, that were used in each set. One wire was stripped of insulation from 1 to 1.5 mm away from the knot. The other wire was stripped from 2 to 2.5 mm away from the knot. On the opposite side of the knot, the ends of these two wires on the were soldered into a 12-pin miniature connector (11P3828, Newark). Each muscle’s electrode had its own specific wire length between the knot and the connector: 2 cm for trapezius, 3.5 mm for biceps and triceps, 4.5 cm for extensor digitorum communis and palmaris longus, and 5.5 cm for pectoralis. To facilitate insertion into the muscle, the stripped wires were twisted together and clamped into a 27-gauge needle.

EMG electrodes were implanted during headplate attachment. First, both the mouse’s neck and right forelimb were shaved. Then, incisions were made above the muscle to be implanted. Finally, a needle was used to guide electrodes pairs, under the skin, from the incision site on the scalp to the particular muscle they would be recording. The distal portion of the electrode was knotted, allowing for the needle and excess wire to be cut away. The connector was affixed to the posterior edge of the headplate with dental cement and the incisions were sutured.

The EMG recordings were amplified with a differential amplifier (RHD2216, Intan Technologies) after which the data was digitized and acquired at 30 kHz using the Intan Technologies software (Intan Technologies).

*Neuronal Recordings Using Neuropixels*

Neuronal recordings were made using state-of-the-art multielectrode arrays called Neuropixels (Jun et. al, 2017). Together, the probes are capable of recording up to 700 neurons at a time whilst tracking their position in relation to the probe and maintaining low influence from noise. We used two probes: one recorded the RFA and the other the CFA.

The probes were inserted after the mice were head-fixed. The caudal insertion occurred at a 30 degree angle from medial to lateral and a 10-15 degree angle from posterior to anterior. It was inserted at a depth of 4mm from a position of anterior/posterior (AP) 0.0 medial/lateral (ML) 1.0 for the first two mice and AP 0.25 ML1.25 for the rest of the mice. The rostral probe was inserted at a depth of 2mm at no angle. For the first two mice, the position was AP2.0 ML1.5 which was changed to AP 2.25 ML .75 for the rest of the mice.

*Data Preprocessing and Synchronization*

The data collected from EMG recordings was high-pass filtered at 250 Hz, convolved with a Gaussian having a 10 ms standard deviation, and rectified. The recordings were normalized to their standard deviation by calculating z scores with the median being set at 0 and the standard deviation at 1. Altogether, these processes normalize the EMG recordings from each individual muscle to its established baseline activity in order to control for varying baseline activity amongst the muscles and isolate activity changes.

The Neuropixel recordings were processed using the spike-sorting Kilosort2 software (Pachitariu et al., 2016). The software distinguishes neuronal spiking from background noise as well as assigns all spikes from one neuron to that specific neuron. Kilosort2 is thus able to classify neuronal spiking units in a quick and accurate manner.

Additionally, the EMG measurements needed to be aligned temporally with the Neuropixel recordings. This was done by using a 1Hz square wave as a sync signal. This sync signal is generated by a sync channel that is connected to both the intan data stream, which records EMG data, and the stream that records Neuropixel data. By syncing the square waves from both streams, we were able to temporally align the neuronal and muscular measurements. During this step, we also downsize the data to 1kHz. The result of this is a matrix where the first column is the neuron index, the second column is the spike time relative to the EMG recording, and the third column is the depth of that spike relative to the probe insertion. This allows us to create a spike time series for each neuron that can be compared to the activity seen in EMG measurements.

Some cells were thrown out during preprocessing due to inter-spike interval (ISI) violations. This metric can be used to inform on the quality of spike clusters. ISI violations were found by calculating a refractory violation percentage by averaging the autocorrelation value, the correlation of a signal with a delayed version of itself, of the refractory window (.3-1ms) and dividing it by the autocorrelation value of the flank edges (10ms-50ms). Neurons with refraction violation percentages of over 18% are flagged and removed. 2% of the neurons were removed during this step.

**Data Analysis:**

All data and statistical analysis was done on Matlab\_R2020a and Matlab\_R2020b.

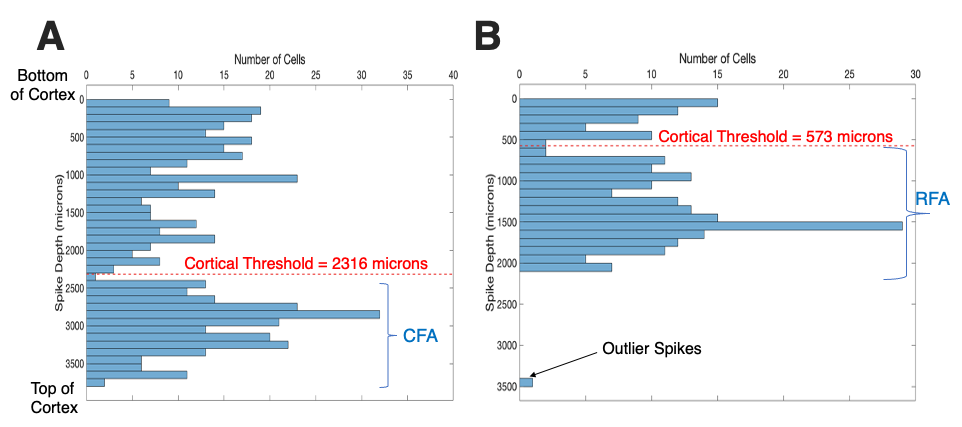
*Identifying Cortical Neurons*

The Neuropixel probes were inserted deep enough to also record striatal neurons.

Moreover, the exact depth varied slightly with each recording session. Therefore, it was necessary to establish a depth threshold for defining the cortical region, as opposed to striatal regions, that was unique to each recording session. Histograms of the depth of spikes in relation to the Neuropixel for the caudal region showed a distinct decrease in spiking 1500 microns below the highest position value (**Figure 2A**). This nadir was assumed to be the location of white matter between the cortical and striatal regions where there is often a decrease, or sometimes absence, of neuronal spiking. Since the tip of the probe, and therefore the deepest recorded spikes, is position 0, a threshold for the cortical region was placed 1500 microns below the highest position value for each recording session. It was assumed that the highest neuron was near pia and below it would be the cortex. Importantly, 1500 microns was also consistent with the calculated approximate depth of the cortex based on the angle of insertion.

Spike-depth histograms for the rostral region neuronal recordings showed spikes at superficial position values that were not part of a cluster of neurons indicating that it was not in the cortical region (**Figure 2B**). Each histogram of recorded spike depths was examined in order to determine a threshold for the outliers such that the cortical region would consist of positions below it. Additionally, for the rostral region, there was often a more clear divide between the cortical and striatal regions due to a complete absence of spiking for hundreds of micrometres. This clear strip was used as a threshold for most recording sessions. However, some rostral sessions did not have this strip; therefore, the 1500 microns below the highest position of a recorded spike value, after removing the outliers, was used.

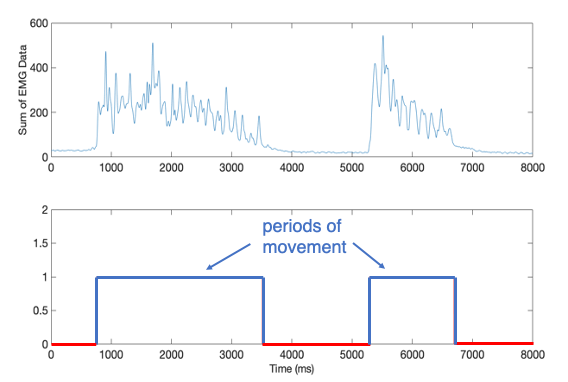
**Figure 2**: *Recorded Spike Depths Using Neuropixel Probes. (A) Recorded spike depths for caudal Neuropixel probe. Cortical threshold is set at 1500 microns below the top of the cortex (highest spike depth value) which is consistent with the angle and position of entry. (B) Recorded spike deaths for rostral Neuropixel probe. Cortical threshold is set 1500 microns below the top of the cortex once outlier spikes have been removed.*



*EMG analysis and processing*

In order to perform the following statistical analysis, it was necessary to separate periods of movement from trial breaks where the mouse is not moving limbs (**Figure 3**). To do so, we created an algorithm that could classify periods of a recording session as during movement or not. First, all the separate EMG electrode recordings were summed. Then, a period of approximately 1 second of non-movement was identified by observing a lack of change in the summed EMG value over a time window. A threshold of movement was set at the average value of this period plus 7 times the standard deviation of this period. To correct for irregular classifications due to noise in the EMG recordings, any period of non-movement of 100 ms or less was reclassified as movement. Additionally, periods of movement less than 10ms were reclassified as non movement since this was unlikely to be significant movement.

**Figure 3**. *Classification of Movement Epochs. Sum of EMG data is used to classify periods of movement and non-movement based on a constant baseline.*



**Statistical Analysis**

*Jittered-Corrected Cross Correlogram (JCCG) Analysis of Spike Trains*

JCCG analysis identifies functional influence between pairs of neurons by looking at patterns in their spike times. If one neuron is consistently spiking at a certain time delay after another neuron spikes, this would be indicative of significant information flow between them. This connection then manifests in a behavioral output, which in this experiment, is movement. By looking at thousands of pairs of neurons between two regions, one can get a sense of if there is a significant functional connection between them and in what direction it is flowing.

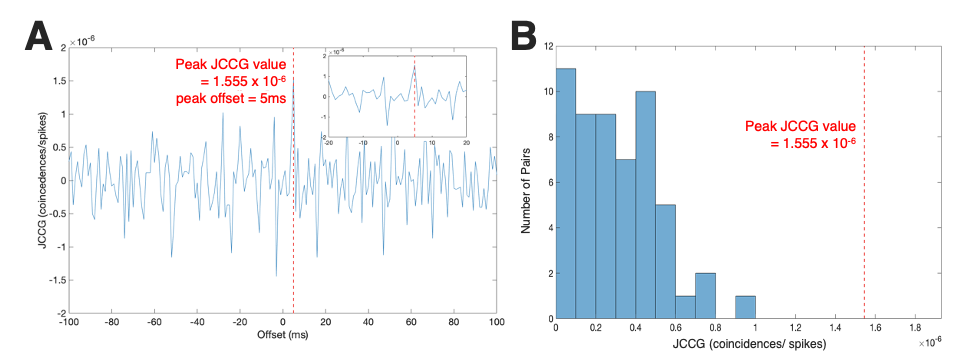
Neurons from RFA and CFA were analyzed using JCCG techniques both between the two regions and within a region . Functional connectivity was measured using a previously studied CCG algorithm (Gerstein and Perkel, 1972; Jia et al., 2013**;** Smith and Kohn, 2008; Siegle et al., 2019). This algorithm measures average delays in spike times between two neurons. A consistent delay between two neurons is indicative of a causal relationship. CCG is illustrated in the following equation:

M is the number of trials. represents the time lag relative to the reference spike. N is the number of time lags being tested. xi1 and xi2 represent spike trains on trial i with xi1 operating as the reference spike train. Θ() is a triangular function, absolute value of the offset subtracted from the duration of the recording, that corrects the CCG value from overlapping time bins as a result of the sliding window. and are the firing rates of the first spike train and second spike train respectively. Correcting using the geometric mean of the firing rates counteracts firing rate dependency. This calculation was done for every neuron pair both within region and between RFA and CFA at all time delays () from -100 to 100 ms at increments of 1 ms for each individual recording session. Moreover, only spikes during movement were used as reference spikes in the calculation.

A jitter-corrected method that has been previously described in other studies focused on hierarchical cortical relationships was used (Smith and Kohn, 2008). The jitter-correction removes slow temporal correlations as well as stimulus-locked correlations. This entails jittering the spike trains within a particular time window that would mimic natural variability in spike timing. An average CCG for five jittered spike trains was calculated and this value was subtracted from the original CCG calculation, resulting in one JCCG value at each time offset (**Figure 4A**). Based on previous studies, we used a 25 ms jitter window (Jia et al., 2013;Zandvakili and Kohn, 2015).

Test statistics for each neuron pair were generated by dividing the peak JCCG value within a short latency window (<10ms) by the standard deviation of the JCCG values of the higher magnitude time delays (-100 to -50 and 50 to 100ms) (**Figure 4B**). The magnitude of the test statistic is directly related to the significance of functional connection between two neurons. We determined connected pairs by setting a threshold for a significant test statistic. For within-area pairs, we were successful in using a mouse-based permutation method for determining this threshold for significance. But due to a relatively weaker connectivity between areas, we needed to use a more statistically powerful method that relied on pair-based permutations.

**Figure 4**: *JCCG Analysis for One Pair of Neurons. (A) JCCG results for one pair of neurons at time offsets from -100 to 100ms. Peak value occurs at the maximum JCCG value within 10ms. The peak offset is the time lag where this occurs. (B) Comparing the values of the flanks (-100 to -50ms and 50 to 100ms) to the peak value. Test statistics are derived from dividing the peak value by the standard deviation of the flank values.*

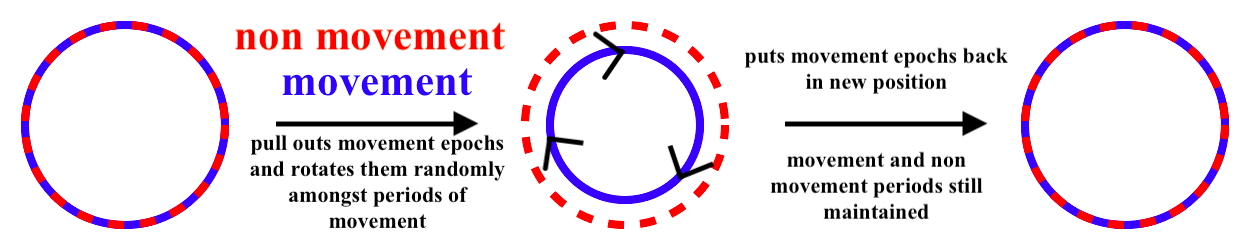


*JCCG Significance Analysis: Mouse-Based Permutations*

To test for significance for both within region and between regions JCCG pairs, we generated 10,000 permuted spike pairs per mouse using a circular permutation of the movement epochs of one spike train per pair (**Figure 5**). Every spike during a movement epoch was rotated a random value greater than 3 seconds and less than the duration of the trial. This would effectively break any functional connection on a millisecond if there was one.

It was observed that some test statistics were artificially inflated. Neurons with low firing rates were shown to have fewer nonzero values in the flanks of their JCCG resulting in the observed inflated test statistic. To account for this, we used only JCCGs that had a nonzero value at every offset tested. Of the total 341,735 cross-regional neuron pairs analyzed, only 1709 (.50%) had no zeros in the JCCG and were therefore included in this particular significance assessment. Therefore, each recording session had a proportional contribution to the 10,000 permuted pairs depending on the number of pairs it contributed to the overall analysis.

These permuted pairs were then analyzed using the same JCCG algorithm and their test statistics were used to generate a graph of expected test statistics under the null hypothesis of no connection between the two regions. The distribution of the test statistics of permuted pairs was compared to the experimental test statistics to look for a test statistic threshold for determining significant pairs.

**Figure 5**: *Circular Permutation of Movement Epochs. Permutations are done by classifying movement and non-movement periods. Then only movement is rotated randomly so that the periods of movement are maintained but any functional connectivity between two spikes is broken.* 

The threshold for significance for experimental test statistics in this method was determined by calculating the expected rate of false positives using the permuted test statistics. At each potential threshold from 3 to 5 at increments of .1, the fraction of permuted test statistics above the threshold was divided by the fraction of experimental test statistics above the threshold, generating a false discovery rate (FDR). The FDR indicates what percent of the experimental pairs above that test statistic are falsely categorized as significant. The threshold of significance for the test statistics of a mouse was set at the lowest value where the FDR reached less than 10%. This means that less than 10% of the pairs that would be classified as significant are being done so falsely.

Finally, for each significant pair, the time delay where the JCCG peaked within a -10 to 10ms window was recorded as the offset. An offset represents the average time delay between spiking of one neuron and another. Offsets for all neuron pairs for a mouse were averaged. An average offset of zero means that two neurons are spiking at the exact same time. A negative or positive offset implies connection between two regions with the sign indicating the direction of information flow. Therefore, an average positive offset in our analysis means that RFA was consistently spiking at a specific time before CFA. This implies an RFA to CFA directionality within the hierarchy, while a negative average offset would imply the opposite. The ratio of pairs with positive offsets to negative ones as well as the overall average offset among significant pairs were used to determine a directionality of the information flow and therefore hierarchy.

*Further Analysis Using Mouse-Based Permutations*

In order to get a better understanding of directionality between regions, we employed another method of statistical analysis on the JCCG calculations between RFA and CFA regions. For each test statistic, the fraction of the 10,000 permuted test statistics from the initial analysis above that value is recorded. If there is significant functional connectivity, there will be more test statistics with greater magnitude. This means there should be a bias towards smaller fractions since less of the permuted test statistics would be above the experimental test statistics. The amount of test statistics with a fraction above .5 and below .5 were recorded. Subtracting the amount of test statistics with less than .5 from the number of test statistics with fractions greater than .5 estimates significant connectivity. By taking this value and dividing it by the total number of test statistics we were able to get a fraction of the excess that represents significant connections. This value was analyzed using a one-tailed t test for significance.

Additionally, we are able to get an estimate of the time lag of connectivity from this fractional method. First, we find the average offset of all the 1709 pairs in this analysis. We then divide this average offset by the fraction of the excess. This gives an estimate of the offset value between two regions and can inform on both the existence and direction of connectivity. Average offset values for each mouse were analyzed using a one-tailed t test for significance.

*JCCG Significance Analysis: Pair-Based Permutations*

For JCCG pairs between regions, it was necessary to perform an additional significance analysis that allowed for more statistical power by analyzing more pairs. First, instead of using only pairs with no zeros in their JCCGs, we used pairs with higher firing rates to combat the same problem encountered in the previous method in regards to inflated test statistics . Second, we use permutations that were created from each specific pair to allow for a more accurate comparison. While statistically more powerful, this method is also much more computationally intensive. Due to limited time, this process was done in four of the six mice and only for pairs where both neurons had a firing rate of over 10Hz during movement. In total, 1720 pairs were analyzed using this pair-based permutation method.

For each pair analyzed, we generated 300 permuted spike pairs using the same circular permutation method. We then calculated the test statistics, in the same way, for these permuted pairs and compared them to the expected test statistic for that particular pair in order to judge significance. We also employed a similar method to the mouse-based permutation method for finding a threshold for significant test statistics amongst experimental neuron pairs.

For this analysis, we set a threshold of significance based on the p-value of a test statistic when compared to its permuted pairs. We used a built in MATLAB function that estimates the p-value and FDR for every experimental test statistic compared to the permuted ones. We constructed a graph of p-value vs fdr for all the test statistics for a mouse. We estimated the p-value from this graph where the fdr is .1. This p-value is used as a threshold for significance. Every test statistic with a p-value less than this threshold is therefore categorized as significant.

A similar directional analysis was performed as well. All the offsets where the significant pairs peaked within a 10ms window were recorded. All these values were averaged to calculate the average directional flow. Additionally, the amount of significant pairs with positive offsets was compared to ones with negative offsets for directional analysis. Together, these two metrics give a measure for directional information flow.

*Transfer Entropy Analysis*

As opposed to JCCG which focuses on individual connected pairs of neurons, transfer entropy provides a more broad analysis of information flow. Transfer entropy is a measure of one's ability to predict that a neuron will spike at a given time. The analysis compares the level of uncertainty in this prediction if given information from a second neuron as opposed to just the history of the neuron itself. If the level of uncertainty consistently decreases to a significant degree when given information about a neuron pair between two regions, there is significant information flow between these two regions.

Transfer entropy analysis was performed using an algorithm made publicly available by Michael Hansen and Shinya Ito (Ito et al., 2011). The algorithm is based on the following equation:

J is the first neuron which may have an effect on neuron I. it and it+1 can either be 0 or 1 depending on whether neuron I is spiking at time t and t+1 respectively. d is time delay being tested. jt+1-d represents the status of neuron J at time t + 1 - d. p represents the probability of what happens within the following parenthesis with everything to the right of the | denoting the conditional probability. Transfer entropy is determined between all possible pairs of neurons, above a firing rate threshold of 1 Hz, of RFA and CFA both within and between the two regions. We tested information delays from 0 to 30ms at increments of 1ms.

In order to determine significant information flow within a neuron pair, the experimental values were compared to generated expected values. The expected transfer entropy values were made by shifting recorded spike trains within a 3ms window. This creates randomized spike trains that account for natural variability in spike times. For every pair of neurons, 100 expected transfer entropies are calculated from 100 different shifted spike trains. A p-value is calculated by comparing observed transfer entropy to the 100 generated transfer entropies. Significance of the information flow was determined by the magnitude of the p value.

REFERENCES

Akay T, Acharya HJ, Fouad K, Pearson KG (2006) Behavioral and Electromyographic Characterization of Mice Lacking EphA4 Receptors. J Neurophysiol 96:642-651.

Alaverdashvili M, Whishaw IQ (2008) Motor cortex stroke impairs individual digit movement in skilled reaching by the rat. Eur J Neurosci. 28(2):311-22.

Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behav. Genet., 32: 435-443

Brodmann, K. (1909). Vergleichende Lokalisationslehre der grosshirnrinde [Comparative localization in the cerebral hemispheres]. Leipzig, Germany: J. A. Barth.

Bucy PC (1933) Electrical excitability and cyto-architecture of the premotor cortex in monkeys. Arch Neurol Psychiat 30:1205-1225.

Butler AB, Hodos W (2005) Comparative Vertebrate Neuroanatomy: Evolution and Adaptation. Hoboken, NJ: John Wiley and Sons.

Cai DJ, Aharoni D, Shuman T, Shobe J, Biane J, Song W, Wei B, Veshkini M, La-Vu M, Lou J, Flores SE, Kim I, Sano Y, Zhou M, Baumgaertel K, Lavi A, Kamata M, Tuszynski M, Mayford M, Golshani P, Silva AJ (2016) A shared neural ensemble links distinct contextual memories encoded close in time. Nature 534:115–118.

Campbell, A.W. (1905). Histological studies on the localization of cerebral function. Cambridge, UK: Cambridge University Press.

Cheney PD, Fetz EE (1985) Comparable patterns of muscle facilitation evoked by individual corticomotoneuronal (CM) cells and by single intracortical microstimuli in primates: evidence for functional groups of CM cells. J Neurophysiol 53:786-804.

Coogan TA, Burkhalter A. Conserved patterns of cortico-cortical connections define areal hierarchy in rat visual cortex. *Exp Brain Res.* 1990;80:49–53

Darling WG, Pizzimenti MA, Morecraft RJ (2011) Functional recovery following motor cortex lesions in non-human primates: experimental implications for human stroke patients. *J Integr Neurosci*. 10(3):353-384

Deffeyes JE, Touvykine B, Quessy S, Dancause N (2015) Interactions between rostral and caudal cortical motor areas in the rat. J Neurophysio 113(10):3893-3904.

Dombeck D, Graziano, Tank D (2009) Functional Clustering of Neurons in Motor Cortex Determined by Cellular Resolution Imaging in Awake Behaving Mice. J Neurosci 29:13751-13760.

Ellens DJ, Gaidica M, Toader A, Peng S, Shue S, John J, Bova A, Leventhal DK (2016) An automated rat single pellet reaching system with high-speed video capture. Journal of Neuroscience Methods. 271: 119-127.

Estebanez L, Hoffmann D, Voigt BC, Poulet JFA (2017) Parvalbumin-expressing GABAergic neurons in primary motor cortex signal reaching Cell Rep., 20: 308-318

Ferrier, D (1873) Experimental researchers in cerebral physiology and pathology. West Riding Lunatic Asylum Medical Reports 3:30-96.

Fogassi L, Gallese V, Fadiga L, Luppino G, Matelli M, Rizzolatti G (1996) Coding of peripersonal space in inferior premotor cortex (area F4). J Neurophysiol 76:141-157.

Fritsch G, Hitzig E (1870) Electric excitability of the cerebrum (Uber die elektrische Erregbarkeit des Grosshirns). Epilepsy Behav 15:123-130.

Fulton J (1934) Forced grasping and groping in relation to the syndrome of the premotor area. Arch Neurol Psychiat 31:221-235.

Galiñanes G, Bonardi C, Huber D (2018) Directional Reaching for Water as a Cortex-Dependent Behavioral Framework for Mice. Cell Reports 22:2767-2783.

Garofalo, M., Nieus, T., Massobrio, P., and Martinoia, S. (2009). Evaluation of the performance of information theory-based methods and cross-correlation to estimate the functional connectivity in cortical networks. PLoS ONE 4:e6482.

Gerstein, G.L., and Perkel, D.H. (1972). Mutual temporal relationships among neural spike trains. *Biophysical Journal* 12, 453–473.

Ghosh KK, Burns LD, Cocker ED, Nimmerjahn A, Ziv Y, Gamal AE, Schnitzer MJ (2011) Miniaturized integration of a fluorescence microscope. Nat Methods 8:871–878.

Graziano MSA (2008) The Intelligent Movement Machine: An Ethological Perspective on the Primate Motor System. Oxford University Press.

Graziano MSA, Hu XT, Gross CG (1997) Coding the locations of objects in the dark. Science 227:239-241.

Graziano MSA, Reiss LA, Gross CG (1999) A neuronal representation of the location of nearby sounds. Nature 397:428-430.

Graziano MSA, Taylor C, Moore T (2002) Complex Movements Evoked by Microstimulation of Precentral Cortex. Neuron 34:841-851.

Guo JZ, Graves AR, Guo WW, Zheng J, Lee A, Rodríguez-González J, Li N, Macklin JJ, Phillips JW, Mensh BD, Branson K, Hantman AW (2015) Cortex commands the performance of skilled movement. Elife 4:e10774.

Guo ZV, Hires SA, Li N, O’Connor DH, Komiyama T, Ophir E, Huber D, Bonardi C, Morandell K, Gutnisky D, et al. (2014a) Procedures for behavioral experiments in head-fixed mice. PLoS ONE 9:e88678.

Hasegawa M, Majima K, T. Itokazu T, T. Maki T,. Albrecht UR, Castner N, Izumo M, Sohya K, Sato TK, Kamitani Y, Sato TR (2017) Selective suppression of local circuits during movement preparation in the mouse motor cortex. Cell Rep. 18: 2676-2686

Hira R, Ohkubo F, Tanaka YR, Masamizu Y, Augustine GJ, Kasai H, Matsuzaki M (2013) In vivo optogenetic tracing of functional corticocortical connections between motor forelimb areas. Front Neural Circuits 7(55):1-10.

Jia X, Tanabe S, Kohn A. 2013. Gamma and the Coordination of Spiking Activity in Early Visual Cortex. *Neuron*77:762–774.

Jun, J.J, Steinmetz, N.A., Siegle, J.H., Denman, D.J., Bauza, M., Barbarits, B., Lee, A.K., Anastassiou, C.A., Andrei, A., Aydin, C., et al. (2017). Fully integrated silicon probes for high-density recording for neural activity. Nature *551,* 232-236.

Kawai R, Markman T, Poddar R, Ko R, Fantana AL, Dhawale AK, Kampff AR, Ölveczky BP (2015) Motor cortex is required for learning but not for executing a motor skill. Neuron, 86:800-812

Kunori N, Takashima I (2016) High-order motor cortex in rats receives somatosensory inputs from the primary motor cortex via cortico-cortical pathways. Eur J Neurosci 44(11):2925-2934.

Latham, N. and Mason, G. (2004) From house mouse to mouse house the behavioural biology of free-living *Mus musculus* and its implication in the laboratory. Applied Animal Behavioral Science *86,* 261-289.

Lewis CM, Bosman CA and Fries P (2015) Recording of brain activity across spatial scales. *Curr. Opin. Neurobiol.* 32: 68–77

Leyton ASF, Sherrington CS (1917) Observations on the Excitable Cortex of the Chimpanzee, Orangutan, and Gorilla. Exp Physiol 11(2):135-222.

Li CX, Waters RS (1991) Organization of the mouse motor cortex studied by retrograde tracing and intracortical microstimulation (ICMS) mapping. *Can J Neurol Sci.* 18:28–38.

Lytton WW, Sejnowski TJ. Simulations of cortical pyramidal neurons synchronized by inhibitory interneurons. *J Neurophysiol.* 1991;66:1059–1079.

Mathis MW, Mathis A, Uchida N (2017) Somatosensory Cortex Plays an Essential Role in Forelimb Motor Adaptation in Mice. Neuron 93:1439-1503.e6.

Maunsell JH, van Essen DC. The connections of the middle temporal visual area (MT) and their relationship to a cortical hierarchy in the macaque monkey. *J Neurosci.* 1983;3:2563–2586.

McFarland NR, Haber SN (2002) Thalamic Relay Nuclei of the Basal Ganglia Form Both Reciprocal and Nonreciprocal Cortical Connections, Linking Multiple Frontal Cortical Areas. J Neursci 22(18):8117-8132.

Miri A, Warriner CL, Seely JS, Elsayed GF, Cunningham JP, Churchland MM, Jessell TM (2017). Behaviorally selective engagement of short-latency effector pathways by motor cortex. *Neuron* 95(3):683-696.

Mohammed H, Jain N (2013) Two Whisker Motor Areas in the Rat Cortex: Evidence from Thalamocortical Connections. J Comp Neurol 522(3):528-545.

Mohammed H, Jain N (2016) Ipsilateral Cortical Inputs to the Rostral and Caudal Motor Areas in Rats. J Comp Neurol 524(15):3104-3123.

Moore GP, Segundo JP, Perkel DH, Levitan H (1970) Statistical signs of synaptic interactions in neurons. *Biophys J* 10: 876-900

Morandell K, Huber D (2017) The role of forelimb motor cortex areas in goal directed action in mice. Sci Rep 7(15759).

Morita K, Kalra R, Aihara K, Robinson HPC. Recurrent synaptic input and the timing of gamma-frequency-modulated firing of pyramidal cells during neocortical “UP” states. *J Neurosci.* 2008;28:1871–1881.

Neafsey E, Bold E, Haas, G., Hurley-Gius K, Quirk G, Sievert C (1986). The organization of the rat motor cortex: a microstimulation mapping study. *Brain Res*. 11: 77–96.

Nudo RJ, Milliken GW (1996) Reorganization of movement representations in primary motor cortex following focal ischemic infarcts in adult squirrel monkeys. J Neurophysiol. 75(5):2144-9.

Pandarinath C, Ames K, Russo AA, Farshchian A, Miller LE, Dyer EL, Kao JC (2018) Latent Factors and Dynamics in Motor Cortex and Their Application to Brain-Machine Interfaces. J Neurosci 38:9390-9401.

Park MC, Belhaj-Saif A, Cheney PD (2004) Properties of primate motor cortex output to forelimb muscles in rhesus macaques. J Neurophysiol 92:2968-2984.

Parkes SL, Balleine BW (2013) Incentive Memory: Evidence the Basolateral Amygdala Encodes and the Insular Cortex Retrieves Outcome Values to Guide Choice between Goal-Directed Actions. J Neurosci 33(20):8753-8763.

Pearson KG, Acharya H, Fouad K (2005) A new electrode configuration for recording electromyographic activity in behaving mice J Neurosci Meth 148:36-42.

Penfield W, Boldrey E (1937) Somatic motor and sensory representation in the cerebral cortex of man as studied by electrical stimulation. Brain 60:389-443.

Penfield W, Welch K (1951) The supplementary motor area of the cerebral cortex: A clinical and experimental study. Am Med Ass Arch Neurol Psychiat 66:289-317.

Piecharka DM, Kleim JA, Whishaw IQ (2005) Limits on recovery in the corticospinal tract of the rat: partial lesions impair skilled reaching and the topographic representation of the forelimb in motor cortex Brain Res. Bull., 66: 203-211,

Pronichev IV, Lenkov DN (1998) Functional mapping of the motor cortex of the white mouse by a microstimulation method. *Neurosci Behav Physiol.* 28:80–85.

Rathelot JA, Strick PL (2006) Muscle representation in the macaque motor cortex: an anatomical perspective. Proc Natl Acad Sci USA 103:8257-8262.

Rizzolatti G, Scandolara C, Mateli M, Gentilucci M (1981) Afferent properties of periarcuate neurons in macaque monkeys. II. Visual responses. Behav Brain Res 2:147-163.

Roland PE, Larsen B (1976) Focal increase of cerebral blood flow during stereognostic testing in man. Arch. Neurol. 33: 551-558.

Roland PE, Larsen B, Lassen NA, Skinhog E (1980) Supplementary Motor Area and Other Cortical Areas in Organization of Voluntary Movements in Man. Journal of Neurophysiology 43(1): 118: 136.

Roland PE, Skinhoj E, Lassen NA, Larsen B (1980) Different cortical areas in man in organization of voluntary movements in extra- personal space. J. Neurophysiol. 43: 137-150.

Rouiller EM, Moret V, Liang F (1993) Comparison of the Connectional Properties of the Two Forelimb Areas of the Rat Sensorimotor Cortex: Support for the Presence of a Premotor or Supplementary Motor Cortical Area. Somatosens Mot Res 10(3):269-289.

Saiki A, Kimura R, Samura T, Fujiwara-Tsukamoto Y, Sakai Y, Isomura Y (2014) Different modulation of common motor information in rat primary and secondary motor cortices. PloS One 9:e98662.

Schreiber T. Measuring information transfer. Physical review letters. 2000;85(2):461–464

Schwartz AB, Kettner RE, Georgopoulos AP (1988) Primate motor cortex and free arm movements to visual targets in three-dimensional space. I. Relations between single cell discharge and direction of movement. J. Neurosci., 8: 2913-2927

Sherrington CS (1939) On the motor area of the cerebral cortex. In: Selected writings of Sir Charles Sherrington. Denny-Brown, D (Ed). London: Hamish Hamilton Medical Books, pp. 397-439.

Shmuelof L, Krakauer JW (2011) Are We Ready for a Natural History of Motor Learning? Neuron 72(3):469-476.

Siegle, J.H., Jia, X., Durand, S., Gale, S., Bennett, C., Graddis, N., Heller. G., Ramirez, T.K., Choi, H., Luviano, J.A., et al. (2019). A survey of spiking activity reveals a functional hierarchy of mouse corticothalamic visual areas.

Smith, M. A. and Kohn, A. (2008). Spatial and Temporal Scales of Neuronal Correlation in Primary Visual Cortex. J. Neurosci. *12591–12603*

Sul JH, Jo S, Lee D, Jung MW (2011) Role of rodent secondary motor cortex in value-based action selection. Nat Neurosci 14(9):1202-1208.

Tennant KA, Adkins DL, Donlan NA, Asay AL, Thomas N, Kleim JA, Jones TA (2011) The Organization of the Forelimb Representation of the C57BL/6 Mouse Motor Cortex as Defined by Intracortical Microstimulation and Cytoarchitecture. Cereb Cortex 21(4):865-876.

Van Acker III GM, Amundsen SL, Messamore WG, Zhang HY, Luchies CW, Kovac A, and Cheney PD (2013) Effective intracortical microstimulation parameters applied to primary motor cortex for evoking forelimb movements to stable spatial end points. *Journal of Neurophysiology*. 110 (5): 1180- 1189.

Veuthey TL, Derosier K, Kondapavulur S(2020) Single-trial cross-area neural population dynamics during long-term skill learning. *Nat Commun* 11: 4057.

Vogt C, Vogt O (1926) The comparative architectonic and physiologic divisions of the cerebral cortex with particular emphasis on the human (Die vergleichend-architektonische und die vergleichendreizphysiologische Felderung der Grosshirnrinde unter besonderer Berucksichtigung der menschlichen). Naturwissenchaften 14:1190-1194.

Walker, B.L.; Newhall, K.A. Inferring information flow in spike-train data sets using a trial-shuffle method. PLoS ONE 2018, 13, e0206977.

Wang X, Liu Y, Li X, Zhang Z, Yang H, Zhang Y, Williams PR, Alwahab NSA, Kapur K, Yu B, Zhang Y, Chen M, Ding H, Gerfen CR, Wang KH, He Z (2017) Deconstruction of Corticospinal Circuits for Goal-Directed Motor Skills. Cell 171(2):440-455.

Warriner CL, Fageiry SK, Carmona LM, Miri A. (2020) Towards cell and subtype resolved functional organization: the mouse as a model for the cortical control of movement.

Welniarz Q, Dusart I, Roze E (2017) The corticospinal tract: Evolution, development, and human disorders. Dev Neurobiol 77(7):810-829.

Zandvakili, A., and Kohn, A. (2015). Coordinated neuronal activity enhances corticocortical communication. *Neuron*87, 827–839.