FUNCTIONAL ORGANIZATION OF NEURAL CIRCUITS

IN RODENT MOTOR CORTEX

A Dissertation in
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by
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ABSTRACT

Rodents are being used increasingly as a model for understanding sensorimotor integration and related mechanisms, but the degree to which motor cortical organization is conserved across rodents and primates has not been well established. Primary and secondary sensory cortical areas, as well as multi-modal associative regions, are well-delineated in both rodents and primates, but the functional subdivisions of motor cortex are not as well understood in non-primate mammals. In rodents, motor cortex consists of three cytoarchitectonic regions: the agranular lateral (AGl), agranular medial (AGm) and cingulate (Cg) cortical areas. Intracortical microstimulation (ICMS) within these subdivisions indicates a somatotopic organization in which the limbs and body are represented in AGl, the face and whiskers in AGm, and the eyes in Cg. The anatomical and physiological experiments described here demonstrate functional distinctions in the different parts of the whisker motor area. Inputs from the whisker representations in somatosensory cortex terminate in the transition region between AGm and AGl, which consequently shows sensory evoked neuronal responses to whisker deflections. By contrast, multimodal inputs from posterior parietal cortex (PPC) terminate more medially in a narrow part of AGm proper, and was found to elicit whisker movements at much lower thresholds of ICMS compared to the adjacent sensory-input zone in the AGm-AGl transition region. Tracing studies further demonstrate that AGm is the source of projections to the ipsilateral superior colliculus consistent with its role in motor-output. The whisker region in AGm was also found to be involved in a number of interhemispheric projections, including a newly described circuit via the claustrum, that support its role in coordinating activity between the whisker motor cortex in each hemisphere to mediate the bilateral coordination of whisking. Collectively, these findings indicate that rodent motor cortex can be functionally divided into multiple subregions, similar to primate motor cortex.
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...And if the body were not the Soul, what is the Soul?"

- Walt Whitman, *Leaves of Grass*
For my Mother, my inspiration,

and my Father, my support.
Chapter 1

Literature Review
1.1 Introduction

Since the discovery by Hitzig and Fritsch in the 1870s of an electrically excitable region of frontal cortex in dogs that evokes muscle twitches, the organization of the motor cortex has been a highly investigated topic in neuroscience. In addition to Hitzig and Fritsch, John Hughlings Jackson had proposed a body map topography based in his observations of epileptiform activity in human patients (Gross, 2007). However, it was not until Wilder Penfield's experiments using electrical stimulation to map the cortical surface in humans that more precise maps of the sensory and motor regions were created (Penfield and Boldrey, 1937). These studies showed a loose body topography in frontal cortex in which stimulation of different cortical sites within each body representation (e.g. the forelimb or hindlimb region) evoked qualitatively different movements of that limb (Schieber, 2001).

In humans and primates, it has been well established that the motor cortex contains multiple subregions, including the primary motor cortex (MI) as well as multiple supplementary- and pre- motor areas (SMA and PMA, respectively). Cytoarchitecturally defined divisions of frontal cortex and modern microstimulation mapping studies have complimentarily demonstrated the boundaries of these different motor areas in terms of both structure and function (Belmalih et al., 2007). These different motor areas have also been demonstrated by neuroanatomical tracing studies that reveal segregated pools of forelimb corticospinal neurons (projecting to the cervical enlargement) throughout SMA and PMA that overlap with neurons projecting to the forelimb representation in MI (Dum and Strick, 2002). Additional studies have further divided the motor cortex based on the specificity of cortical afferents into parieto-dependent and pre-fronto-dependent motor areas (Rizzolatti and Luppino, 2001). Together these studies demonstrate a complex hierarchy of motor processing across functionally distinct areas of frontal cortex in primates.
These same techniques for mapping motor cortex (cytoarchitectonics, stimulation mapping, and neuroanatomical tracing) have been used in other mammalian species, specifically in rodents. However, the subdivisions of the motor cortex are highly debated, specifically regarding the presence of supplementary motor regions in rodents (Cenci et al., 2002; Krubitzer et al., 2011). One of the main difficulties with rodents is that the motor and sensory regions are not in separate gyri as in primates, due to rodent brains being lysencephalic. As in other species where motor and somatosensory regions are still diverging, the forelimb and hindlimb representations of motor cortex partially overlap with somatosensory cortex in the rat, so they are difficult to concretely dissociate (Nudo and Frost, 2007). However, the whisker representation in somatosensory and motor cortex are separated by the forelimb and hindlimb representations. Therefore the whisker system is a viable model for studying organizational principles of motor cortex in rodents.

The presence of higher order motor areas in rodents, or more generally the degree to which organizational principles are conserved between rodents and primates, is a pertinent issue in neuroscience. Rodents are frequently chosen as a model species for biomedical research because they are phylogenetically close to primates, both belonging to the superorder Euarchontoglires (Murphy et al., 2004). Furthermore, rodents are small, inexpensive, breed quickly and allow for a greater variety of experimental tools (e.g. genetic intervention); making them ideal for biomedical investigations. Rodent motor cortex is already being used to study a wide range of neurological disorders including Huntington's Disease (Miller et al., 2011), chronic neuropathic pain (Lucas et al., 2011), ischemic stroke (Lapash Daniels et al., 2009), amyotrophic lateral sclerosis (Van Den Bosch, 2011), Parkinson's Disease (Kita and Kita, 2011), and others (Lane and Dunnet, 2011). Thus, the extent of homology between rodents and primates is a primary concern. The goal of this dissertation is to address this issue using the rat vibrissal system to analyze the organization of rodent motor cortex in terms of cytoarchitecture, stimulation
mapping, sensory evoked neuronal activity, and anatomical connectivity. Our data reveal the presence of subregions within the whisker representation in motor cortex, which demonstrates functional parcellation of the motor cortex similar to primates. This suggests homologous organization of sensorimotor processing between the two mammalian groups, which indicates they are a suitable animal model for studies of motor cortex function.

1.2 Whisker-Barrel System

The whisker-barrel system of rodents is an extensively used model for studying sensorimotor processing due to its isomorphic representation of each of the vibrissa throughout the ascending neuraxis (see Figure 1-1). This precise somatotopy is largely invariant across individuals, making this system ideal for use in the study of sensorimotor disease models, genetics studies, and investigation of functional connectivity. In addition to its well-described anatomical organization, whisking behavior itself is highly stereotyped, allowing investigators to make robust structure-function relationships.

1.2.1 Whisking behavior

Rodents actively move their mystacial vibrissa to gain sensory information about their immediate environment. This behavior coincides with rodents being largely nocturnal and subterranean, relying on active touch in lieu of visual guidance (Burn, 2008). Rhythmic back and forth movement of the macrovibrissa is known as "whisking" and has been seen in a number of different rodent species (Mitchinson et al., 2011). Whisking behavior is highly correlated with other exploratory behaviors such as sniffing and head orienting (Semba and Komisaruk, 1984; Cao et al., 2012; Towall and Hatrmann, 2006). Together, these behaviors allow the animal to
gather sensory information pertinent to navigation, texture identification, prey capture, and social activities (Brench, 2007; Carvel and Simons, 1990; Favaro et al., 2011; Hartman, 2011).

During exploratory whisking, the whiskers on each side of the face are rhythmically protracted and retracted in the rostro-caudal plane in 1-2 second epochs, at frequencies from 5-15 Hz (Berg and Kleinfeld, 2003). This is accomplished by active control of three different sets of muscles in the whisker pad. Initially the rostral extrinsic muscles contract, causing the whiskers to protract. This is followed by contraction of the intrinsic muscles, which further pivot the whiskers rostrally. Finally, these two muscle groups are relaxed and the caudal extrinsic muscles are contracted, pulling the entire whisker pad caudally (Hill et al., 2008). Together, the activity of these muscle groups creates the rhythmic motion of the whiskers during open-air, exploratory whisking.

Behavioral studies have shown that whisker contract with external objects can disrupt the whisking cycle. If an object is contacted during the protraction sweep of the whisking cycle, the animal responds by suddenly ceasing the anterior sweep of the vibrissa (Grant et al., 2009). Evidence from electromyography (EMG) recordings of the extrinsic and intrinsic muscles shows that each phase of whisking is actively controlled by the specific muscle groups mentioned previously (Berg and Kleinfeld, 2003). Furthermore, it has been shown that each of these muscles is controlled by separate pools of motor neurons in the facial nucleus (Takatoh et al., 2013). This suggests that sensory information can alter the activity of these different pools of whisker motor-neurons, to modify the whisking cycle in response to the whisker contact.

Stimulus induced modification of whisking behavior also disrupts the bilateral coordination of whisking. When the animal engages in bouts of rhythmic whisking, the vibrissa on each side of the face are simultaneously engaged. However, while exploratory whisking in free air is generally symmetrical (Gao et al., 2003), unilateral contact of the vibrissa and horizontal head movements can cause asymmetries in whisking behavior (Towall and Hartman, 2006).
Following unilateral whisker contact, the whisking amplitude on the contacted side are dampened, whereas the whiskers on the contralateral side are swept in a wider amplitude arc (Mitchinson et al., 2007). However, the relative frequency of whisking is still conserved. Thus, whisking in rodents, similar to movements of the eyes in primates, is a bilaterally coordinated behavior that is modulated by sensory feedback.

1.2.2 Ascending sensory pathways

The rodent whisker-to-barrel system is a well studied sensory system due to the highly organized topography of the ascending projections from the vibrissa up to the cortical level. The large whiskers in rodents are organized into a specific pattern of rows and arcs that is for the most part invariant across individuals. As shown in Figure 1-1, a nomenclature has been adopted where the whisker rows are labeled "A" through "E" from dorsal to ventral, respectively, and the arcs increase in number in the anterior direction. The organization of the whiskers is maintained in its neuronal representation throughout the ascending neuraxis, creating "barrel"-like structures referred to as "barrelettes" in the principal and spinal trigeminal (PrV and SpV) nuclei of the brainstem, "barreloids" in the ventroposterior medial (VPM) nucleus of the thalamus, and "barrels" in layer IV of primary somatosensory cortex (SI). These isomorphic representations contain a one-to-one relationship from whisker to barrel, creating an easily identifiable structure-function relationship, which is why this system is an ideal model for sensory processing.
**Figure 1-1.** Ascending projections of the whisker-barrel system. The ascending projections of the whisker-barrel system are topographically organized throughout the neuraxis. Left column shows examples of cytochrome-oxidase (CO) dense blobs that match the row-like organization of the vibrissa on the face. Each CO dense region is a neuronal aggregate that processes a single whisker; known as "barrelettes" in the brainstem (Source: image adapted from Toki et al., 1999), "barreloids" in the ventroposterior medial (VPM) thalamus (Source: image adapted from Haidarliu and Ahissar, 2001), and "barrels" in primary somatosensory (SI) cortex (Source: image adapted from Alloway, 2008). Right column shows the parallel pathways of ascending sensory projections: single-whisker lemniscal(1) in red, multi-whisker lemniscal(2) in orange, extralemniscal in magenta, and paralemniscal in green (Source: image of Sprague-Dawley rat courtesy of Glenn Watson; sagittal image of trigeminal nuclei in the brainstem adapted from Paxinos and Watson, 2007). Organization of ascending projections is adapted from Kleinfeld and Deschenes, 2011; Feldmeyer, 2012; Mao et al., 2012; and data from our laboratory.

Abbreviations: principal trigeminal (PrV) nucleus; spinal trigeminal (SpV) oralis (o), interpolaris (i), and caudalis (c) nuclei; ventroposterior medial (VPM) dorsomedial (dm) and ventrolateral (vl) nuclei; posterior medial (POm) nucleus; primary (SI) and secondary (SII) somatosensory cortex; primary motor (MI) cortex; rostral (R); caudal (C); lateral (L); dorsal (D); dorsolateral (DL).

In addition to the precise somatotopic organization, well-defined parallel processing streams convey the sensory information up the neuraxis. Information from the mechanoreceptors wrapped around the follicle of the whiskers is passed via the trigeminal ganglion into the principal and spinal trigeminal nuclei in the brainstem. As shown in Figure 1-1, the relays conveying this information are organized into 4 parallel pathways: the single-whisker lemniscal (1), the multi-whisker lemniscal(2), the extralemniscal, and the paralemniscal pathway (Yu et al., 2006; reviewed in Feldmeyer, 2012). The two lemniscal pathways convey whisking and touch information. They begin in PrV, but innervate separate regions of the VPMdm. The lemniscal(1) pathway arises from the 'core' region of VPMdm, where the barreloids are located, and then innervates layers IV, Vb, VIa of the barrel columns. The lemniscal(2) pathway passes through the 'head' region of VPMdm, before innervating the septal compartment of layer IV, and is thought to be responsible for the multi-whisker receptive fields of septal neurons (Furuta et al., 2009). The extralemniscal pathway, by comparison, only conveys information about whisker contacts, and has a separate pathway starting in the interpolar division of the spinal trigeminal nucleus (SpVi) and continuing through the VPMvl onto SII. The paralemniscal pathway, contrary to common knowledge, originates from neurons in PrV, SpVo, and SpVi (Veinante and Deschenes, 1999;
Veinante et al., 2000). This pathway only processes whisking (e.g. motion) kinematics, and is markedly different from the other pathways due to its innervation of the posterior medial (POm) nucleus, instead of VPM. Projections from POm innervate layers I and Va of both septal and barrel columns with weak innervation of the layer IV septa. Together these parallel pathways represent an organized mixture of information about whisker touch and motion in the ascending whisker sensory system.

1.2.3 Beyond primary somatosensory cortex

From SI barrel cortex, information is transmitted to several other sensory cortical areas and subcortical structures. Beyond SI, these lemniscal and paralemniscal pathways continue to be divided by the projections from barrel and septal columns, maintaining their specificity in processing whisker motion and contact. In addition to subcortical projections to the striatum, thalamus, pons and superior colliculus (Hoffer et al., 2005), SI projects to other cortical areas including secondary somatosensory (SII), primary motor (MI), posterior parietal (PPC), parietal ventral (PV), perirhinal (PR) and the contralateral SI (Fabri and Burton, 1991; Koralek et al., 1990). However, there are differences in the origin of these projections relative to the barrel and septal columns. Though both barrel and septal columns project to SII, it is mainly the septal neurons that innervate MI and PPC (Alloway et al., 2004; Lee et al., 2011). This difference in projections from barrel and septa compartments to these regions is thought to relate to processing whisker contact and whisker motion, respectively, as conveyed by the different ascending pathways (Alloway, 2008). Each of these sensory regions (SI, SII and PPC namely) has been shown to project to MI (Colechio and Alloway, 2009), indicating multiple higher order pathways for sensory information to modify activity in motor cortex.
1.3 Rodent Motor Cortex

1.3.1 Cytoarchitecture and mapping.

The motor cortex in rodents is located in the frontal cortex, rostral and medial to the primary somatosensory cortex. The most common approach to investigate the organization of the motor cortex has been to use intracortical microstimulation (ICMS) to map how cortical locations relate to evoked muscle twitches in the periphery. Generally, an electrode is inserted into layer V, the main output layer containing corticobulbar and corticospinal neurons, and a brief train of electric pulses (<100msec) is administered causing the corticofugal neurons to discharge. The result is brief contractions of small groups of muscles, which results in visible movement of body parts (e.g. the whisker retraction, elbow flexion, etc.). By marching the electrode across a wide region of cortex, many studies have observed a loose somatotopic organization similar to that observed in SI (Hall and Lindholm, 1974; Donoghue and Wise, 1982; Sanderson et al., 1984; Neafsey et al., 1986; Miyashita et al., 1994; Hoffer et al., 2003; Brecht et al., 2004; Tandon et al., 2008; Tennant et al., 2011). Panel A in Figure 1-2 demonstrates this general musculotopic organization in MI of a rat relative to the somatotopic organization of SI seen in layer IV of CO processed tissue. Another prominent feature of the body representation found in MI is its mirrored relationship to the somatotopy seen in SI.
Figure 1-2. Functional organization of rat sensorimotor cortex. A: Tangential section from layer IV of rat cortex processed for cytochrome oxidase (CO) showing the representation of the different body parts in the primary somatosensory (SI) and motor (MI) cortex. B: Nissl stained section of cortex corresponding to the arrow and dotted line in A depicting cytoarchitectural regions relative to their functional representations. C: Myelin stained section of cortex corresponding to area showed in B depicting higher density of myelin in AGm. (Source: image of myelin stained section adapted from Brecht et al., 2004). Abbreviations: cingulate (Cg), agranular medial (AGm), agranular lateral (AGl), primary somatosensory cortex (SI), primary motor cortex (MI), secondary somatosensory cortex (SII), posterior parietal cortex (PPC), auditory cortex (AC), visual cortex (VC), hindpaw (Hp), forepaw (Fp), whisker (Wh), rhythmic whisking (RW), nose (N), lower jaw (Lj), upper jaw (Uj), jaw (J), eye (E).

An alternate theme of motor cortex organization has emerged from microstimulation mapping studies using long pulse trains. In both rodents and primates, long duration ICMS (>500 msec in duration) has been shown to elicit complete behaviors of body parts in contrast to the brief muscle twitches evoked by short duration ICMS. Administering long duration ICMS in the forelimb area of primates results in movements of the arm that bring the hand to the mouth, as in feeding, or making defensive gestures, to block the face from attack (Grazziano and Aflalo, 2007). Long pulse train ICMS experiments have also been performed in rodents, showing target
guided movements of the forelimb (Bonazzi et al., 2013). In the whisker region of the rodent motor cortex, long duration ICMS mapping has been used to demonstrate two subregions. Long pulse train stimulation throughout most of wMI results in a sustained whisker retraction, whereas stimulation of the caudal-medial portion of the MI-whisker representation (see Figure 2-2, panel A) causes rhythmic whisking (RW) similar to exploratory whisking of the awake-behaving animal (Haiss and Schwartz, 2005; Cramer and Keller, 2006). These results have been further confirmed by ICMS and optogenetic stimulation in wMI of mice (Matyas et al., 2010). These findings suggest that in addition to the loose body map, motor cortex in mammalian species may also be organized in a behavior specific manner.

In addition to microstimulation maps, motor cortex can also be divided based on cyto- and myelo- architectural information. As shown in panel B of Figure 1-2, rodent motor cortex contains three different regions as defined my cytoarchitecture: agranular lateral (AGl), agranular medial (AGm), and cingulate (Cg) cortical areas (Donoghue, 1982). These regions have been shown to correspond with ICMS mapping such that the representation of the eyes is found in Cg, the whiskers in AGm, and the forelimb in AGl (Brecht et al., 2004). Whisking is a highly precise, rapid behavior and thus requires rapid neural conduction to control such a fast behavior. Thus, the motor representation in AGm is heavily myelinated (figure 1-2, panel C), further distinguishing the whisker representation in the frontal region of cortex. Together, the use of histological staining and ICMS mapping have aligned to show structure function relationships for describing the organization of rodent motor cortex.

1.3.2 Electrophysiological recordings in whisker motor cortex.

The whisker representation in MI (wMI) has been further explored by electrode recordings of neuronal activity while monitoring whisking behavior. Recordings of both local
field potentials (LFP) as well as single-unit activity in wMI have been shown to cohere with electromyographic (EMG) activity recorded from the musculature of the whisker pad (Ahrens and Kleinfeld, 2004; Friedman et al., 2012). Specifically, a rise in LFP power and increases in neuronal firing rates in MI have been shown to increase just prior to whisking (Carvell et al., 1996; Friedman et al., 2006). Furthermore, spike rates in wMI correlate with many different parameters of whisking, including the amplitude of the whisk (Friedman et al., 2012) or the coding of whisker angle (Hill et al., 2011). ICMS mapping and electrode recordings lead to the conclusion that wMI plays a significant role in control of whisking behavior and have identified the regions of MI involved in different aspects of whisker motor control.

In contrast to motor related neuronal activity, some studies have shown somatosensory evoked responses in wMI. In both humans and primates, neurons in MI have been shown to respond to somatosensory stimulation (Hatsopolous and Suminski, 2011; Shaikhouni et al., 2013). Based on the coordinates from ICMS mapping studies and tracing from SI barrel cortex, neuronal recordings in the wMI of anesthetized rats are characterized by increased discharges in response to both electrical and mechanical stimulation of the whiskers (Farkas et al., 1999; Chakrabarti et al., 2008). These sensory evoked responses are driven by septal column neurons in SI, and are thought to code for the frequency of whisking (i.e. the kinematics of whisking). Single unit responses in vivo were found to be strongest in the deep layers of MI. Conversely, optogenetic activation of SI barrel cortex afferents to wMI in vitro revealed stronger synaptic drive in layers II/III and Va, though this study did not record action potentials (Mao et al., 2011). Voltage-sensitive dye imaging has also been used to characterize the spatial and temporal response pattern of somatosensory evoked activity (all voltage fluctuations, not just action potentials) in the superficial layers of MI (Ferezou et al., 2007). These physiology studies have characterized the spatial localization of somatosensory responses in wMI, however, they have not been directly compared to the motor activity.
1.3.3 Cortical connectivity of whisker motor cortex.

Understanding the afferent and efferent connectivity of the motor cortex is crucial for interpreting physiology studies and ultimately understanding its role in modifying behavior. These connections are generally organized into three groups: reciprocal loops with other cortical areas, subcortical loops, and motor output projections to the brainstem and spinal cord. Studying these projections is accomplished using either anterograde (from neuron to terminal) or retrograde (from terminal to neuron) tracers injected into specific physiological or cytoarchitectural regions of the brain and then reconstructing the resulting labeling patterns. These experiments have demonstrated the multitude of afferent pathways through which sensory information reaches the motor cortex to alter behavior, and the equally myriad efferent pathways by which motor information ultimately produces behavior.

Sensory afferents to the motor cortex largely arise from parietal regions of cortex. The main source of sensory information in wMI comes from SI barrel cortex (Izraeli and Porter, 1995; Colechio and Alloway, 2009; Aronoff et al., 2010; Mao et al., 2011), specifically from neurons aligned with the septal columns (Alloway et al., 2004; Chakrabarti et al., 2006). These reciprocal projections are organized in an anisotropic scheme to facilitate integration of barrel projections within a specific row (Hoffer et al., 2003). This is behaviorally relevant given that rodents whisk in the rostrocaudal axis and thus are more likely to contact the same object with whiskers in the same row and not across arcs. As illustrated in Figure 1-1, projections from SI barrel cortex originate in layers II, III, and V₃ but terminate throughout all the layers of wMI (Hoffer et al., 2005; Mao et al., 2011). The "with-in" row convergence of projections from SI is markedly pronounced in the deep layers of MI (Hoffer et al., 2003), indicating that the supragranular and infragranular SI to MI projections are organized by different schemes for processing sensorimotor transformations. Convergence of SI projections in infragranular MI may explain the strong
stimulus response observed in MI recording studies (Farkas et al., 1999; Chakrabarti et al., 2008). Understanding the spatial extent and laminar specificity of SI barrel cortex projections to MI provides important clues not only for understanding MI sensory processing, but for helping to demarcate the extent of wMI in terms of anatomical connectivity.

In addition to inputs from SI barrel cortex, wMI (AGm) also receives projections from other sensory and associative cortices. As discussed earlier, SI projects to a number of other higher order cortical regions, such as SII, PPC, PV, and PR. Retrograde tracing studies have shown that wMI receives projections from each of these regions, as well as, cortical areas associated with memory (entorhinal cortex, EC) and executive (prefrontal cortex, PFC) functions (Reep et al., 1990; Colechio and Alloway, 2009). This suggests that in addition to processing purely somatosensory related information, wMI may integrate multimodal, associative, and executive processes as well. In primates, different cortical projections from parietal and prefrontal areas are distributed across MI, PMA and SMA and are easily distinguishable due to the wide cortical area and separate gyri encompassing these different motor areas (Rizzolati, 2002). However, in lysencephalic mammals like rodents, no study has yet employed anterograde tracers to determine the extent to which different cortical areas (SI, SII, PPC, PFC etc.) converge or remain segregated in motor cortex. Divergence of these cortical projections would suggest the presence of higher order motor areas homologous to primates.
1.3.4 Efferent projections from whisker motor cortex.

The efferent projections to the brainstem and spinal cord allow motor cortex to exert control of the skeletal muscles during behavior. Multiple pathways exist in both rodents and primates to control limb movements via corticospinal projections and intervening brainstem regions such as the superior colliculus, red nucleus, reticular formation and others. Motor cortex control of whisking is achieved solely via oligosynaptic pathways through these brainstem regions that then project to the facial nucleus (Isokawa-Akesson and Komisaruk, 1987; Hattox et al., 2002; Alloway et al., 2010). This motor efferent organization is similar to frontal eye field control of saccades in primates (Shook et al., 1990). Though there is some evidence of a direct cortical projection to the facial nucleus, the number of corticofacial neurons is small and they make very sparse terminations indicating they are weak projections (Grinevich et al., 2005; Alloway et al., 2010). Ultimately, it is motor neurons in the lateral subnucleus of the facial nucleus that control the extrinsic and intrinsic muscles of the whisker pad via the facial nerve (Takatoh et al., 2013). The activity of the motor neurons that control whisking is determined by a confluence of brainstem inputs, which act in concert to create both the rhythmic nature of whisking and the precise stimulus evoked alterations discussed above.
Figure 1-3. Illustration of the efferent projections of the MI-whisker region (AGm) to brainstem nuclei projecting to the lateral facial nucleus to control whisking. Line thickness represents strength of connection. Green projections are putatively glutamatergic whereas projections in red are serotonergic. Projections shown in blue represent pathways by which superior colliculus controls whisking.

The route by which motor cortex alters activity in the lateral facial nucleus has been studied by anatomical tracing studies from physiologically defined areas of motor cortex (Miyashita et al., 1994; Hattox et al., 2002; Alloway et al., 2010). As summarized in Figure 1-3, these studies have revealed bilateral projections of varying density from wMI to brainstem regions including the superior colliculus, red nucleus, deep mesencephalic nucleus, periaqueductal grey (PAG), as well as pontine and medullary reticular formations. In turn, each of these brainstem regions provide bilateral inputs to the lateral facial nucleus. In addition, serotonergic innervation from the raphe nucleus and the lateral paragigantocellularis reticular formation (LPGi) have been shown to induce the rhythmicity of exploratory whisking (Hat
The superior colliculus is an important relay structure in the control of whisking (see projections in blue in Figure 1-3). In addition to receiving the strongest projections from wMI, the superior colliculus has direct projections to the facial nucleus, with additional widespread connectivity to other brainstem regions that project to the facial nucleus (Alloway et al., 2010; Miyashita and Mori, 1995). Thus, rodent control of whisking is not only similar to primate saccade regulation in terms of multiple oligosynaptic output pathways via brainstem regions projecting to motor neurons, but also due to its strong involvement of the superior colliculus (Schiller and Tehovnik, 2005).

1.3.5 Subcortical loops.

In addition to cortical connectivity and motor output projections, motor cortex is involved in several subcortical processing loops. Specifically, motor cortex is a part of four main subcortical loops via projections to the striatum, claustrum, thalamus, and basal pons. These subcortical projections either directly or indirectly (via motor thalamus) project back to the motor cortex, creating processing loops that are involved in modifying motor output and motor skill learning (Doyon et al., 2003). The striatum is the main input nucleus of the basal ganglia and is thought to be involved in action selection (during both stimulus-response and goal directed behaviors) in addition to sequential learning during the acquisition of motor skills (Redgrave et al., 2010). The basal pontine nucleus is the main input to the pontocerebellar system and is also involved in motor control and motor skill learning. This loop is thought to regulate motor adaptation; the fine precision of motor control via detection and correction of motor errors (Doyon et al., 2003; Ramnani, 2006). Little is known however about the claustrum in terms of either its connectivity or its general function (Crick and Koch, 2004). However, all these loops have one commonality: they feed back to the motor cortex.
One important function of these subcortical loops is sensorimotor integration. In addition to their direct connections, SI and MI in rats send overlapping projections to some thalamic nuclei (namely POm), the striatum, and the basal pons (Hoffer and Alloway, 2001; Leergard et al., 2004). These different structures relay back to motor cortex either directly, as is the case with POm (suggesting fast time-scale sensorimotor integration), or via multisynaptic connections terminating in the motor thalamus. Just as in primates, the basal ganglia and pontocerebellar loops target separate nuclei in the motor thalamus. The basal ganglia output from the globus pallidus internal (GPi) targets the ventromedial (VM) and rostroventral region of the ventroanterior-ventrolateral (VA-VL) nuclear complex, whereas the deep cerebellar nuclei projections terminate in the dorsocaudal region of the VA-VL complex (Kuramoto et al., 2010). These different regions of the VA-VL complex then project to different layers of the motor cortex, with basal ganglia thalamic output targeting layer 1 and cerebellar thalamic output terminating in layer V of motor cortex (Kuramoto et al., 2009). Interestingly, POm projections to MI terminate in layer II/III, such that each of the major thalamic nuclei target different layers of MI, suggesting different roles in modifying motor output (Ohno et al., 2012). Together the basal ganglia, sensorimotor thalamus, and pontocerebellar systems are involved in subcortical sensorimotor integration circuits essential for sensory guided action selection and motor modulation.
Figure 1-4. Illustration of the difference in interhemispheric projections from the MI-whisker region (AGm) and the MI-forepaw region (AGl). The MI-whisker region was found to have stronger projections to the contralateral striatum, claustrum, thalamus and pons (red arrows) compared to the MI-forepaw projections (green arrows). These subcortical structures feed into the motor cortex via mono- and multi-synaptic projections (black arrows).

In addition to sensorimotor integration, these subcortical structures have been shown to be heavily involved in the interhemispheric coordination of the motor cortex in each hemisphere. Previous studies in our lab tracing the bilateral projections from ICMS defined whisker (AGm) and forepaw (AGl) regions of MI have shown strong interhemispheric projections from wMI to the striatum, claustrum, thalamus and pons, as shown in Figure 1-4 (Alloway et al., 2008; 2009; 2010). These projections are significant given the evidence noted previously that whisking, as opposed to forelimb movements, is a bilaterally coordinated behavior. As illustrated in Figure 1-4, the striatal and pontocerebellar loops have a multisynaptic feedback to MI via the motor
thalamus. The projections from wMI to the contralateral thalamus, however, directly feedback to wMI of that hemisphere. These small, modulator type terminals originate in layer VI and cross through the thalamus itself (as opposed to crossing in the corpus callosum) and target the intralaminar nuclei (PC/CL), anterior medial nucleus (AM), VA-VL complex, and VM (Alloway et al., 2008). This monosynaptic loop likely demonstrates a faster route of interhemispheric communication in comparison to multisynaptic basal ganglia and pontocerebellar loops. The interhemispheric claustral projections, prior to this doctoral work had just been discovered (Alloway et al., 2009) and not fully characterized as functional loops. These subcortical loops as a whole, though regulating interhemispheric communication may serve to synchronize or desynchronize activity wMI of each hemisphere, however, little to nothing is known about their physiological characteristics, nor their influence on behavior.

1.4 Contribution of Doctoral Research

The goal of my doctoral work was to characterize motor cortex neural circuitry in rats that has received little or no attention and draw comparisons with known organization of primate motor cortex. The aim of the first study was to characterize the interhemispheric connections between different regions of motor cortex and the claustrum. Preliminary work in the lab discovered that wMI had stronger projections to the claustrum in the contralateral hemisphere, but that the claustrum projections back to wMI remained in the ipsilateral hemisphere (Alloway et al., 2009; Colechio and Alloway, 2009). To demonstrate a functional interhemispheric loop, bilateral injections were made into physiologically-defined homotopic representations in MI (whisker retraction, rhythmic whisking, or forepaw); an anterograde fluorescent tracer in one hemisphere and a retrograde fluorescent tracer in the other. The bilateral claustral labeling was then quantitatively analyzed in terms of terminal/neuron counts, terminal density, and terminal/neuron
overlap. A final set of experiments was conducted to inject a retrograde tracer into the claustrum, to identify both the laminar origin of corticoclastral neurons as well as the relative interhemispheric balance. These studies identified a unique interhemispheric circuit, connecting the wMI regions of each hemisphere.

The goal of the second study in the doctoral work was to assess the involvement of the claustrum in sensorimotor interactions. Though the striatal, thalamic and pontocerebellar systems have received a great deal of attention regarding sensorimotor integration, few studies to date have investigated the potential involvement of the claustrum. To elucidate the neural circuitry regulating sensorimotor interactions, a series of neuronal tracing experiments were conducted. In the first set of animals, two anterograde tracers were injected into physiologically defined whisker regions of SI and MI. In the second set of animals, these same regions of SI and MI were injected with two retrograde tracers. Our results showed that only MI sent afferent projections to the claustrum, however, the claustrum projected back to both regions of cortex. In a final set of animals, anterograde tracers were placed bilaterally into wMI and a retrograde tracer was injected into wSI. These experiments demonstrated that wMI projects to claustral neurons that project to wSI. The results of this study show that the claustrum is involved in sensorimotor coordination, but not sensorimotor integration.

The goal of the final study was to ascertain the degree to which sensory and motor processing overlap in wMI. Observations from previous studies in the lab had indicated that wSI projections and sensory responses were most robust in the lateral region of MI (Hoffer et al., 2005; Chakrabarti et al., 2008), whereas low current ICMS evoked the strongest whisker movements from medial regions (Brecht et al., 2004; Alloway et al., 2008; 2009; 2010). To ascertain the separation of sensory and motor functions in wMI, ICMS maps and cortical afferents from wSI were compared in the same animal and were verified to be in medial (AGm) and lateral (AGm-AGl transition zone) compartments as suspected. In subsequent tracing
experiments, one or two anterograde tracers were injected in physiologically defined whisker regions of SI, SII, or PPC and their terminal labeling in MI was analyzed. Tracer labeling revealed that SI and SII converge in the lateral, sensory compartment of MI, whereas PPC projects more medially showing little overlap with SI projections. These tracing results were further verified via two electrode recordings in the medial and lateral subdivisions of MI. The strongest ICMS evoked whisker movements (as measured by EMG) were located medially, whereas sensory evoked responses were only found in the lateral region. A final set of animals received tracer injections in the whisker regions of the superior colliculus, the main output target of wMI (Alloway et al., 2010). Retrogradely labeled neurons were preferentially located in AGm, the motor output region of wMI, and seemed to avoid the sensory input region. These experiments demonstrate functional subregions within the whisker representation in motor cortex, indicating parcellation of motor cortex is a homologous feature in both rats and primates.
Chapter 2

Functional Specificity of Claustrum Connections in the Rat: Interhemispheric Communication between Specific Parts of Motor Cortex

2.1 Introduction

The claustrum is an elongated neuronal structure that extends rostrocaudally through the telencephalon of all mammals including rats (Kowianski et al., 1999). The claustrum receives inputs from multiple cortical areas (Pearson et al., 1982; Carey and Neal, 1985; Clarey and Irvine, 1986), but multimodal integration is minimal because separate parts of the claustrum process different modalities (Olson and Graybiel, 1980; Remedios et al., 2010). Despite this topographic organization, many neurons in the claustrum project to widely separated cortical targets (Minciacchi et al., 1985; Li et al., 1986). These and other findings indicate that the claustrum may coordinate widespread cortical regions for specific behavioral and cognitive functions (Edelstein and Denaro, 2004; Crick and Koch, 2005).

Consistent with its role in orchestrating neural processes in separate cortical areas, the claustrum has connections with the motor cortices in both hemispheres (Minciacchi et al, 1985; Sloniewski et al, 1986; Li et al, 1986). In cats, electrical stimulation of the claustrum excites the frontal eye fields in both hemispheres, and this suggests that the claustrum could synchronize cortical regions that subserve bilaterally coordinated behaviors such as eye movements (Cortimiglia et al, 1991). Like eye movements, rats actively move their whiskers on both sides of the face, and these simultaneous movements are bilaterally coordinated during different behavioral situations (Towal and Hartman, 2006; Mitchinson et al., 2007). In view of the inherent bilateral nature of eye and whisker movements, similar claustral circuits might mediate the interhemispheric coordination that regulate these behaviors.

Our laboratory has shown that the rat claustrum receives bilateral projections from the whisker region in primary motor (MI) cortex, but the contralateral projections are more prominent (Alloway et al., 2009). Furthermore, the rat claustrum projects to the ipsilateral MI whisker region, but it does not project to the contralateral hemisphere (Colechio and Alloway, 2009). In
comparison, the MI forepaw region has few afferent or efferent connections with the claustrum in either hemisphere. These results suggest that one function of the claustrum and its interhemispheric connections is to coordinate the motor areas that regulate specific types of bilateral movements.

The present study assessed the plausibility of this view by injecting an anterograde tracer into motor cortex of one hemisphere and a retrograde tracer into the corresponding motor region of the other hemisphere. This bilateral two-tracer approach tested the hypothesis that corticoclaustral and claustrocortical neurons form interhemispheric circuits between specific motor regions. In addition to injecting the motor forepaw (MI-Fp) regions in one group of rats, two other groups received tracer injections in motor regions that evoke specific types of whisker movements. One whisker region, located in medial agranular cortex, evokes whisker retractions (MI-Re) when microstimulated (Brecht et al., 2004). The second, more caudal whisker region, evokes protractions or rhythmic (5-15 Hz) whisking (MI-RW) movements when microstimulated (Sanderson et al., 1984; Haiss and Schwarz, 2005). The results revealed a cortico-claustro-cortical interhemispheric circuit that interconnects the MI whisker regions of both hemispheres, especially the RW regions.

2.2 Materials and Methods

All procedures on adult male Sprague-Dawley rats (Charles River Co., Wilmington, MA) conformed to National Institute of Health guidelines and were approved by our Institutional Animal Care and Use Committee.
2.2.1 Animal surgery.

Rats were initially anesthetized by an intramuscular injection of ketamine HCl (20 mg/kg) and xylazine (6 mg/kg) followed by intramuscular injections of atropine methyl nitrate (0.5 mg/kg), dexamethasone sodium phosphate (5 mg/kg), and chloramphenicol (50 mg/kg). Each rat was intubated, placed in a stereotaxic frame, and ventilated with oxygen. A homeothermic heating blanket maintained body temperature at 37.0°C, ophthalmic ointment was used to prevent corneal drying, and vital signs such as heart rate, specific oxygen, and end-tidal carbon dioxide (Surgivet) were monitored continuously. A midline incision was made over the cranium and lidocaine (2%) was injected into the wound margins. Craniotomies were made bilaterally over the motor cortex of each hemisphere at coordinates consistent with previous studies (Hall and Lindholm, 1974; Hoffer et al., 2003; Brecht et al., 2004; Haiss and Schwarz, 2005).

2.2.2 Tracer injections in MI.

Intracortical microstimulation (ICMS), was administered to MI cortex as short (80 ms) and long (1400 ms) pulse trains via a glass electrode filled with hypertonic (3M) saline (0.4 -1.3 MΩ). Short stimulus trains were administered at 250 Hz (0.7 ms pulse separated by intervals of 3.3 ms), whereas long stimulus trains were tested at both 250 and 100 Hz (0.7 ms pulses separated by intervals of 9.3 ms). The MI cortex was briefly mapped to identify sites that evoked movements of the forepaw or whiskers. Mapping of MI commenced when the rat spontaneously began making just-noticeable whisking movements because this indicated an anesthetic plane that allowed ICMS to evoke muscle twitches.
The microstimulation electrode entered cortex orthogonal to the pial surface, and ICMS was administered at depths of 1.0 – 1.7, which is where corticospinal and corticobulbar projections originate. Multiple depths were stimulated along the electrode penetration, especially in the medial bank of MI where the functional topography can change suddenly (see Figure 3 in Brecht et al., 2004). Short ICMS trains at low currents (< 50 μA) evoked brief dorsoflexion of the forepaw in the MI-Fp region, whisker retractions in the MI-Re region, and whisker protractions in the MI-RW region. In contrast, long ICMS trains in the MI-Fp region produced sustained contraction of the forelimb muscles, sustained retraction of the whiskers in the MI-Re region, and low-amplitude (~2-3 mm) rhythmic whisking movements in the MI-RW region. These observations are consistent with other reports (Sanderson et al., 1984; Haiss and Schwarz, 2005). Mapping started in the MI-Re region and continued caudally until the MI-RW region was located, and then continued laterally and rostrally to locate the MI-Fp region. Stimulation sites were separated by at least 500 μm, and four to seven sites were tested in each hemisphere so that tracer injections were greater than 500 μm away from ICMS sites that had different functional representations.

After locating a suitable injection site, the stimulating electrode was removed and an injection pipette was inserted at that site. Tracer injections were made bilaterally in the Fp, Re, or RW regions of MI at depths of 1.1 to 1.7 mm below the pial surface. In one hemisphere, a 2% solution (in physiological saline) of the retrograde tracer FluoroGold (FG, Fluoro-Chrome, LLC, Denver, CO, H-22845) was iontophoretically administered through a glass pipette (30-60 μm outer tip) at 2 μA for 15 minutes using a 7-s on/off duty cycle. The other hemisphere was pressure injected with 100 nL of a 15% solution (in 0.01M PBS) of the anterograde tracer FluoroRuby (FR, Molecular Probes, Eugene, OR; D-1817) via a Hamilton syringe with a glass pipette (60-80 μm tip) cemented on the end. As shown by Table 2.1, tracer injections for each functional region in MI were placed in a limited range of stereotaxic coordinates as follows (in
mm): MI-Fp region, 1.6-2.5 rostral and 2.1-2.8 lateral to bregma; MI-Re region, 1.8-2.2 rostral and 1.2-2.1 lateral to bregma; and the MI-RW region, 1.0-2.0 rostral and 0.8-1.4 lateral to bregma.

### 2.2.3 Retrograde tracer injections in claustrum.

In a separate group of rats, the retrograde tracer (FG) was iontophoretically injected into the claustrum or surrounding regions. After an incision was made along the face between the eye and the ear, the tissue was retracted to allow access to the skull just caudal to the orbital socket (1.4 mm rostral to bregma and 6.9 mm ventral to the surface of the skull at bregma). After a craniotomy, a glass pipette oriented orthogonal to the temporal bone (i.e., parallel to the stereotaxic ear bars) was inserted into the brain while a retention current (-5 μA) was applied to prevent unwanted labeling of the insular cortex. The retrograde tracer was then injected at a depth of 1.75 mm from the pial surface with +5 μA pulses for 20 minutes using a 7-s on/off duty cycle. Compared with MI injections, larger currents were needed for FG injections into the claustrum because the pipette was oriented horizontally and gravitational force could not facilitate expulsion into the claustrum. The retention current was reapplied while withdrawing the pipette.

### 2.2.4 Histology.

After tracer injections, the wound margins were sutured and the rat received supplemental doses of atropine, dexamethasone, and chloramphenicol before returning to its home cage for 7 days. After this, each rat was deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and transcardially perfused with saline, 4% paraformaldehyde, and 4% paraformaldehyde with 10% sucrose. The brain was removed and refrigerated in 4%
paraformaldehyde with 30% sucrose for 2-3 days. The olfactory bulbs, brainstem, and cerebellum were removed and a small slit was made in the left hemisphere, ventral to the rhinal fissure, to distinguish the two sides of the brain. The brain was sectioned coronally (60 μm) and sections were saved in 0.1M phosphate buffered saline. Serially ordered sections were used to make three series, one for fluorescent labeling, one for thionin staining, and one for processing cytochrome oxidase. Sections from the fluorescent series were mounted on gelatin-coated glass slides and dried overnight in an incubator. The next day these sections were dehydrated in 100% ethanol, defatted in xylene, and coverslipped with Cytoseal. The remaining two series, which were used to reveal cytoarchitectonic borders, were stained with thionin or processed for the presence of cytochrome oxidase (Land and Simons, 1985).

2.2.5 Anatomical analysis.

Alternate sections in the fluorescence series, which were obtained at intervals of 360 μm during frozen sectioning, were used to reconstruct the locations of labeled terminals and neurons. The remaining intervening sections in the fluorescence series were used for photomicrography of the labeling patterns. The locations of labeled varicosities and neurons were reconstructed with an BH-2 microscope (Olympus) equipped with a reconstruction system (Accustage) in which optical transducers were mounted on the x–y axes of the microscope stage to construct digital records of FR-labeled varicosities and FG–labeled neuronal soma, as well as other anatomical features (MD-Plot software, version 5.1, AccuStage.com). The microscope contained a tetrarhodamine isothiocyanate (TRITC) filter for visualizing the axonal varicosities of the FR-labeled terminal arbors and a UV (Chroma Technologies Corp; 51004v2) filter for visualizing FG-labeled neuronal soma. Plotting of fluorescent labeling was done with a 10X eyepiece in combination with a 10X or 20X objective. For each reconstruction, the anterogradely labeled
(FR) terminals were plotted first. Beaded varicosities along the labeled axons were plotted because axonal enlargements are known to contain synaptic vesicles that are colocalized with markers for synaptophysin, and thus represent en passant synapses (Voight et al., 1993; Kincaid and Wilson, 1996; Meng et al., 2004). Subsequently, the filters were switched so that the retrogradely labeled (FG) soma could be plotted in the same claustral area.

Based on the recent identification of a protein, Gng2, that is expressed in the claustrum and distinguishes it from adjacent structures (Mathur et al., 2009), analysis of claustral labeling did not extend beyond the most rostral coronal section that contained the striatum because such regions do not contain Gng2. Neuronal labeling (cell bodies or terminal varicosities) in sections located caudal to the rostral edge of the striatum was plotted if it appeared adjacent to the external capsule at the level of the rhinal sulcus. Tracer injection sites, neuronal labeling, and cytoarchitecture were documented by images acquired with a Retiga EX CCD digital camera (Q-imaging) mounted on the Olympus light microscope.

Plotted reconstructions were analyzed for the number of labeled varicosities and labeled soma, the areal extent of labeling, and the spatial overlap of the labeled terminals and cell bodies. For these purposes, the reconstructions were converted into color-coded density diagrams in which the number of plotted processes (neurons or varicosities) within a spatial unit (e.g., 25, 50, or 100 μm²) could be represented by a specific color. These procedures were performed by specific modules in the MD-Plot software (ver. 5.1).

The density of the terminal varicosities varied substantially across sections and from one region to another within the claustrum. In places where terminal labeling was sparse-to-moderate, the Accustage system enabled accurate reconstructions of varicosity density. In the most densely labeled regions, however, each labeled varicosity could not be individually visualized and plotted using conventional light microscopy. Therefore, an accurate measurement of the density of anterogradely labeled varicosities in the most densely labeled regions required
confocal microscopy. For this purpose, a confocal microscope (TCS SP2 AOBS, Leica Microsystems,) equipped with a 63X oil emersion objective was used with an optical zoom (120X) to acquire high-resolution images of both anterogradely labeled (FR) terminals (543 nm excitation, 565-630 nm detection) and retrogradely labeled (FG) neurons (405 nm excitation, 520-600 nm) in the claustrum.

To determine the maximum density of anterogradely labeled varicosities in the claustrum a square region (125 μm²) or counting box was obtained from the center of the ventral claustrum as defined by Paxinos and Watson (2005). Multiple images were taken through the depth of the section, such that the final image contained a sum of luminance values for each pixel extending 15 μm through the z-plane (maximum projection). These images were imported into a software drawing program (Deneba Systems, Inc., Canvas X), and two individuals (KDA and JH) blind to the experimental details for each image were responsible for counting the number of labeled varicosities. For this procedure, a green dot was placed on each anterogradely labeled varicosity, and, when finished, the drawing program counted the number of green dots placed on the image. This produced an accurate count of the labeled varicosities, which was then used to calculate their density in this focal region. Consistent with previous criteria for calculating the density of en passant axonal varicosities (Reep et al., 2009; Alloway et al, 2010), only axonal enlargements twice the diameter of the emerging axon on each side were counted.

Differences in terminal and neuronal labeling were analyzed with several statistical procedures including ANOVA, as well as paired and independent t tests. The statistical computations and determination of significance levels were performed by in Origin software (version 8.0; OriginLab).
2.3 Results

The bilateral connections between MI and the claustrum were analyzed in all nine rats that received bilateral tracer injections. As shown in Table 2.1, injections in each rat were bilaterally placed in homotopic sites located either in the MI-Fp (n = 3), MI-Re (n = 3), or MI-RW (n = 3) regions as defined by movements evoked by short (80 ms) and long (1400 ms) trains of ICMS. Figure 2-1 illustrates examples of bilateral tracer injections in the RW, Re, and Fp regions of MI. Photomicrographs of the tracer injections were compared with photomicrographs of adjacent thionin-stained sections to locate the injection site with respect to the cytoarchitecture of MI cortex. Consistent with previous reports (Brecht et al., 2004; Alloway et al., 2008, 2009), tracer injections in MI-Re were always in medial agranular (AGm) cortex, whereas tracer injections in MI-Fp were always in lateral agranular (AGl) cortex.

Tracer injections in sites that evoked rhythmic whisking movements were concentrated in the part of AGm that is closest to cingulate (Cg) cortex. Iontophoretic administration of the retrograde tracer (FG) produced sphere-shaped deposits in which the ventral portion of the tracer injection crossed the boundary between AGm and Cg. Microstimulation of this part of Cg cortex evokes eye movements (Brecht et al, 2004), but we did not observe eye twitches during our MI mapping procedures. Compared with maps reported by Brecht et al., (2004), in which eye twitches were evoked by ICMS at depths 2 mm and deeper from the pial surface, ICMS in our study was administered at depths of ≤ 1.7 mm or less from the pia, and this explains why eye twitches were not observed in our experiments. Nonetheless, the ventral portion of our retrograde injections in the RW region extended into the dorsal part of Cg cortex.
Figure 2-1. Representative examples of bilateral injections of FR and FG into the RW, Re, and Fp regions of MI cortex. A: FR injection in the RW region of the left hemisphere of case CL21. B: Thionin-stained section depicting MI cortical cytoarchitecture at the level of the tracer injections. C: FG injection in the RW region of the right hemisphere. D-I: FR and FG injections in the Re (CL05) and Fp (CL03) regions depicted as in A-C. Dashed lines and arrowheads indicate the cytoarchitectonic borders of AGm cortex. Numbers (bottom right) in B, E, and F indicate distance from bregma. Scale bar: 0.5 mm for all panels.

In comparison, pressure injections of FR caused this anterograde tracer to flow upward along the injection pipette, thereby forming an elongated tracer deposit that extended towards the superficial layers of MI. Although the ventral edge of the FR injections approached the medial border of AGm, very little, if any, of the tracer diffused into Cg cortex (Figure 2-1 A).
2.3.1 Location of claustral labeling.

The nomenclature for the claustrum has changed over the past two decades, and most of the labeling that we observed in the claustrum was concentrated in the ventral subnucleus as outlined in a recent atlas (Paxinos and Watson, 2005). As shown in Figure 2-2, the ventral claustrum is characterized by an oval-shaped region of small to medium neurons that is capped by a small region of densely packed neurons that form the dorsal claustrum. Located dorsal to the deep endopiriform nucleus, the ventral claustrum adjoins the part of the external capsule that becomes noticeably narrower as it proceeds ventrally near the rhinal fissure.

Recent evidence indicates that the neuropil in the rat claustrum is associated with a membrane-linked receptor protein known as Gng2 (Mathur et al., 2009). This protein is not expressed in sections located beyond the rostral edge of the striatum, and, consistent with this pattern of protein expression, the amount of labeling declined steeply in sections located immediately rostral to the striatum. Therefore, our analysis of anterograde and retrograde labeling in the claustrum commenced at the first section that contained the rostral striatum (~2.5 mm rostral to bregma) and continued caudally until all labeling in the claustrum was no longer present.
**Figure 2-2.** Bilateral labeling patterns in the claustrum produced by FR and FG injections in the MI-RW regions of CL21 (see Figure 2-1 A, C). **A, C:** Magnified views of the left and right claustrum; dorsal and ventral subnuclei are indicated by arrows and arrowheads, respectively. **B:** Coronal section indicating the claustral areas (rectangles) depicted in A and C. As indicated, FR was injected on the left, and FG was injected on the right. The number indicates the distance from bregma. **D–G:** Claustral boundaries and plotted locations of FR-labeled varicosities (D, G, small red dots) and FG-labeled neurons (E, F, large yellow dots). Boxes indicate regions depicted in the bottom row. **H–K:** Photomicrographs of FR-labeled terminals (H, K) and FG-labeled neurons (I, J) in the claustrum. The number in K indicates distance from bregma. Scale bars: 250 µm in A, C–G; 1 mm in B; 100µm in H–K. ec, external capsule; dCl, dorsal claustrum; NS, neostriatum; rf, rhinal fissure; vCl, ventral claustrum.

In the nine rats that received bilateral injections in MI cortex, labeling in the claustrum consistently appeared within a limited rostrocaudal range that extended from 2.5 mm rostral to 1.4 mm caudal to bregma. Because reconstructions were performed on coronal sections obtained
at intervals of 360 µm (see Methods), exactly 11 sections within this rostrocaudal range were plotted in each rat.

2.3.2 Functional specificity of claustral labeling.

Our bilateral tracer injections in the MI-RW regions produced distinct asymmetric patterns of claustral labeling across the two hemispheres. As indicated in Figure 2-2, an injection of the retrograde tracer (FG) into the RW region of the right hemisphere labeled many neuronal soma in the right claustrum but labeled very few neurons in the left claustrum. In the same rat, an injection of the anterograde tracer (FR) into the RW region of the left hemisphere produced dense terminal labeling in the right claustrum but noticeably less terminal labeling in the left claustrum. Hence, the claustrum located ipsilateral to the retrograde tracer (FG) injection contained a sizeable region in which high densities of FR-labeled terminals and FG-labeled soma were intermingled in overlapping areas. In contrast, the other claustrum contained a much smaller region of tracer overlap in which anterograde and retrograde labeling was less intense.

Bilateral tracer injections in the Re region of MI produced asymmetric patterns of claustral labeling that resembled the patterns produced by the MI-RW injections, but the overall intensity and spatial extent of the labeling was reduced. An injection of retrograde tracer into the MI-Re region of the right hemisphere revealed dozens of labeled neurons in the right claustrum but very few neurons in the left claustrum (Figure 2-3 A-H). An injection of anterograde tracer into the left MI-Re region of the same rat produced moderately dense terminal labeling in the right claustrum, but the labeling in the left claustrum was relatively sparse. Consequently, when compared with the MI-RW injections, the bilateral tracer injections in the MI-Re regions produced a small region of tracer overlap in the claustrum located ipsilateral to the retrograde tracer injection but very little tracer overlap in the other claustrum.
Figure 2-3. Bilateral labeling patterns in the claustrum produced by FR and FG injections in the Re (CL05) and Fp (CL03) regions of MI (see Figure 2-1). Claustral labeling is displayed as in Figure 2-2 (D-K). A-H: Plotted reconstructions and photomicrographs of FR-labeled varicosities and FG-labeled neurons in the claustrum after tracer injections in MI-Re. I-P: Reconstructions and photomicrographs of a small amount of claustral labeling after tracer injections in MI-Fp. Large FR-labeled cells in E and M are pericytes associated with blood vessels. Scale bars: 250 μm in A; 100 μm in E.

Tracer injections in the MI-Fp regions produced almost no claustral labeling. As shown by Figure 2-3 (panels I through P), very few anterogradely labeled terminals or retrogradely
labeled neurons were observed in the claustrum of either hemisphere. A few labeled varicosities were scattered in the claustral region where the dorsal and ventral subnuclei adjoin each other, but the density was too low to produce noticeable labeling in photomicrographs obtained at low magnification (see Figure 2-3, panels M and P). Labeled neurons were virtually absent from the claustrum located contralateral to the retrograde tracer (FG) injection (see Figure 2-3 J, N), and only a sparse group of labeled soma appeared in the ventral and dorsal subnuclei on the ipsilateral side (see Figure 2-3 K, O). Hence, because tracer injections in the MI-Fp regions produced very little claustral labeling, tracer overlap was not apparent in the claustrum of either hemisphere.

Statistical analysis confirmed that the number of retrogradely labeled neurons in the claustrum was affected by the functional locations (RW, Re, Fp) of the FG injections in MI ($F = 15.7, p < 0.0001$). Furthermore, the hemispheric location (contralateral or ipsilateral) of the claustrum also had a significant effect on the number of FG-labeled neurons ($F = 68.2, p < 0.0001$). When the number of labeled claustral neurons on each side was compared section-by-section (11 sections analyzed in each rat), a matched-sample analysis showed that the neuronal labeling was significantly higher in the hemisphere that received the retrograde tracer, regardless of whether the tracer was placed in the RW (paired $t = 6.5; p < 0.000001$) or Re (paired $t = 5.4; p < 0.00001$) regions of MI.

Similar analyses confirmed that the number of anterogradely labeled varicosities plotted in the claustrum was affected by the MI location of the FR tracer injections ($F = 51.1, p < 0.0001$) and by the hemispheric location of the claustrum ($F = 22.7, p < 0.0001$). A matched-sample section-by-section analysis demonstrated that the number of plotted varicosities was significantly larger in the contralateral claustrum when the anterograde tracer was injected into the RW (paired $t = 6.3, p < 0.000001$) or Re (paired $t = 5.6, p < 0.00001$) regions of MI.
2.3.3 Relative strength of claustral connections with the MI whisker regions.

For the six rats in which tracers were injected into the MI whisker regions (RW or Re), asymmetries in the relative balance of labeling across the two hemispheres were apparent in all claustral sections that contained labeling. As shown by Figure 2-4, regardless of which MI whisker region was injected, labeled neurons were mainly in the claustrum located ipsilateral to the retrograde tracer injection (see Figure 2-4 A). Conversely, labeled terminals were consistently more numerous in the claustrum located contralateral to the anterograde tracer injection (see Figure 2-4 B).

Figure 2-4. Rostrocaudal profile of the bilateral distribution of claustral labeling produced by tracer injections in the RW, Re, and Fp regions of MI. A: Distribution of FG-labeled neurons plotted contralateral and ipsilateral to the FG injection site. B: Distribution of FR-labeled varicosities plotted contralateral and ipsilateral to the FR injection site. Symbols represent the mean number of plotted processes at each rostrocaudal position; error bars represent SEM.

Although tracer injections in both MI whisker regions produced similar labeling patterns, several observations indicate that the claustrum is more strongly connected with the RW than with the Re region. At each rostrocaudal position (Figure 2-4 A), retrograde tracer injections in MI-RW revealed more labeled neurons in the ipsilateral claustrum than similar tracer injections in
the MI-Re region. Statistical analysis confirmed that these differences were significant \((t = 2.4; p < 0.05)\). Furthermore, despite the fact that exactly 100 nL of the anterograde tracer (FR) was pressure injected in each rat, the total number of labeled varicosities in the claustrum of both hemispheres was highest when the tracer was injected into the MI-RW region \((t = 4.0; p < 0.001)\). When each hemisphere was analyzed separately, anterograde tracer injections in the RW region produced more labeled varicosities in the claustrum than similar injections in the Re region for both the ipsilateral \((t = 2.9; p < 0.01)\) and contralateral \((t = 4.1; p < 0.001)\) hemispheres.

We also analyzed the areal extent of terminal labeling in the claustrum after anterograde injections in the RW and Re regions. For this purpose, the plotted reconstructions of the claustral labeling patterns were subdivided into a grid of 25 um\(^2\) bins, and variations in the number of plotted varicosities in each bin was represented by a color-coded map of labeling density. As shown by these color-coded reconstructions in Figure 2-5, projections from the RW region innervated a larger claustral area than the projections from the Re region. Analysis of the spatial extent of anterograde labeling in the claustrum, as measured by the sum of the colored bins (25 um\(^2\) each) across all sections, indicated that the RW region innervated a larger portion of the claustrum than the Re region \((t = 2.0, p < 0.05)\). This difference was mainly caused by the increased innervation of the contralateral claustrum \((t = 2.0, p < 0.05)\), as the innervated area in the ipsilateral claustrum was not significantly different for injections in the RW or Re regions \((t = 1.8, p = 0.07)\).
Figure 2-5. Relative density of corticoclaustral projections from FR injection sites in the MI-RW (CL21) and MI-Re (CL05) regions. A,C: Confocal images of FR-labeled varicosities in the claustrum of the ipsilateral and contralateral hemispheres after an FR injection into the RW region. B: Color-coded reconstructions of the density of plotted varicosities in the claustrum of each hemisphere. The number of plotted varicosities in each 25 μm² bin is indicated by the color scale on the right. Inset, Arrowheads indicate the region from which the confocal images in A and C were obtained. D-F: Density of FR-labeled varicosities after injecting the tracer into the MI-Re region; images and reconstructions are presented as in A and C. Scale bars: 25μm in A,C-E; 1 mm B,E.

The color-coded reconstructions indicated variations in the density of anterogradely labeled varicosities across different parts of the claustrum. As indicated in Figure 2-5, B and E, labeled varicosities were densest in the dorsal half of the labeled area. The high density of labeling in this region, however, made it impossible to plot each individual varicosity using conventional fluorescent microscopy. To obtain an accurate count of the labeled varicosities in these densely labeled regions, confocal images were acquired from the sites where our color-coded reconstructions indicated that anterograde labeling was densest (Figure 2-5 B,E arrowheads). Consistent with the rostrocaudal distribution of terminal labeling (Figure 2-4 B), confocal images were obtained from three coronal sections located 0.70, 1.40, and 2.10 mm rostral to bregma. For each section, a confocal image was obtained from both hemispheres as
indicated in Figure 2-5. The number of labeled varicosities in each image was counted by two individuals who were unaware of the image identity, and these counts were averaged for each image (see Materials and Methods).

Figure 2-6. Bar graphs depicting the maximum density of labeled varicosities in the claustrum after FR injections into the RW or Re regions. Labeled varicosities were counted from confocal images obtained from the claustral regions with the most FR labeling. Each bar represents the mean varicosity density calculated from nine confocal images obtained from three rats. Error bars represent SEM; asterisks indicate that RW injections produced a significant increase in the maximum density of FR labeling (***p < 0.01; **p < 0.001).

Visual inspection of the confocal images in Figure 2-5 suggests that the maximum density of labeled varicosities was highest when the anterograde tracer was placed in the MI-RW region. In fact, statistical analysis confirmed that the density of labeled varicosities was significantly higher when the anterograde tracer was injected in the MI-RW region. As indicated in Figure 2-6, differences in the maximum density of labeled varicosities produced by tracer injections in the RW and Re regions were significant for both the contralateral (t = 5.1; p = 0.001) and ipsilateral (t = 3.4; p = 0.01) hemispheres.
2.3.4 Interhemispheric cortico-claustro-cortical circuits.

Simultaneous visualization of both tracers indicated that a large portion of the anterogradely labeled terminals were intermingled with retrogradely labeled neurons when the tracers were injected into MI-RW or MI-Re. In fact, as shown by the confocal images in Figure 2-7, C and F, the high density of labeled terminals prevented complete visualization of many of the labeled cell bodies. Although ultrastructural evidence is needed to confirm synaptic connectivity, the dense intermingling of the anterogradely labeled terminals and retrogradely labeled neurons indicates that an interhemispheric cortico–claustral–cortical circuit could be mediated by monosynaptic connections in the claustrum.

**Figure 2-7.** Bilateral distribution of tracer overlap in the claustrum after injections of FR and FG into MI-RW and MI-Re. **A,C:** Confocal images of FR-labeled varicosities and FG-labeled neurons in the claustrum of the left and right hemispheres after tracer injections in the RW region. **B:** Color-coded reconstruction of tracer overlap in the MI-RW case. Bins (50 μm²) colored red contain four or more FR-labeled varicosities, yellow bins contain one or more FG-labeled neurons, and white bins contain four or more FR-labeled varicosities with one or more FG-labeled neurons. **D-F:** Tracer overlap produced by tracer injections in the MI-Re regions; images and reconstruction are depicted as in **A-C.** Arrowheads (B,E) indicate claustral regions from which confocal images were obtained. Scale bars: 50 μm in **A, C, D, F;** 1 mm in **B,E. ec,** External capsule; ac, anterior commissure; cc, corpus callosum.
To assess potential differences in the amount of tracer overlap produced by tracer injections in the RW or Re regions, the plotted reconstructions of the two-tracer labeling patterns were subdivided into 50 µm² bins. Compared with our spatial analysis of labeled terminals, which was based on grids containing 25 µm² bins (Figure 2-5), spatial analysis of tracer overlap was initially based on grids containing larger bins because neurons are much larger than axonal varicosities. Furthermore, because multiple varicosities are more likely to produce postsynaptic effects, at least four or more labeled varicosities had to reside in a bin with a labeled neuron to represent a bin with overlapping tracers. Therefore, as shown in Figure 2-7, B and E, bins in which we plotted at least one retrogradely labeled neuron and four or more anterogradely labeled varicosities were colored white. In comparison, bins with four or more FR-labeled varicosities but no FG-labeled neurons were colored red, whereas bins that contained at least one FG-labeled neuron but fewer than four FR-labeled varicosities were colored yellow. All of the remaining bins were colored black. The white bins in each hemisphere were counted to provide an index of the spatial extent of terminal–soma overlap in the two groups (RW and Re) of rats that received tracer injections in the MI whisker regions.

As shown in Figures 2-7 and 2-8, this methodology revealed clear hemispheric differences in the amount of tracer overlap. Consistent with the fact that the vast majority of labeled neurons were located ipsilateral to the retrograde tracer injection, the areal extent of tracer overlap (i.e., number of white bins) was always highest in the claustrum located in the hemisphere that received the retrograde tracer. This hemispheric difference in tracer overlap was significant regardless of whether the tracers were injected into the RW (paired t = 6.16; p < 0.001) or Re (paired t = 3.97; p < 0.01) regions.
Figure 2-8. Bar graphs depicting the spatial extent of terminal–soma overlap in the claustrum after tracer injections in the RW or Re regions. The overlap area was calculated from the number of 50 \( \mu \text{m}^2 \) bins that were colored white, as shown in Figure 2-7. Each bar represents the mean area of tracer overlap in three rats. Error bars represent SEM; asterisks indicate that RW injections produced significantly more overlap than Re injections (*\( p < 0.05 \); *** \( p < 0.001 \)).

Furthermore, tracer injections in MI-RW produced more overlap in the claustrum than injections in MI-Re. As indicated by the reconstructions in Figure 2-7, tracer overlap was more extensive in the RW than in the Re case. When the white bins were summed across both hemispheres, statistical analysis confirmed that total tracer overlap in the RW cases was significantly greater than the overlap produced by injections in the Re regions (\( t = 2.9; p < 0.01 \)). In fact, tracer overlap was significantly higher for the RW cases regardless of whether the analysis was applied to the hemisphere that received the retrograde tracer (\( t = 2.8; p < 0.01 \)) or the one that received the anterograde tracer (\( t = 2.6; p < 0.05 \)).

The difference in tracer overlap between the RW and Re cases did not depend on bin size or the threshold number of labeled varicosities in each bin. Although the absolute amount of tracer overlap varied with bin size (25, 50, or 100 \( \mu \text{m}^2 \)) and with the threshold number of labeled varicosities required for each bin (one, two, four, or six), the relative extent of tracer overlap was always higher for rats in which both tracers were injected into the RW regions (data not shown).
2.3.5 Retrograde labeling of corticoclastral projection neurons.

In a separate set of rats, the retrograde tracer (FG) was placed in the claustrum or surrounding regions. These injections were targeted at the rostrocaudal levels of the claustrum (1.0–1.5 mm rostral to bregma) that received the greatest number of projections from the MI whisker region (Figure 2-4). Among 11 rats that received tracer injections aimed at the claustrum, tracer deposits in 9 rats were either completely outside the claustrum or involved both the claustrum and extensive parts of the surrounding regions. When the tracer was injected entirely outside the claustrum (n = 6), either into the insular cortex or into the ventrolateral striatum, very few retrogradely labeled neurons appeared in MI cortex. This result is consistent with the fact that our anterograde tracer injections in the RW and Re regions produced terminal labeling in the claustrum but not in the insular cortex or ventral striatum.

Injecting the claustrum alone proved difficult, and only two rats received retrograde tracer deposits that were largely restricted to the claustrum. In the case shown in Figure 2-9, the tracer filled most of the ventral claustrum, a small portion of the dorsal claustrum, and possibly the surrounding edges of the insular cortex. The other case involved tracer that was mainly in the dorsal claustrum, the upper part of the ventral claustrum, and in the lateral edge of the ventral striatum. These retrograde tracer deposits were relatively compact, and histologic inspections indicated that the tracer diffused <400 µm in the rostral and caudal directions.
Figure 2-9. Intraclaustral labeling patterns produced by a focal tracer injection in the claustrum. A,C: Rostral (A) and caudal (C) sections illustrating the longitudinal extent of FG-labeled soma after the retrograde tracer was placed in the claustrum. B: Location of the FG injection in the claustrum. D-F: Adjacent sections illustrating cytoarchitecture of the claustrum at each rostrocaudal level depicted in A-C. Numbers (bottom right) indicate rostrocaudal distance from bregma; arrowheads indicate same blood vessels in adjacent sections. Scale bar, 250 μm for all panels. ec, External capsule; NS, neostriatum.

In the cases in which the FG was placed almost entirely in the claustrum, hundreds of retrogradely labeled neurons appeared throughout elongated portions of the ipsilateral claustrum (Figure 2-9 A,C). Neuronal labeling did not appear in the claustrum, however, when large deposits of the retrograde tracer were injected in the insular cortex or in the ventral striatum (data not shown). Hence, these collective results indicate extensive longitudinal axonal projections within the claustrum itself.

Injections of FG into the claustrum revealed large numbers of retrogradely labeled cortical neurons in Cg, AGm, and AGl cortices and related frontal areas, but not in other cortical areas. Despite suggestions that the claustrum integrates multimodal information received from different cortical areas (Edelstein and Denaro, 2004; Crick and Koch, 2005), our tracer injections in the claustrum failed to produce neuronal labeling in somatosensory barrel cortex or other
somatosensory regions such as the secondary somatosensory cortex or the parietal ventral area. Nor did any labeling appear in more caudal regions that contain visual or other sensory-processing areas. The total lack of labeling in these nonmotor cortical areas is consistent with physiological data in the primate showing that local regions in the claustrum are modality specific and do not respond to multimodal inputs (Remedios et al., 2010).

As shown in Figures 2-10 and 2-11, the cortical labeling produced by retrograde tracer injections in the claustrum was greatest in the AGm and Cg cortices of the contralateral hemisphere. A section-by-section analysis indicated that the number of labeled neurons was significantly higher in the contralateral than in the ipsilateral hemisphere for both the AGm (paired t = 4.1; p < 0.01) and Cg (paired t = 2.6; p < 0.05) cortical areas. We did not detect any hemispheric differences among the smaller number of labeled neurons in AGl cortex (paired t = 0.8; p < 0.4).

The neuronal labeling in AGm of the contralateral hemisphere was extremely dense in the medial part of this region that adjoins the Cg cortex. Photomicrographs of this densely labeled site illustrate high concentrations of brightly labeled neurons in layer V near the border separating the medial edge of AGm cortex from the underlying Cg cortex (Figure 2-10 F,G). The plotted reconstructions also indicate that the density of neuronal labeling was highest at this site (Figure 2-11). To portray more precisely the spatial variations in the density of neuronal labeling, adjacent pairs of these reconstructions were superimposed and subdivided into a grid of 100 μm² bins that were color coded according to the number of labeled neurons in each bin. As indicated in Figure 2-12, this procedure indicated that neuronal labeling was densest at the medial edge of AGm cortex that borders the Cg cortex. This focal region of dense labeling was apparent at the same location in all sections obtained 1.0–2.5 mm rostral to bregma, a region that corresponds to the same MI area that we and others have microstimulated to evoke rhythmic whisking movements (Haiss and Schwarz, 2005).
Figure 2-10. Labeled neurons in MI cortex produced by the tracer injection in the left claustrum as shown in Figure 2-9. A,C: Thionin-stained sections show the cytoarchitecture of area AGm in the left and right hemispheres, respectively. Arrowheads indicate cytoarchitectonic borders of AGm cortex. B: Adjacent unstained sections used for fluorescent microscopy. Rectangles indicate the regions in E and F; the number indicates the distance from bregma. D,G: Magnified views of labeled neurons in AGm cortex of the left and right hemispheres, respectively. E,F: Low-power views of neuronal labeling with respect to the borders of AGm cortex (arrowheads) and layer V (dashed line). Rectangles indicate regions depicted in D and G. Scale bars: 500μm in A-C; 100 μm in D,G; 250μm in E,F.
**Figure 2-11.** Reconstructions illustrating the distribution of retrogradely labeled cortical neurons after focal injection of FG into the left claustrum as shown in Figure 2-9. Each dot represents the location of an FG-labeled neuron. Numbers indicate distance from bregma; lines indicate borders between cortical areas Cg, AGm, and AGl determined from the cytoarchitecture of adjacent thionin-stained sections. Scale bar: 1 mm.

**Figure 2-12.** Relative density of labeled neurons in motor and cingulate cortex produced by the FG tracer injection in the left claustrum as shown in Figure 2-9. Color-coded reconstructions depicting spatial variations in the density of labeled cortical neurons. Each section is subdivided into 100μm² bins that encode the number of labeled neurons summed across two adjacent plotted reconstructions shown in Figure 2-11. Numbers (bottom left) indicate the rostrocaudal range of each pair of sections. Scale bar: 1mm. ec, external capsule; NS, neostriatum.
Changes in the laminar pattern of neuronal labeling were apparent at the cytoarchitectonic transitions between AGm cortex and its neighboring cortical areas. In contrast to AGm cortex, in which labeled neurons appeared in layer V and more superficial layers, the density of neuronal labeling dropped sharply at the border between AGm and AGl cortices. Interestingly, neuronal labeling in AGl cortex had a distinct laminar pattern in the rostral parts of the ipsilateral hemisphere but showed less laminar organization in the contralateral hemisphere.

In both hemispheres, the border between AGm and Cg cortices was marked by a distinct change in the laminar organization of neuronal labeling. In contrast to AGm cortex, in which labeled neurons appeared in layer V and more superficially, most labeled neurons in Cg cortex were concentrated in layer V. Layer V is relatively thick in AGm cortex but becomes substantially thinner in Cg cortex. Consequently, because layer III in Cg cortex contains few projections to the claustrum, the high density of layer V labeling in both areas accentuates the border between them as this layer becomes compressed in the transition from AGm to Cg cortex.

2.4 Discussion

Our bilateral tracer injections revealed an interhemispheric cortico–claustro–cortical circuit that connects corresponding parts of MI cortex. This interhemispheric circuit connects MI regions associated with whisker retractions or rhythmic whisker movements, but not the MI regions linked to forepaw movements. Quantitative analysis indicates that interhemispheric connections between the MI-RW regions are stronger than the connections between the MI-Re regions. This finding was confirmed by retrograde tracer injections in the claustrum showing that the densest neuronal labeling was in the medial part of the contralateral AGm cortex that represents the MI-RW region. Furthermore, the tracer injections in the claustrum revealed extensive interconnections along its rostrocaudal length.
2.4.1 Cortico-claustro-cortical circuit connections.

We previously used anterograde tracers to demonstrate that the MI whisker region projects bilaterally to the claustrum, but with more numerous projections to the contralateral than to the ipsilateral side (Alloway et al., 2009). In another report (Colechio and Alloway, 2009), injections of retrograde tracers in MI established that the vast majority of clastral projections to the MI whisker region originate from the ipsilateral side. The present study used bilateral combinations of both types of tracers to demonstrate that corticoclaustral projections to the contralateral hemisphere terminate in regions that overlap the neuronal soma that form the origin of the claustrocortical pathway in that hemisphere. Indeed, the high density of terminal and neuronal labeling in overlapping regions makes it highly probable that monosynaptic connections in the claustrum convey information from the contralateral MI cortex to the corresponding motor region in the ipsilateral hemisphere.

Previous work indicates that MI cortex projects bilaterally to several structures that could coordinate the motor cortical areas in both hemispheres. Several studies, for example, demonstrate that MI cortex projects bilaterally to the striatum (Wilson, 1986, 1987; Reiner et al., 2003; Reep et al., 2008; Alloway et al., 2009). The MI whisker region also projects bilaterally to the thalamus, especially the ventrolateral, ventromedial, and intralaminar nuclei (Molinari et al., 1985; Rouiller et al., 1991; Alloway et al., 2008).

The MI projections to the claustrum, however, are different from the MI projections to the thalamus and striatum. Although the MI whisker region projects more strongly to the contralateral claustrum, corticothalamic and corticostriatal projections from MI cortex terminate predominantly in the ipsilateral hemisphere [Alloway et al., their Figure 9A (2008) and Figure 12 (2009)]. In addition, corticoclaustral projections to the contralateral hemisphere have a higher density of terminal varicosities than MI projections to the contralateral striatum or thalamus.
[Alloway et al. (2009), their Figure 7]. This is consistent with the fact that MI whisker projections to the claustrum are concentrated in a limited territory, whereas corticothalamic and corticostriatal projections are less dense and innervate much larger regions.

Other aspects of the cortico–claustral–cortical circuit distinguish it from the interhemispheric circuits that involve the striatum or thalamus. Information processed in the striatum is conveyed across multiple synapses in the basal ganglia and thalamus before it reaches MI cortex. Hence, the basal ganglia–thalamocortical loop is longer and, undoubtedly, slower than the claustral circuit in coordinating MI regions in both hemispheres. Furthermore, the MI-Fp region sends few projections to the claustrum in either hemisphere but projects strongly to the ipsilateral thalamus and to the striatum in both hemispheres (Alloway et al., 2009). Hence, the connectional data suggest important functional distinctions in the content of information and its speed of transmission through the cortico–claustral–cortical circuit and other interhemispheric circuits originating from MI.

2.4.2 Intraclaustral connections.

Whereas many anatomical studies have revealed similarities in the afferent and efferent connections of the claustrum in a number of mammalian species (for review, see Sherk, 1986; Edelstein and Denaro, 2004), there is little information regarding neuronal connectivity within the claustrum. The claustrum contains a limited number of neuronal cell types, and several studies have characterized claustral neurons according to their size, shape, and neurochemical features (Eiden et al., 1990; Druga et al., 1993; Kowianski et al., 2001, 2008). Yet, no study has characterized the axonal processes of claustral neurons to determine the extent of intraclaustral connectivity. The present study, however, established extensive interconnections along the claustrum’s rostrocaudal axis. Placement of a retrograde tracer in the claustrum revealed
hundreds of labeled neurons throughout its longitudinal extent, but the surrounding cortex was devoid of labeled neurons. When large retrograde tracer injections were placed in the insular cortex adjacent to the claustrum, we observed labeled neurons in the cortical regions that surround the claustrum throughout its rostrocaudal extent but saw very few labeled neurons in the claustrum. Hence, the extensive claustral labeling observed after a retrograde tracer injection in the claustrum must be attributable to long-range intraclaustral connections that were labeled by the bulk of the tracer deposit in the claustrum and not by the small quantity of tracer that may have diffused into the surrounding cortex.

These long-range connections indicate that information travels widely throughout the claustrum. The precise nature of this information is not known, however, and our data do not indicate whether these long claustral projections represent communication between separate parts of the same motor-based claustral region or between parts of the claustrum that process different cortical modalities. Nonetheless, intraclaustral connections could provide a mechanism for binding near-coincident inputs from cortical areas that process related aspects of the same behavioral activity (Crick and Koch, 2005).

The presence of extensive intraclaustral connections, coupled with the convergence of inputs from large parts of Cg and AGm cortices, has important implications for the transmission of information through the claustrum and its impact on postsynaptic cortical targets. Regardless of whether the intraclaustral connections represent the axons of interneurons or the collaterals of claustrocortical projections, either type of connection could synchronize extensive parts of the claustrum. This view is consistent with data indicating that the claustrum is susceptible to kindling and plays a role in the development of epileptiform activity (Mohapel et al., 2000, 2001). If many claustral neurons converge on overlapping cortical regions, synchronization of these neurons should effectively activate their cortical targets. In this context, it is worth noting that our relatively small injections of retrograde tracers in the MI-RW region produced widespread
neuronal labeling throughout the rostrocaudal extent of the claustrum. Hence, the high density of overlapping afferent (FR-labeled) and efferent (FG-labeled) claustral connections, along with the its extensive intrinsic connectivity and the likelihood of neuronal synchronization, demonstrates that the claustrum has connectional properties that should optimize the transmission of information from one MI region to its counterpart in the other hemisphere.

2.4.3 Neural and behavioral functions of interhemispheric claustral circuits.

The MI whisker regions in each hemisphere are directly interconnected by dense sets of axons that project through the corpus callosum (Donoghue and Parham, 1983; Reep et al., 1987; Miyashita et al., 1994). The afferent and efferent connections of the claustrum provide another route, probably monosynaptic, that complements the direct callosal connections between the MI whisker regions in each hemisphere. By including an intervening synapse along this second route, the claustrum offers an opportunity for integrating cortical information from the ipsilateral hemisphere with information received from the MI cortex in the contralateral hemisphere. The claustrum projects to both the Re and RW regions in MI, and this could coordinate their interactions during whisking behavior. Depending on the details of the synaptic organization in both the claustrum and MI cortex, the information transmitted by this interhemispheric circuit could reinforce the bilateral symmetry whisker movements or, alternatively, decouple the whisker movements in response to head movements or other sensory inputs (Towal and Hartmann, 2006; Mitchinson et al., 2007). Determining the specific actions mediated by claustral circuits must await future research that examines the behavioral effects of manipulating the claustrum.

From a systems perspective, the functional significance of the interhemispheric claustral circuit is inferred from the fact it is structured to coordinate MI cortical areas that regulate eye and whisker movements (i.e., Cg and AGm), but not the MI areas associated with forelimb
movements (i.e., AGI). Although forelimb movements are bilaterally coordinated to support the body and move it through space, the eyes and whiskers are bilaterally coordinated to acquire sensory information for perceptual awareness of a wide portion of the surrounding environment, especially as it relates to spatial orientation and directed attention. In contrast to forelimb movements, which have a limited ability for quickly sampling multiple stimuli, bilateral whisking movements can rapidly detect nearby stimuli throughout the three-dimensional space surrounding the head. Consistent with this, the MI whisker regions receive inputs from the posterior parietal cortex (PPC), but the MI forepaw region does not (Colechio and Alloway, 2009). The PPC processes multimodal sensory inputs that are needed to modulate MI and guide motor output for spatial orientation and directed attention (Dijkerman and de Haan, 2007; Reep and Corwin, 2009). Hence, the interhemispheric claustral circuit described in the present study probably coordinates the motor cortical regions that regulate movements involved in the acquisition of sensory information for global, large-scale perception.
<table>
<thead>
<tr>
<th>Case</th>
<th>Region</th>
<th>Left Hemisphere</th>
<th>Right Hemisphere</th>
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<td>Tracer</td>
<td>Coordinates (R,L)</td>
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<tr>
<td>CL01</td>
<td>MI-Re</td>
<td>FR</td>
<td>2.3, 1.2</td>
</tr>
<tr>
<td>CL02</td>
<td>MI-Re</td>
<td>FR</td>
<td>2.7, 1.3</td>
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<tr>
<td>CL03</td>
<td>MI-Fp</td>
<td>FR</td>
<td>2.9, 2.8</td>
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<tr>
<td>CL04</td>
<td>MI-Fp</td>
<td>FR</td>
<td>2.5, 2.3</td>
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<tr>
<td>CL05</td>
<td>MI-Re</td>
<td>FR</td>
<td>2.3, 1.4</td>
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<tr>
<td>CL06</td>
<td>MI-Fp</td>
<td>FR</td>
<td>2.5, 2.7</td>
</tr>
<tr>
<td>CL21</td>
<td>MI-RW</td>
<td>FR</td>
<td>1.6, 1.0</td>
</tr>
<tr>
<td>CL22</td>
<td>MI-RW</td>
<td>FR</td>
<td>1.7, 1.0</td>
</tr>
<tr>
<td>CL23</td>
<td>MI-RW</td>
<td>FR</td>
<td>1.6, 1.2</td>
</tr>
</tbody>
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Coordinates are rostral (R) and lateral (L) to bregma in millimeters

Table 2-1. Summary of Bilateral Tracer Injections.
Chapter 3

Rat Clastrum Coordinates But Does Not Integrate Somatosensory and Motor Cortical Information

2 This chapter appears as previously published in the journal article: Smith JB; Radhakrishnan H; Alloway KD (2012) Rat clastrum coordinates but does not integrate somatosensory and motor cortical information. J. Neurosci. 32(25): 8583-8588.
3.1 Introduction

Our anatomical work in rats has revealed an interhemispheric cortico-claustro-cortical pathway that supplements the direct callosal interconnections between the motor whisker representations in the two hemispheres (Alloway et al., 2009; Smith and Alloway, 2010). This finding is functionally significant because rodent whisking behavior displays a great deal of bilateral coordination (Mitchinson et al., 2007; Towell and Hartman, 2006). In cats, microstimulation of the claustrum exerts bilateral influences on the motor cortical neurons that control eye movements (Cortimiglia et al., 1991). Together, these findings suggest that the claustrum is important for regulating the bilateral coordination of certain behavioral movements. Consistent with this view, the claustrum is connected with the motor areas in both hemispheres (Sloniewski et al., 1986; Li et al., 1986), and one study has reported that some claustral neurons have widely-divergent axonal collaterals that allow them to innervate the motor region in each hemisphere (Minciacchi et al., 1985).

Rodent tracing studies indicate some overlap among populations of claustral neurons that project to the somatosensory and motor cortical areas, but claustral neurons that project to both SI and MI are not frequently observed (Sloniewski et al., 1986). These previous retrograde tracing studies, however, relied exclusively on stereotaxic coordinates to locate injection sites in SI and MI. Consequently, because physiology-based mapping techniques were not used to determine tracer placement, it is unlikely that corresponding representations in SI and MI were injected.

To characterize the sensorimotor input-output connections of the claustrum, we injected different combinations of anterograde and retrograde tracers in physiologically-defined sites in the SI and MI whisker representations of rats. Paired injections of different anterograde tracers in these cortical regions indicate that the claustrum receives inputs from wMI, especially from the contralateral hemisphere, but not from the wSI region of either hemisphere. Paired injections of
different retrograde tracers revealed overlapping retrograde labeling in the claustrum of each hemisphere with many double-labeled neurons. When anterograde tracer deposits in wMI of both hemispheres were coupled with a retrograde tracer deposit in wSI, corticoclaustral projections from wMI terminated densely around claustral neurons that project to wSI. These data indicate that the rat claustrum transmits information to both SI and MI, but it does not integrate information from both of these regions.

3.2 Materials and Methods

Anatomical tracer injections were made in adult male Sprague-Dawley rats (Charles River Co., Wilmington, MA) weighing 300-800g. All procedures conformed to NIH guidelines and were approved by our Institutional Animal Care and Use Committee.

3.2.1 Animal surgery.

Rats were initially sedated by an IM injection of ketamine HCl (40 mg/kg) and xylazine (12 mg/kg), and then received IM injections of atropine methyl nitrate (0.5 mg/kg), dexamethasone sodium phosphate (5 mg/kg), and chloramphenicol (50 mg/kg). Each rat was intubated, placed in a stereotaxic frame, and ventilated with oxygen. A homeothermic heating blanket maintained body temperature at 37.0°C, and ophthalmic ointment was applied to avoid corneal drying. Heart rate, specific oxygen, and end-tidal carbon dioxide were monitored throughout each surgery. A midline incision was made over the cranium, and lidocaine (2%) was injected into the wound margins. A hole was drilled over the cerebellum to allow insertion of a ground screw. Craniotomies were made over the somatosensory (1-4 mm caudal, 3-7 mm lateral)
and motor (1-3 mm rostral, 0.5-3 mm lateral) cortical areas based on previous reports (Brecht et al., 2004; Hall and Lindholm; 1974; Haiss and Schwarz; 2005; Hoffer et al., 2003).

3.2.2 Tracer injections.

Rats received combined injections of different anterograde and retrograde tracers in physiologically-defined cortical regions (i.e., wSI and wMI). The first set of rats received paired injections of anterograde tracers, namely 15% Fluororuby (FR; D-1817; Invitrogen) and 15% biotinylated dextran amine (BDA; D-7135; Invitrogen). The second set of rats received paired injections of retrograde tracers, namely 2% True Blue (TB; T-1323; Invitrogen) and 2% Fluorogold (FG; H-22845; Fluoro-Chrome). The final group of rats received two anterograde tracer injections, 15% FR in the left wMI and 15% Alexa Fluor 488 (AF; D-2290; Invitrogen) in the right wMI, and an injection of a retrograde tracer, 2% FG, in the wSI of the right hemisphere.

Most tracers (BDA, FR, AF, and TB) were pressure injected via Hamilton syringes with pulled glass pipettes (~60 μm tip diameter) cemented on the tip of the syringe needle. A volume of 75 nL was injected at each of two depths (1.7 and 1.0 mm below pial surface). Deposits of FG were iontophoretically injected using positive current pulses at 2-5 μA for 10-20 minutes (with a 7s on/off duty cycle) at multiple depths (1.6, 1.0, and 0.4 mm below the pia).

To identify wMI, a pipette filled with 3M NaCl (0.5-1.0 MΩ) was inserted orthogonal to the pial surface to a depth corresponding to layer V (~1.5 mm deep). Low amplitude current (< 50 μA) was administered as 0.7-ms pulses at a rate of 250 Hz for an 80-ms period to elicit muscle twitches. The stimulating electrode was moved to map the extent of wMI (both retraction and rhythmic whisking zones) relative to other representations including forelimb, neck, and nose representations, as described in previous reports (Brecht et al., 2004; Haiss and Schwarz, 2005; Sanderson et al., 1984; Smith and Alloway, 2010). After removing the stimulating electrode,
tracers were injected at multiple sites within wMI, but were kept at least 500 μm from other functional representations.

Injection sites in wSI were determined by receptive field mapping using a saline-filled pipette. The pipette was tilted at an angle of 25° to the sagittal plane and stereotaxically positioned above regions known to correspond to wSI (Hall and Lindholm, 1974; Hoffer et al., 2003). A dural slit was made and the pipette was lowered to 400-800 μm below the pial surface, corresponding to cortical layer IV. A silver wire with a male gold pin was placed in the pipette and was connected to a cable attached to the headstage of the amplifier. Extracellular potentials were band pass filtered (300-3000 Hz) and amplified by a Dagan amplifier (Model 2200, Minneapolis, MN). Neuronal activity was visualized on a digital oscilloscope (Tektronix DPO4034; Tektronix, Beaverton, OR) and played through an audio monitor. Neuronal receptive fields were identified by manual deflection of individual whiskers with a wooden probe. Multiple sites in wSI were mapped and injected so that the tracers would fill SI barrel cortex.

3.2.3 Histology.

Following tracer injections, wound margins were sutured and rats were returned to their home cage for 7-10 days to permit tracer transport. Subsequently, rats were deeply sedated with IM injections of ketamine (60mg/kg) and xylazine (18mg/kg), and were transcardially perfused with heparinized-saline, 4% paraformaldehyde, and 4% paraformaldehyde with 10% sucrose. Brains were removed and refrigerated overnight in 4% paraformaldehyde with 30% sucrose. Olfactory bulbs and the hindbrain were removed and a slit was made ventrally in the left hemisphere to orient sections for mounting. Brains were sectioned by a frozen microtome and stored in 0.1M phosphate buffered saline (PBS). Sections were divided into three series. The first series was mounted onto gelatin-coated slides and dried overnight before staining with thionin.
The second series was processed to visualize tracer labeling. Brains containing only fluorescent tracers were mounted and dried overnight before dehydrating in ethanol, defatting in xylene, and coverslipping with Cytoseal. For cases injected with BDA, sections were processed to visualize the tracer using heavy metal-enhanced peroxidase immunohistochemistry as previously described (Alloway et al., 1998; Kincaid and Wilson, 1996). Briefly, sections were agitated in 0.3% H2O2 and then 0.3% Triton X-100, 0.1M PBS before being incubated for 2-4 hours in activated avidin-biotinylated horseradish peroxidase solution (Vector Novocstra Laboratories, Burlingame, CA). After rinses in 0.1M PBS, sections were incubated in 0.05% diaminobenzidine (DAB), 0.0005% H2O2, 0.04% NiCl2 in 0.1M tris buffer (pH = 7.2) for 9-12 minutes. The reaction was then stopped by subsequent washes in 0.1M PBS. Following immunohistochemical processing to reveal BDA labeling, sections were mounted, dried over night, and coverslipped. The third series was processed for cytochrome oxidase (Land and Simons, 1985).

3.2.4 Anatomical analysis.

Tracer labeling was analyzed with conventional brightfield and fluorescent microscopy using an Olympus BH-2 microscope and photomicrographs were obtained with a Retiga EX CCD digital camera (Q-imaging) mounted on the microscope. One UV filter was used to visualize TB and FG (11000v2; Chroma Technologies), whereas a combined fluorescein isothiocyanate-tetarhodamine isothiocyanate (FITC/TRITC) filter (51004v2; Chroma Technologies) was used to visualize FR and AF labeling. Conventional brightfield microscopy was used to see BDA labeling. Digital reconstructions of tracer labeling relative to anatomical landmarks were made using an MDPlot system (Accustage, St Paul, MN) interfaced with the stage of the microscope. Retrogradely-labeled neurons were required to display at least one prominent dendritic process to
be plotted. Anterogradely-labeled beaded varicosities (en passant and terminal) were plotted to indicate putative synapses (Kincaid and Wilson, 1996; Voight et al., 1993).

Additional images were captured by a laser confocal microscope (TCS SP2 AOBS; Leica Microsystems) with a 63x-oil emersion objective. Each tracer was scanned separately with its own excitation/detection specifications per optical slice and maximum projection composites were constructed after imaging. In dual retrograde cases, TB (405nm excitation, 410-460nm detection) and FG (405nm excitation, 520-600nm detection) were imaged to reveal double-labeled neurons. Cases that received three tracer injections were visualized for AF-labeling (488nm excitation, 500-565nm detection), FR-labeling (543nm excitation, 565-630nm detection), and FG-labeled neurons as described above.

3.3 Results

Connections between wMI, wSI, and the claustrum were analyzed in three sets of experiments. Paired injections of the anterograde tracers FR and BDA in wMI and wSI (n=3) were performed to determine if these sensorimotor cortical regions project to overlapping parts of the claustrum. Paired injections of retrograde tracers TB and FG in wMI and wSI (n=3) were made to determine if the same part of the claustrum projects to both cortical regions. Finally, the anterograde tracers AF and FR were separately injected into corresponding parts of wMI in the two hemispheres, and these were coupled with an injection of the retrograde tracer FG into wSI (n=2) to ascertain whether wMI in both hemispheres converge onto claustral neurons that project to wSI.

Our analysis of tracer labeling was limited to sections located within 2.5 mm of bregma that also contained the striatum because these sections are known to be associated with...
claustrum specific protein called Gng2 (Mathur et al., 2009). Nissl staining was used for one
series of sections to visualize the claustrum as described elsewhere (Paxinos and Watson, 2007;
Smith and Alloway, 2010).

3.3.1 Dual anterograde injections.

To ascertain if the claustrum integrates whisker related information from
sensorimotor cortex, FR and BDA were injected into wMI and wSI, respectively, of the same
hemisphere. Consistent with previous work (Brecht et al., 2004; Alloway et al., 2008, 2009;
Smith and Alloway, 2010), FR injections in wMI were in agranular medial cortex (AGm) as
illustrated in Figure 3-1. Tracer injections in wSI filled all layers of cortex and, as expected,
revealed dense labeling in the thalamus, including the ventroposterior medial (VPM) and
posterior medial (POm) nuclei (Hoffer et al., 2001).

The claustrum contained many labeled projections from wMI, but projections from wSI
were not apparent. As described before (Alloway et al., 2009; Smith and Alloway, 2010), labeled
projections from wMI were densest in the claustrum located contralateral to the injection site.
These MI projections terminated mainly in the anterior portion of the claustrum and slowly
tapered off in posterior regions.
By contrast, virtually no projections from wSI were seen in the claustrum (see Figure 3-1). Despite the presence of extremely dense BDA-labeled axon terminals in the thalamus, especially in VPM and POm, BDA-labeled projections from SI were largely absent from the claustrum. Stray fibers were occasionally observed in the claustrum located ipsilateral to the injection site, but these faintly-labeled fibers did not contain varicosities indicative of en passant synapses. The lack of anterograde labeling in the claustrum following tracer injections in wSI is consistent with our previous report in which focal injections of retrograde tracers into the claustrum of two rats failed to produce retrogradely-labeled neurons in somatosensory cortex (Smith and Alloway, 2010).
3.3.2 Dual retrograde injections.

Different retrograde tracers were injected into wMI and wSI to determine if the same part of the claustrum projects to these cortical areas. An example of these injections in wMI and wSI appears in Figure 3-2. Retrogradely-labeled neurons produced by the wMI injections were found in the claustrum of both hemispheres, but were more numerous in the ipsilateral claustrum, which on average contained 88.0% (± 3.8%) of total claustral labeling. Furthermore, retrograde labeling was strongest in the anterior claustrum and gradually declined in more posterior sections. Injections in wSI revealed labeling in the claustrum of both hemispheres, but also favored the ipsilateral hemisphere (78.5 ± 1.9% of total claustral labeling) as described previously (Li et al., 1986; Sloniewski et al., 1986).

Figure 3-2. The claustrum projects to wMI and wSI. A,A': Injection of True Blue (TB) in wMI. B,B': Injection of Fluoro-Gold (FG) in wSI. D: Reconstruction of retrogradely-labeled neurons in the left and right claustrum showing intermingled TB (blue) and FG (gold) labeled neurons. Double-labeled neurons are plotted as green. Arrows correspond to confocal images obtained from the claustrum in the left (C) and right (E) hemispheres. Double labeled neurons indicated by arrowheads. Scale bars: 0.5 mm in A; 50 μm in C; 1 mm in D. ac, anterior commissure; cc, corpus callosum; ec, external capsule.
The photomicrographs and plotted reconstructions of the claustrum revealed substantial intermingling of TB- and FG-labeled neurons, as well as some double-labeled neurons, in the rostral half of the claustrum. The double-labeled neurons comprised a small portion of total claustral labeling (4.0 ± 0.5%), which roughly matches the small proportion of double-labeled neurons observed after tracer injections in other widely-separated cortical regions (Minciacchi et al., 1985). Our confocal images of the claustrum in both hemispheres depict the double-labeled neurons as blue soma with granules of FG. Some double-labeled neurons were exceptionally bright, as indicated by Figure 3-2C.

### 3.3.3 Triple tracer injections

In the final two experiments, we combined anterograde and retrograde injections to characterize a serially-connected cortico-claustral-cortical circuit that could be involved in coordinating wMI and wSI. The anterograde tracers AF and FR were injected into wMI of the left and right hemispheres (see Figure 3-3A-C), respectively, with injections of the retrograde tracer FG in wSI of the right hemisphere (see Figure 3-3D-E’). The bilateral injections of anterograde tracers in wMI produced dense bilateral claustral labeling in which labeled terminals were denser in the hemisphere contralateral to the injection site. FR-labeling was more prominent than AF, and this obscured visualization of the full extent of AF-labeling using conventional fluorescent microscopy. Nonetheless, confocal microscopy revealed both FR- and AF-labeled terminals in the claustrum of each hemisphere, especially in the anterior and central portions of this structure. These labeled terminals overlapped with the FG-labeled neurons produced by the tracer injections in wSI. This result suggests a cortico-claustral-cortical circuit in which information received from the wMI of each hemisphere is transmitted ipsilaterally to the wSI region.
Figure 3.3. The same claustral region receives wMI inputs from both hemispheres and projects to the ipsilateral wSI. A-C: AF- and FR-injections in wMI of the left and right hemispheres, respectively. D-E': FG-injection in wSI. G: Bilateral reconstructions of tracer labeling in the claustrum. AF- and FR-labeled terminals shown as green and red dots, respectively; FG-labeled neurons shown as gold circles. Arrowheads indicate sites for confocal images from left (F) and right (H) hemispheres. Dense AF and FR terminal labeling surrounds FG-labeled neurons on both sides. Scale bars, 0.5 mm in A,E; 1 mm in D; 50 μm in F; 1 mm in G. ac, anterior commissure.

3.4 Discussion

This neuroanatomical tracing study revealed three major findings. First, injections of different anterograde tracers in wMI and wSI revealed that the claustrum does integrate information from these sensorimotor regions; in fact, wSI does not project to the claustrum of either hemisphere. Second, injections of retrograde tracers in wMI and wSI revealed overlapping populations of labeled neurons with many double-labeled neurons. This indicates the claustrum transmits information to both cortical regions, and suggests that the claustrum in rodents plays a
role in sensorimotor coordination. Finally, anterograde tracer injections in wMI of each hemisphere coupled with a single retrograde tracer injection in wSI revealed that wMI projects to claustral regions that project to wSI. This demonstrates an interhemispheric cortico-claustral-cortical circuit that could supplement the ipsilateral corticocortical projections from MI to SI in each hemisphere.

Many studies report that cortex is reciprocally connected with the claustrum (see Edelstein and Denaro, 2007 for review). However, the present study did not reveal any projections from wSI to the claustrum. Furthermore, anatomical tracing studies in the rat visual system did not identify any corticoclastral afferents from Area 17 or 18 (Carey and Neal, 1985). In addition, tracing experiments in macaques also failed to find projections from V1 to the claustrum (Sherk, 1986). Together, these data indicate that not all cortical areas project to the claustrum, and the claustrum may project to cortical regions that do not reciprocate.

Another theory of claustral function centers on sensorimotor integration (Pearson et al., 1982; Edelstein and Denaro, 2007). Our data indicate that the rat claustrum does not integrate somesthetic and motor inputs. However, our retrograde tracing experiments do suggest a role for the claustrum in transmitting information to both the somatosensory and motor cortical areas. Simultaneous neuronal recordings in wSI and wMI have revealed correlated discharges in these regions during both spontaneous and stimulus-evoked activity (Chakrabarti and Alloway, 2008). Therefore, divergent projections from the claustrum could represent a substrate for coordinating the flow of motor-related information to both of these cortical regions.

In addition to motor control, AGm (wMI) has been implicated with a role in selective attention and memory-guided orienting (Reep and Corwin, 2009; Erlich et al, 2011). This view is consistent with tracing data showing that AGm receives input from multiple somesthetic cortical areas and association cortices (PPC, PV) that are involved in spatial attention and memory (Colechio and Alloway, 2009; Reep et al., 1987). This is significant when considered with our
current data showing that AGm densely innervates that claustrum, which can then modulate activity across other sensorimotor cortical regions. This pattern of neural connectivity suggests that the claustrum could be a useful rodent model for investigating the neural basis of spatial attention.
Chapter 4

Rat Whisker Motor Cortex Is Subdivided Into Sensory-Input and Motor-Output Areas

\[3\] This chapter appears as previously published in the journal article: Smith JB; Alloway KD (2013) Rat whisker motor cortex is subdivided into sensory-input and motor-output areas. *Front. Neural Circuits* 7:4.
4.1 Introduction

The functional organization of rodent motor cortex (MI) has traditionally been defined by using intracortical microstimulation (ICMS) to evoke peripheral movements (Hall and Lindholm, 1974; Donoghue and Wise, 1982; Sanderson et al., 1984; Neafsey et al., 1986; Miyashita et al., 1994; Brecht et al., 2004; Tandon et al., 2008). Studies using ICMS to map MI cortex generally find that the whisker region is located medially, whereas the forelimb and hindlimb representations are located, respectively, more laterally and caudally. When ICMS is combined with cytoarchitectonic analysis, the whisker region is linked to the medial agranular (AGm) area, whereas limb representations are in lateral agranular (AGl) cortex (Brecht et al., 2004).

Several investigators have sought to define the MI whisker representation by using neuronal tracing techniques to identify the motor cortical regions that receive projections from primary somatosensory (SI) cortex (Hoffer et al., 2003, 2005; Aronoff et al., 2010; Mao et al., 2011). By placing tracers in the SI whisker region and then reconstructing the terminal labeling in agranular cortex, these studies demonstrate that SI barrel cortex projects to a strip of MI cortex located approximately 1-2 mm anterior and lateral to bregma.

No study has ever used ICMS and neuronal tracing in the same animal to determine if both techniques produce corresponding results. Although many sensory cortical regions project to MI cortex (Colechio and Alloway, 2009; Reep et al., 1990), the relative topography of their projections to MI has not been examined. Both the secondary somatosensory cortex (SII) and the posterior parietal cortex (PPC), for example, convey sensory information to MI, but how these projections relate to SI inputs or the motor maps produced by ICMS remains unknown.

In this study, we used multiple techniques to characterize the whisker representation in rat MI cortex. In some rats, one or two anterograde tracers were placed in the SI, SII, or PPC whisker regions to compare the topography of their projections to MI with respect to the
cytoarchitectonic boundary that separates AGm from AGl. In some rats, the sensory innervation patterns were compared to the ICMS sites that evoked whisker movements. In other rats, two electrodes were placed in MI cortex, one at the border between AGm and AGl, and another more medially within AGm proper. Each electrode recorded isolated MI neuronal activity during controlled deflections of the peripheral whiskers, and then ICMS was administered at each electrode to compare their effectiveness at evoking whisker movements. The final set of animals received tracer injections in whisker-responsive parts of the superior colliculus (SC) to determine if corticotectal projections correspond to the motor output region defined by ICMS.

The results show that projections from SI and SII terminate in a narrow cortical region located at the border between AGm and AGl. By comparison, the PPC projects to a narrow region in AGm that adjoins the area that receives projections from SI and SII. Consistent with this anatomical specificity, neurons in the transitional region between AGm and AGl are more responsive to whisker stimulation than neurons located more medially in AGm proper. By comparison, ICMS in the AGm is more effective in evoking whisker movements than at the AGm-AGl transition region. Finally, corticotectal projections that convey motor information from MI are preferentially clustered in AGm and AGl, but are sparsely represented in the transitional region between these areas.

4.2 Material and Methods

Neuronal tracer injections and physiology experiments were conducted in male Sprague-Dawley rats (Charles River Co., Wilmington, MA) ranging in weight from 240 - 475 g. All procedures complied with NIH guidelines and were approved by our Institutional Animal Care and Use Committee.
4.2.1 Animal surgery.

Rats were anesthetized with an IM injection of ketamine HCl (40mg/kg) and xylazine (12mg/kg), and were subsequently administered atropine methyl nitrate (0.5mg/kg), dexamethasone sodium phosphate (5mg/kg), and enrofloxacin (2.5mg/kg). Following an initial dose of ketamine and xylazine, rats were maintained in a lightly-anesthetized state by isoflurane (0.5-1.0%) for the remainder of the surgery. Anesthetic state was monitored by electrocorticography (ECoG) via a screw over the frontal cortex contralateral to the experimental hemisphere. Online Fourier analysis of the ECoG signal was used to identify the dominant frequency of cortical activity, and isoflurane levels were adjusted throughout the procedure to maintain the animal in a III-2 or III-3 stage of anesthesia (Friedburg et al., 1999).

Rats were intubated and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and ventilated with oxygen. Heart rate, blood oxygen, and end-tidal carbon dioxide were monitored continuously during the procedure. Body temperature was maintained at 37.0⁰C, and ophthalmic ointment was applied to the eyes to prevent drying. A 2% solution of mepivacaine was injected into the scalp for local anesthesia, a midline incision exposed the cranial surface, and a ground screw was placed in the posterior cranium. Relative to bregma, craniotomies were made at coordinates that exposed SI (0-4 mm caudal, 3-7 mm lateral), SII (0-4 mm caudal, 6-9 mm lateral), PPC (1-5 mm caudal, 3-8mm lateral), or SC (5-8 mm caudal, 1-3 mm lateral).

4.2.2 Tracer injections.

Anterograde tracers were placed in the whisker regions of SI, SII, or PPC. The tracers were either a 15% solution of biotinylated dextran amine (BDA, Invitrogen) or a 15% solution of
Fluoro-Ruby (FR, Invitrogen). Locations of corticotectal projection neurons in MI were determined by injecting a combined solution of 2% Fluorogold (FG) and 15% BDA into whisker responsive sites in the SC.

To identify whisker-responsive sites in SI and SII, a glass pipette (~1MΩ) filled with 3M saline was inserted into cortical layer IV (400-800 μm deep) at 25° from the sagittal plane. A recording wire was inserted into the saline and then connected to the headstage of an extracellular amplifier (Dagan 2200; Dagan Corp., Minneapolis, MN) to monitor neuronal activity (filtered 300 – 3000 Hz, 60 Hz notch) on a digital oscilloscope (Tektronix DPO4034; Tektronix Beaverton, OR) and an audio monitor. Receptive field mapping was performed with a wooden rod to identify SI barrel cortex as described in previous reports (Hall and Lindholm, 1974; Hoffer et al., 2003). To identify SII, the pipette was moved lateral to the A-row representation in SI. Similar to previous findings (Hoffer et al., 2003), we observed a mirror image representation of the SI whisker rows, and more ventral whisker representations were encountered as the electrode was marched further laterally.

To identify PPC, the pipette was moved caudal to the α, β, γ, and δ whisker arc in SI, where another mirror-image reversal of somatotopic organization was observed. Compared to SI, in which a principal whisker was identified at each recording site, multi-whisker receptive fields were observed in SII and PPC. Early reports on the PPC area in rats called it the parietal medial (PM) region to be consistent with prior descriptions of the somatosensory areas in the squirrel (Koralek et al., 1990; Fabri and Burton, 1991; Krubitzer et al., 1986), but subsequent studies emphasized the similarity of this region with the PPC region in primates (Reep and Corwin, 2009).

Another set of rats received tracer injections in whisker-responsive regions of the SC. A tracer-filled pipette was inserted orthogonal to the pial surface, and neuronal responses to mechanical whisker stimulation were recorded 4-5 mm deep. Additionally, ICMS was used to
identify SC sites that evoke whisker movements as in other studies (McHaffie and Stein, 1982; Hemelt and Keller, 2008).

Deposits of BDA and FG/BDA were made using iontophoretic currents (2.5-5.0 µA, 10-20 min) administered on a 7s on/off duty cycle through glass pipettes with tip diameters of 30-50 µm. Small volumes (120 nL) of FR were pressure injected through a glass pipette cemented onto the end of a Hamilton syringe. Following tracer injections, the rats were sutured and allowed to recover for 7-9 days before being sacrificed.

4.2.3 Intracranial microstimulation.

In MI cortex, saline-filled pipettes or tungsten electrodes were inserted orthogonal to the pial surface to a depth of ~1.5 mm, which corresponds to layer V. Trains of cathodal current pulses (0.7 ms duration) were administered at 250 Hz for 20, 40, or 80 ms at current levels ranging from 10 – 250 µA. In all rats, these current parameters produced brief muscle twitches that were easily visualized. When ICMS was tested at MI sites that evoked whisker movements, the stimulation always evoked rapid biphasic excursions characterized by retraction and then movement back to the resting position. When evoked responses were visualized, the identity of the responsive whiskers was recorded in the experimental protocol.

The EMG responses that accompany whisker movements evoked by ICMS were recorded in some rats. These recordings were obtained from two needle electrodes in the muscles of the whisker pad and a ground electrode in the musculature of the hindlimb. A Grass preamplifier (model P5; Grass Instrument Co., Quincy, MA) amplified and filtered the EMG signal (0.3 – 3000 Hz, 60 Hz notch), which was recorded by a DataWave SciWorks acquisition system (SciWorks, ver. 8.0; DataWave Technologies, Broomfield, CO) at a rate of 26 kHz. Raw EMG traces were used to measure latency from stimulus onset to the first motor response that
exceeded baseline. The EMG records were low-pass filtered (250 Hz), rectified, and the mean response was calculated from 10 trials administered at each current level. The areal extent of the EMG envelope was used to quantify the magnitude of the muscle response.

4.2.4 **Extracellular neuronal recordings.**

Extracellular discharges recorded from MI were amplified (Dagan 2200; Dagan Corp., Minneapolis, MN), filtered, (300 to 3000 Hz), and converted into digital signals (DT2839, Data Translation, Marlboro, MA). Each neuronal recording channel was sampled at a rate of 26 kHz using the SciWorks data acquisition system. Waveforms were sorted using conventional parameters (spike height, width, valley time, peak time), time-stamped at a resolution of 0.1 ms, and displayed as peristimulus time histograms (PSTHs).

4.2.5 **Whisker stimulation.**

Whisker deflections were administered by a Galvanometer from a Grass polygraph controlled by a digital waveform generator (ArbStudio; LeCroy, Chestnut Ridge, NY) activated by SciWorks. A small piece of window screen glued to the end of a pen on the Galvanometer was placed next to the rat’s face so that vibrissa in rows A-E and arcs 1-5 protruded through the openings in the window screen. Each deflection consisted of a 50-ms back-and-forth pulse, first in the caudal direction (25 ms) and then back to the resting position (25 ms). In each trial, whisker stimulation consisted of three blocks of four deflections administered at frequencies of 2, 5, and 8 Hz. Because the first deflection in each block was preceded by a 1-s period, responses to the initial stimulus in each block were analyzed separately and classified as 1-Hz responses.
4.2.6 Sacrifice.

After each rat was deeply anesthetized with ketamine (80 mg/kg) and xylazine (6 mg/kg), it was perfused transcardially with heparinized saline, 4% paraformaldehyde, and 4% paraformaldehyde in 10% sucrose. The brain was removed and stored in 4% paraformaldehyde with 30% sucrose in a refrigerator for 1-2 days.

4.2.7 Histology.

Prior to sectioning, the brainstem, cerebellum, and olfactory bulbs were removed, and the two hemispheres were separated. In some cases a cortical slab was removed from the hemisphere and flattened between two slides prior to being sectioned tangentially to enhance visualization of cytochrome oxidase (CO) in the SI barrel field. In other cases, the hemisphere was split into two parts along a coronal plane at bregma; this allowed the rostral forebrain to be sectioned coronally (to visualize MI cytoarchitecture) and the caudal part of the cortex to be sectioned tangentially (to visualize tracer injections relative to CO-labeled barrels in SI). In rats that received tracer injections in the SC, the entire brain was sectioned coronally.

All blocks of neural tissue were cut into 60-μm thick sections and placed in 0.1M phosphate buffered saline (PBS). Tangential sections through the layer IV barrel field of SI were processed for CO (Land and Simons, 1985; Wong-Riley, 1979), and the remaining layers were processed for tracer labeling. For other tissue blocks, alternate sections were processed for tracer labeling and Nissl material.

To reveal BDA labeling, sections were processed using nickel and cobalt enhanced peroxidase immunohistochemistry as described previously (Smith et al., 2012). Tissue sections were rinsed in 0.3% H₂O₂ and then 0.3% Triton X-100 in 0.1M PBS. Sections were subsequently
incubated for 2 hours in an avidin-biotin horseradish peroxidase solution (Vector Novocostra Laboratories, Burlingame, CA) in 0.3% Triton X-100 in 0.1M PBS. Following incubation, sections were rinsed twice in 0.1M PBS and then incubated in 0.06% diaminobenzidine (DAB), 0.0005% H₂O₂, 0.05% NiCl₂, and 0.02% CoCl₂ in 0.1M tris buffer (pH = 7.2) for 10 minutes. The DAB reaction was halted by rinses in 0.1M PBS. The processed sections were next mounted on gel-dipped glass and dried overnight. All sections processed for CO, Nissl material, or tracer labeling were dehydrated in ethanol, defatted in xylene, and then coverslipped.

4.2.8 Anatomical analysis.

An Olympus BH-2 microscope equipped for fluorescent microscopy was used to analyze tracer labeling. An Accustage plotting system (St. Paul, MN) was used to make digital reconstructions of tracer labeling in MI relative to anatomical landmarks. The BDA-labeled terminals were viewed in brightfield illumination, and fluorescent FR-labeled terminals were visualized using a TRITC filter (41002; Chroma Technologies). Axonal varicosities were plotted because these represent en passant synapses (Kincaid and Wilson, 1996; Meng et al., 2004; Voight et al., 1993).

Tracer overlap was analyzed with a module in the Accustage software. Each digital reconstruction was subdivided into a grid of square bins (25 and 50 μm² were both tested), and each bin was color-coded blue if it contained BDA-labeling, red if it contained FR-labeling, or white if it contained both tracers. Tracer overlap was expressed as the proportion of tracer-filled bins that were colored white. Photographs of anatomical landmarks and tracer labeling were acquired using either an Epson V330 flatbed scanner or a Retiga EX CCD digital camera (Q-imaging, Surry, British Columbia, Canada).
4.3 Results

A total of 18 rats were used in this study. The first 10 animals, listed in Table 1, received tracer injections in whisker-sensitive parts of SI, SII, and PPC to characterize the topography of their projections to MI. Another 6 rats were used in electrophysiology experiments in which MI neurons were recorded during whisker stimulation and then the recording sites were tested with ICMS. The final 2 rats received tracer deposits in whisker-related sites in the SC.

4.3.1 Topography of the SI projections to MI.

In 3 rats, the whisker-related projections from SI to MI were characterized by sectioning the entire cortex tangentially. In one case, which is illustrated in Figure 4-1, multiple BDA deposits were placed in the C-row of SI barrel cortex. The BDA deposits were confined entirely within the SI barrel field and, consistent with previous reports (Hoffer et al., 2003; Lee et al., 2010), revealed labeled projections to neighboring whisker regions in SII and PPC. In addition, labeling of the long-range projections from SI produced dense terminal labeling in a strip of MI cortex located approximately 2 mm from the midline.
Figure 4-1. Corticocortical projections from SI barrel cortex terminate at MI sites located lateral to the sites most effective for evoking whisker movements. **A**: Tangential section processed for cytochrome oxidase (CO) shows the spatial distribution of the layer IV barrels in SI cortex. **A’**: An adjacent section processed for biotinylated dextran amine (BDA) shows the location of BDA deposits in SI barrel cortex. Contour lines indicate the primary (SI) and secondary (SII) somatosensory cortical areas as well as the posterior parietal cortex (PPC). Rectangle indicates the region depicted in panel **B**. **B**: Location of two electrolytic lesions (red arrows) marking where intracranial microstimulation (ICMS) was most effective in evoking whisker twitches. Labeled projections (arrowheads) from SI terminate in a strip of MI cortex located caudal and lateral to sites that evoked the best whisker responses. Scale bars: 2 mm in **A**; 500 µm in **B**.

On the day of sacrifice, ICMS was used to locate the MI sites most effective for evoking movements of the C-row whiskers. Microstimulation of MI sites located more than 2 mm lateral to the midline evoked twitches in the forelimb, shoulder, and neck muscles. Medial to these sites, low-threshold (~ 60 µA) currents evoked movements of the E and D-row whiskers. When the stimulating electrode was marched further medially, C-row whisker movements were evoked at the lowest currents (< 50 µA) used in this experiment. Because these ICMS sites evoked movements from the same whiskers whose representations were filled with tracer deposits in SI barrel cortex, electrolytic lesions were made at these MI sites and the electrode penetrations were marked with ink. As indicated by Figure 4-1B, the labeled projections from SI barrel cortex terminated in a strip of MI cortex located caudal and lateral to the MI lesions that marked the locations for evoking movements of the C-row whiskers.
4.3.2 MI cytoarchitecture.

The somatosensory cortical areas that received tracer deposits were always sectioned tangentially, but MI cortex was usually sectioned coronally so that we could examine its cytoarchitecture with respect to labeled projections from SI and its surrounding cortical fields. Figure 4-2 depicts the cytoarchitecture of MI cortex and the laminar changes that characterize the transition from AGm to AGl. Consistent with previous studies of rat motor cortex (Donoghue and Wise, 1982; Brecht et al., 2004), AGm has a relatively thick layer V that is dense with pyramidal neurons. More superficially, layer III is thin and has a noticeable pale appearance. Moving laterally towards the transitional zone between AGm and AGl, layer III gradually becomes much thicker as its neuronal density increases. By comparison, layer V becomes narrower as the bottom of layer III expands ventrally, and the neuronal density of layer V is much lower in AGl than in AGm. Layers III and V have a similar thickness in AGl, and the lateral edge of AGl is delineated by the sudden appearance of a dense granular layer IV, which signifies the medial edge of SI cortex.

Figure 4-2. Photomicrograph depicting the cytoarchitecture of MI cortex located 1.5 mm rostral to bregma. The transitional zone (TZ) between the medial (AGm) and lateral (AGl) agranular areas is indicated by arrowheads. Additional arrowheads indicate the MI borders with the cingulate (Cg) and somatosensory (SI) cortical areas. Thin lines indicate laminar boundaries for layers I through VI.
4.3.3 SI projections and MI topography.

Experimental results depicting the topography of SI projections to MI are illustrated in Figure 4-3. After briefly mapping whisker-responsive sites in SI, multiple BDA deposits were placed so that the tracer infiltrated large portions of the SI barrel region (see Figure 4-3B). Local transport of the tracer revealed dense terminal labeling in SII cortex and in a smaller caudal area that represents PPC.

Figure 4-3. Projections from SI barrel cortex terminate in the cortical area that marks the transition between AGm and AGl. A: CO-processed tangential section through the SI barrel field. The rostral half of the hemisphere is not apparent because it was sectioned coronally. Rectangle depicts the region in panel B. B: Adjacent tangential section shows multiple BDA deposits in SI, as well as transport of BDA into SII and PPC. C, C': Adjacent sections processed for Nissl material and BDA-labeled projections from the SI tracer injections shown in panel B. The border between AGm and AGl is marked by arrowheads. Arrows indicate ICMS sites for evoking peripheral movements. Red arrow indicates the site where the lowest current (10 μA) evoked whisker twitches; black arrows indicate where higher currents (100-250 μA) evoked whisker movements; blue arrow indicates the ICMS site (50 μA) that evoked forelimb movements. D: Plotted reconstructions illustrating labeled SI terminals with respect to the AGm-AGl border. Numbers indicate the distance from bregma in mm. Scale bar = 2 mm in A; 1mm in B,D; 500μm in C.
In MI cortex, a large bundle of labeled projections from SI coursed rostrally through cortical layer VI and abruptly turned toward the pial surface to form a vertical column of labeling (Figure 4-3C'). The labeled column was 400-500 µm in width and extended upwards from upper layer VI to layer I. Inspection of the adjacent Nissl-stained section indicated that this labeling was located in the MI region that represents the transition from AGm to AGl. As noted previously (Brecht et al., 2004), the border between these regions is often characterized by a gradual transition that can extend over several hundred microns.

Microstimulation was used to analyze the functional topography of MI with respect to the labeled projections from SI. After using saline-filled glass pipettes to evoke muscular responses at several sites within a single coronal plan, a tungsten electrode was reinserted at selected sites to verify each ICMS response and make a small electrolytic lesion. The locations of four microlesions that marked ICMS sites associated with whisker or limb movements are illustrated in Figure 4-3C.

The site at which the lowest stimulation current (10 µA) evoked whisker motion was in AGm, approximately 1.25 mm from the midline. Threshold currents for evoking whisker movements increased as the electrode moved to sites 1.75 mm (100 µA) and 2.15 mm (250 µA) lateral to the midline. The most lateral site, located 2.65 mm from the midline, was located in AGl and evoked dorsoflexion of the forepaw when a threshold current of 50 µA was administered. The vertical column of densely-labeled projections from SI, which occupied the AGm-AGl transitional zone, overlapped the two microlesion sites where whisker movements were evoked by moderately large current levels (100-250 µA). The most medial microlesion, which marked the site where whisker movements were evoked by the lowest stimulation currents (10 µA), was located in AGm proper.
Similar topographical responses were observed in other cases in which ICMS was systematically tested at different mediolateral locations in MI cortex. In all cases, low current levels near threshold evoked movements of either the whiskers or the forelimb in AGm and AGl, respectively. When suprathreshold currents were administered, however, movements of both whiskers and forelimb were occasionally observed, but only when the electrode stimulated the border between these functionally-defined regions.

Reconstructions of the terminal labeling patterns indicate that SI projections terminate in vertical columns that gradually move laterally as sections were analyzed from progressively more rostral parts of MI (Figure 4-3D). This medial-to-lateral shift is consistent with the diagonally-oriented strips of labeling observed in our tangential sections of MI cortex (Figure 4-1B), and it matches the systematic changes in AGm width observed by others (Donoghue and Wise, 1982; Tennant et al., 2011).

4.3.4 SII projections and MI topography.

In 3 rats, anterograde tracing was used to determine the topography of SII projections with respect to the cytoarchitecture of MI (see Table 4-1). After whisker-induced responses were recorded at multiple sites in SI barrel cortex, the electrode was marched laterally to establish a reversal in the whisker map as successive sites in SII cortex were recorded. Once the C-row region in SII was located, BDA was injected so that it infiltrated much of SII without invading SI. As seen in Figure 4-4B, the BDA injection did not diffuse into the barrel field of SI, but many retrogradely-labeled neurons were present in both the septal and barrel columns as previously reported (Chakrabarti and Alloway, 2006).
Figure 4-4. Projections from SII cortex terminate in the transitional region between AGm and AGl. A: Tangential section depicting the CO-labeled barrel field in SI. Rectangle indicates the area depicted in panel B. B: Multiple injections of BDA into the SII whisker region located lateral to SI. Locations of the CO-labeled barrels in SI are indicated by contours. Subsequent panels are illustrated as in Figure 4-3. Scale bar = 2 mm in A; 1mm in B,D; 500µm in C.

Inspection of the labeled terminals in MI indicates that SII projects to the transitional zone between AGm and AGl (Figure 4-4C’), which is the same MI region that receives projections from SI (see Figure 4-3C’). However, despite the relatively large tracer injection in SII, the labeled projections to MI appeared weaker and were less extensive than the terminal labeling observed after tracer injections in SI. Compared to projections from SI, the SII projections innervated layers II, III, and the lower part of layer V of MI, but were weaker in upper layer V and did not innervate any part of layer VI. Furthermore, the SII projection terminals were more concentrated in the rostral half than in the caudal half of MI (Figure 4-4D).

To confirm that both SI and SII innervate overlapping regions, paired injections of different anterograde tracers were placed in SI and SII. As shown by Figure 4-5, injections of FR into the C and D rows of SI were paired with a BDA injection in the C-row representation of SII. In both cortical areas, tracer injections were guided by mapping neuronal responses to whisker stimulation. Tracer placements in SI and SII were visualized in tangential sections processed for
CO, and microscopic examination confirmed that corresponding whisker representations were injected in both areas as indicated by the presence of BDA-labeled neurons at the FR injection site (Figure 4-5A',B).

![Figure 4-5](image)

**Figure 4-5.** Projections from the whisker regions in SI and SII terminate in the AGm-AGl transition zone. A,A': Adjacent tangential sections depicting the SI barrel field (A) and the location of BDA deposits in SII (A'). Arrows indicate common blood vessels. Rectangle around the SI barrel field indicates the region depicted in panel (B). B: Deposits of Fluoro-Ruby (FR) in SI barrel cortex. C: Cytoarchitecture depicting the transition zone (arrowheads). C', C'': Adjacent sections depicting the terminal projections from SII and SI with respect to the transition zone (dashed line). D: Reconstructions of SI (red) and SII (blue) labeled terminals throughout the rostrocaudal extent of MI. Scale bar = 500µm in (A,C); 250µm in (B); 1mm in (D).

The labeled projections from SI and SII terminated in an overlapping part of MI located in the transitional region between AGm and AGl (Figure 4-5C',C''). While the labeled projections from SI infiltrated all layers of MI, those from SII were noticeably absent from layer VI. Nonetheless, reconstructions of terminal labeling in MI revealed substantial tracer overlap in the
transitional region between AGm and AGl. This overlap extended throughout MI cortex but was densest in its rostral half (Figure 4-5D).

4.3.5 PPC projections to motor cortex.

To characterize more completely the sensory-related cortical regions that innervate MI cortex, we injected PPC with anterograde tracers and analyzed the labeled projections with respect to the AGm-AGl border. These experiments were prompted by previous studies showing that PPC projects to AGm but not to AGl (Reep et al., 1990; Colechio et al., 2009).

To locate the whisker region in PPC, neuronal responses to whisker stimulation were initially tested in the caudal part of SI barrel cortex. Sites located caudal to SI were then tested, and neurons in this area had multi-whisker receptive fields. As indicated by Figure 4-6, tracer deposits in PPC were caudal to the CO-labeled barrels in SI. Although the tracer did not diffuse into SI, retrogradely-labeled neurons and other processes appeared in the SI septal regions as described earlier (Lee et al., 2011).
Figure 4-6. Projections from posterior parietal cortex (PPC) terminate primarily in the caudal part of AGm. A,B: Adjacent tangential sections showing CO-labeled barrels in SI and the BDA tracer deposit in PPC, which is caudal to SI. Rectangle indicates the magnified image of the PPC injection site and BDA labeling in the SI septa. C,C’: Adjacent sections depicting the labeled projections from PPC with respect to the transition zone. Arrowheads indicate cytoarchitectonic boundaries. D: Plotted reconstructions indicate PPC projections terminate most densely in caudal parts of MI. Scale bar = 2 mm in A; 1 mm in B; 500 µm in C; 1 mm in D.

Virtually all labeled projections from PPC were located in AGm proper (see Figure 4-6C,C’). In each coronal section that was inspected, the PPC projections terminated in the region medial to the AGm-AGl transition zone (Figure 4-6D). The labeled terminals were intermingled with labeled neuronal soma, which indicates that PPC and AGm are reciprocally connected. In contrast to SII, the PPC projections were concentrated in the caudal half of MI.

To verify that SI and PPC innervate adjacent parts of MI, we placed pairs of different anterograde tracers in SI and PPC of three rats (See Table 4-1). Figure 4-7 illustrates a case in which FR and BDA were placed in whisker-sensitive sites in SI and PPC, respectively. Histological inspection revealed that many SI neurons retrogradely-labeled by the PPC tracer injections were at sites that overlapped the tracer injections in SI (Figure 4-7A’,B), thereby indicating that both tracers were injected into corresponding whisker regions.
Figure 4-7. Projections from PPC and SI terminate in adjoining MI regions. A, A’, B: Tangential sections showing CO-labeled barrels and tracer injections in PPC and SI as in Figure 4-5. Arrows indicate common blood vessels. C, C’, C’’: Adjacent coronal sections illustrate labeled projections from PPC (C’) and SI (C’’) with respect to the transition zone. D: Reconstructions of the labeled terminals in MI originating from PPC (blue) and SI barrel cortex (red). Scale bars = 500µm in A, C; 250µm in B; 1mm in D.

These dual tracer experiments indicate that SI and PPC innervate adjoining, partially overlapping regions in MI. The projections from PPC innervate regions located medial to most of the MI sites that receive SI inputs. While SI projections terminate densely in the AGm-AGl transition region (Figure 4-7C’’), the labeled projections from PPC terminate almost exclusively in an adjoining part of AGm proper (Figure 4-7C’). Reconstructions of labeled terminals throughout the rostrocaudal extent of MI depict some overlap among the projections from PPC and SI (Figure 4-7D), but most of the axons labeled by different tracers terminated in separate regions. Consistent with this distinction in projection targets, the PPC projections are densest in caudal MI, whereas those from SI are densest in rostral MI.
4.3.6 Overlap Analysis.

To assess the amount of convergence among SI, SII, and PPC projections to MI, we quantitatively analyzed the labeled overlap produced in the five rats that received paired tracer injections (see Table 4-1). For this purpose, plotted reconstructions of the MI terminal labeling patterns were subdivided into square bins that were color-coded according to the type of terminal labeling present in each bin. Then, in each of the five rats, the proportion of all bins throughout MI that contained tracer overlap was measured so that overlap resulting from SI and SII tracer injections could be compared with the overlap produced by injections in SI and PPC.

The results indicate that projections to SI and SII overlap substantially more than the projections to SI and PPC regardless of whether 25 or 50 μm² bins were used in the analysis. Figure 4-8 illustrates the amount of overlap in tangential sections obtained from two rats in which different tracers were injected either into SI and SII or into SI and PPC. As indicated by these individual sections, which depict 50 μm² bins, projections from both SI and SII were present in nearly 20% of the labeled area, whereas overlapping projections from SI and PPC occupied less than 5% of the labeled area. When tallied across all sections in each rat, MI tracer overlap in the SI and SII cases (n = 2) varied from 16.4 to 26.9%, but ranged from only 2.5 to 6.9% in the rats that received injections in SI and PPC (n = 3). Although the number of dual tracer cases is too small for a rigorous statistical comparison, the large differences in overlap between these groups suggest that PPC projects to an MI area that is separate from the area innervated by SI and SII.
Figure 4-8. Projections from SI and SII overlap in MI, but projections from SI and PPC terminate in adjoining MI areas. Left set of panels depict results from a rat in which SI and SII were injected with FR (red) and BDA (blue), respectively. Right panels depict results from a second rat in which SI and PPC were injected with BDA and FR, respectively. In both cases, the panels depict labeling patterns in tangential sections obtained from layers III or V. For each section, the plotted reconstructions of the labeled terminals were subdivided into square bins (50 μm²) color-coded by whether the bin contains terminal projections from one injection site (blue or red) or overlapping projections from both injections (white). Amount of overlap in each section is indicated by percentages. Scale bar = 1mm. Abbreviations: hindpaw, HP; forepaw FP.

4.3.7 Topography of sensory responses in MI.

The overlap of SI and SII projections to the AGm-AGl transitional zone suggests that this region is specialized for processing sensory inputs. To confirm that SI and SII conveys whisker-related information to this region, we recorded neuronal responses at the AGm-AGl border during controlled whisker deflections. Furthermore, to determine if this region is functionally distinct from AGm proper, we recorded neurons in both areas simultaneously so that
we could compare their whisker-induced responses. For this purpose, two electrodes were independently inserted into layer V of both regions because previous work showed that MI responses to whisker stimulation are maximal at sites located 1-1.5 mm below the pial surface (Chakrabarti et al., 2008). In addition, after recording neuronal responses to whisker deflections at each electrode, we administered ICMS at each recording site to determine if both regions are equally effective in evoking whisker movements.

Peripheral whisker stimulation evoked strong neuronal responses in the AGm-AGl transition zone, but did not activate neurons in AGm proper. As illustrated by Figure 4-9, spontaneous discharge rates were similar for the neurons recorded in each area. Yet, whereas the neuron in the transition zone responded to each tested frequency (2, 5, 8 Hz), the neuron in AGm did not respond to any whisker deflections. Subsequent administration of ICMS at each recording site evoked whisker movements that were easily visualized.

**Figure 4-9.** Neuronal responses in MI during computer-controlled whisker deflections. **A:** Coronal section through MI showing two recording sites (arrows) with respect to the transition zone. Scale bar = 500 μm. **B:** Peristimulus time histograms (PSTHs) show the responses recorded in AGm (top) and the transition zone (bottom). Controlled stimulation consisted of 50-ms deflections applied simultaneously to 15 whiskers at multiple frequencies over 200 trials. Dashed lines represent 99% confidence interval. Waveform scales: 200 μV, 1ms. PSTH bins: 10ms.
In all rats (n=6), histology confirmed that whisker-responsive neurons were recorded in the AGm-AGl transition zone, which coincides with the afferent projections from SI and SII. A total of 9 pairs of MI neurons were recorded, and in each case the AGm neuron failed to show an increase in discharge rate when the whiskers were deflected. One AGm neuron, however, displayed spontaneous activity that was inhibited when the whiskers were stimulated. All neurons in the AGm-AGl transition zone were activated by whisker stimulation. While some responded at each frequency of whisker stimulation (4/9), the rest responded to stimulation up to 5 Hz (5/9). These results indicate that the transitional region between AGm and AGl represents a functionally distinct part of MI that is specialized for processing sensory inputs.

4.3.8 Comparison of sensory and motor responses

Some of the electrophysiology experiments (n = 3) were conducted in rats in which we compared the sensory-input and motor-output functions of AGM and the AGm-AGl transitional zone. As in the other MI recording experiments, one electrode was placed in AGm and the other was placed at the AGm-AGl border to record neuronal responses in both regions at one or two depths during controlled whisker deflections. Subsequently, for ICMS, the electrodes were advanced to deep layer V (1.5 mm) where corticobulbar neurons originate. Microstimulation was alternately administered to each electrode on every trial, and EMG responses were recorded by needle electrodes inserted into the whisker pad (see Methods).

Administration of ICMS evoked detectable differences in EMG responses that varied with the location of the MI stimulating electrode. For the case shown in Figure 4-10, ICMS with currents of 10 µA evoked whisker movements only in AGm on 20% of the trials. When higher current (25 µA) levels were employed, both AGm and the transition zone were effective in evoking EMG responses, but EMG responses evoked from AGm had shorter latencies than the
responses evoked from the transition zone (see Figure 4-10B). The latencies measured from the raw EMG responses (10 trials) was 15.2 ms for AGm and 17.2 ms for the transition zone, consistent with latencies from other studies performing ICMS in MI (Berg and Kleinfeld, 2003). Although the peak amplitudes of the EMG responses evoked by both sites were similar at near threshold current levels, more whiskers responded to ICMS at AGm (whiskers in rows B-E, arcs 1-4) than at the AGm-AGl transition zone (whiskers in rows C-D, arcs 1-2).

Figure 4-10. ICMS-evoked whisker movements are strongest at AGm, but sensory-evoked responses are present only at the transition zone. A: Nissl-stained section showing recording sites marked by lesions (black arrows). Scale bar = 500 μm. B, C: EMG responses to ICMS (bar) administered at two current levels in AGm or the transition zone. Each graph represents the mean of 10 trials. Arrowheads indicate onset of EMG response; dashed lines represent maximum pre-stimulus activity. D, E: PSTHs illustrate sensory-evoked responses as in Figure 4-9. Waveform scales: 200 μV, 1ms. PSTH bins: 10ms.
At higher current levels (50 μA), ICMS at both cortical regions produced whisker retractions, but the EMG responses evoked from AGm had larger amplitudes (see Figure 4-10C). Consistent with this, the latency of the EMG responses evoked from the AGm electrode became even shorter when compared to the latency of the responses evoked from the AGm-AGl border (14.6 vs. 16.7 ms). In fact, in the three cases analyzed, EMG responses evoked by ICMS at AGm had shorter latencies (16.3 ± 0.3 ms) than the EMG responses evoked from the AGm-AGl border (17.3 ± 0.4 ms). Compared to the AGm-AGl transition zone, these findings suggest that AGm proper has stronger functional connections with the periphery whisker pad.

In experiments in which EMG responses were recorded during ICMS, the MI responses to controlled whisker stimulation had already been recorded from neurons encountered before the electrodes reached deep layer V. As before, whisker-induced sensory responses were recorded from neurons in the AGm-AGl transition zone but not from neurons in AGm proper (see Figure 4-10D). Hence, whisker-evoked responses in the AGm-AGl transition zone are consistent with the specificity of the projections from SI and SII. Coupled with the differential EMG responses evoked by ICMS at these two MI regions, these data indicate that rat MI contains distinct sensory-input and motor-output subregions.

4.3.9 Organization of corticotectal projections from MI

In an effort aimed at characterizing a route for conveying MI signals to the facial nucleus, we placed tracers into the whisker representation of the SC. This brain region was chosen because SC is one of the main targets of MI corticofugal projections and the SC projects directly to the facial nucleus (Miyashita et al., 1994; Alloway et al., 2010). In two rats, we injected a combined solution of anterograde (BDA) and retrograde (FG) tracers into SC sites that responded to whisker deflections and were effective at evoking whisker movements when tested with ICMS.
Consistent with previous work (Hemelt and Keller, 2006), neurons in the ventrolateral SC respond to repetitive deflections of the contralateral whiskers as shown in Figure 4-11. A combined injection of BDA and FG into this site produced limited diffusion of both tracers, but revealed many local projections within the SC and to the periaqueductal grey region. In addition to labeled projections to the reticular formation and spinal trigeminal nucleus (data not shown), BDA-labeled terminals also appeared in the contralateral facial nucleus (see Figure 4-11C,C’,D). This result confirms that the tracers were placed in a region that could transmit MI information to the facial nucleus.

**Figure 4-11.** Whisker-responsive region in the superior colliculus (SC) projects to the contralateral facial nucleus. **A:** PSTH illustrating the whisker-induced responses of a SC neuron recorded by a pipette containing FG and BDA. **B:** Nissl-stained coronal section showing the laminar location of BDA (B’) and FG (B'”) injections at the neuronal recording site. **C:** Nissl-stained section showing the facial nucleus contralateral to the SC injection site C’,D: BDA-labeled terminals in the facial nucleus. Waveform scales: 200 µV, 1ms. PSTH bins: 2ms. Scale bars = 500 µm in B and C; 250 µm in D.
Corticotectal neurons labeled by tracer injections in SC were present throughout MI cortex. As seen in Figure 4-12, populations of labeled neurons were easily visualized in tissue sections processed for BDA. Although BDA is normally used as an anterograde tracer, injecting it in solution with FG greatly increases its retrograde transport (Smith et al., 2012). The topographic distributions of BDA-labeled and FG-labeled neurons were identical, but we chose to plot and photograph only BDA-labeled neurons because these neurons were easier to visualize, especially at low magnifications.

Figure 4-12. Most corticotectal projections from MI originate from AGm. A: Nissl-stained section of MI showing boundary between AGm and AGl. A', B: Photomicrographs illustrating MI neurons retrogradely-labeled by the BDA tracer injection shown in Figure 4-11. Corticotectal neurons are densest in AGm. Rectangle depicts region shown in panel B. C: Digital reconstruction of labeled neurons throughout the rostrocaudal extent of MI. Two plotted reconstructions are superimposed in each depiction. Scale bars = 500 μm in A; 100 μm in B; 1mm in C.

The majority of corticotectal neurons labeled by tracer injections in whisker-sensitive parts of the SC are located in AGm proper. As illustrated by our plotted reconstructions, the densest clusters of labeled neurons appeared in AGm proper. Within some coronal sections, these large clusters of labeled neurons were easily visualized in AGm at low magnifications (see Figure 4-12 A'). Secondary clusters of labeled neurons also appeared in AGl at specific rostrocaudal...
coordinates, which is consistent with previous work showing that the forelimb representation in AGl projects to scattered targets in the SC (Alloway et al., 2010). Compared to the main parts of AGm and AGl, the transition zone between these regions contained few labeled neurons. In fact, in many coronal sections the AGm-AGl transition zone was devoid of labeled neurons even though distinct clusters of corticotectal neurons appeared in AGm or AGl.

4.4 Discussion

This study characterized the functional organization of the MI whisker representation by using a combination of techniques that included anterograde tracing from multiple cortical areas, paired neuronal recordings in MI during whisker stimulation, and recordings of EMG signals in the whisker pad during ICMS at different MI sites. The tracing results indicate that projections from SI and SII terminate in the transitional zone between AGm and AGl. Neurons in this region are highly responsive to whisker stimulation, but neurons in AGm proper do not respond to passive whisker deflections. By contrast, AGm is more effective for evoking whisker responses. These findings suggest the existence of functionally-distinct subregions within the whisker representation in MI cortex: a sensory-input region in the AGm-AGl transition zone and a motor-output region in AGm proper.

4.4.1 Sensory processing in MI.

Our findings suggest that MI cortex contains one or more subregions that are specialized for processing sensory inputs. The existence of a sensory processing region in MI was previously suggested by tracing studies showing that whisker-related areas in SI and SII project to a relatively compact part of MI (Hoffer et al., 2003, 2005; Colechio and Alloway, 2009; Aronoff et
al., 2010). Consistent with previous results (Chakrabarti et al., 2008), we confirmed that passive whisker deflections evoke neuronal responses at MI sites that receives sensory inputs from SI and SII.

Our results also suggest the possibility that MI cortex might contain more than one sensory processing region. In addition to the border region between AGm and AGl, the adjoining region in AGm receives inputs from PPC that display minimal overlap with the projections from SI. Tracing studies indicate that PPC receives somatosensory, auditory, and visual cortical inputs (Reep et al., 1994). Consistent with these multimodal sensory inputs, lesions that damage PPC or its connections with frontal cortex produce attention deficits characterized by hemispatial sensory neglect (Burcham et al., 1997; Reep and Corwin; 2009). Compared to projections from SI and SII, the anatomical and functional specificity of the PPC projections suggest that its target region in AGm might represent an additional MI area that is specialized for processing multimodal spatial information.

Axonal projections from SI to MI originate from the septal circuits in SI barrel cortex (Alloway et al., 2004; Chakrabarti et al., 2006; Chakrabarti et al., 2008). Although the functions of the septa remain controversial, substantial evidence suggest that septal circuits encode information about the kinematics of active whisker movements (Alloway et al., 2008). In this context it is noteworthy that PPC derives its SI inputs from the septal circuits (Lee et al., 2010). This prompts the view that the AGm-AGl transition zone is responsible for processing relatively pure kinematic information received from SI, whereas the adjoining subregion in AGm proper processes whisking information that is integrated with visual and auditory inputs in the PPC. Presumably, both of these sensory-related MI regions provide information to AGm proper that helps refine the execution of the cortical motor programs that regulate whisking behavior.
4.4.2 MI control of whisking behavior.

The role of motor cortex in whisking behavior is a contentious topic. Some investigators who used ICMS in awake animals concluded that MI exerts direct control over specific aspects of whisking behavior (Haiss and Schwarz, 2005; Berg and Kleinfeld, 2003; Cramer and Keller, 2006). Consistent with this view, neuronal recordings during whisking behavior indicate that MI neurons “report the absolute angle of vibrissa position” even after the trigeminal nerve has been transected (Hill et al., 2011). By contrast, others have suggested that AGm “plays a role in orienting that is independent from any role in control of whisking” (Erlich et al., 2011). These conflicting views emphasize the need for using multiple approaches to elucidate the functional organization of the MI whisker region.

Our data indicate that ICMS in AGm proper is more effective for evoking whisker movements than ICMS in the AGm-AGl transition zone. Compared to the transition zone, we observed that microstimulation in AGm evokes movements from a larger number of whiskers when the same current levels were administered to both regions. These visual observations were usually confirmed when we compared the amplitudes of EMG responses recorded at the mystacial whisker pad. In addition, EMG responses evoked from AGm appeared earlier than the responses evoked from the AGm-AGl transition zone. Based on these differences in the EMG response latencies, we conclude that the outputs from AGm proper have stronger and more direct connections with the facial nucleus than the outputs from the AGm-AGl transition region.

Short-latency EMG responses evoked from AGm are consistent with tracing studies showing that AGm projects to many brainstem regions, including the SC, that project to the facial nucleus (Grinevich et al., 2005; Alloway et al. 2010, Hattox et al., 2003). This was corroborated by the present study, which used anterograde and retrograde tracing to demonstrate that whisker-sensitive regions in SC have input-output connections that could transmit MI information directly
to the facial nucleus. Although projections originating exclusively from the AGm-AGl transition zone were not characterized, we found that most MI inputs to whisker-responsive sites in the SC originate from the AGm. Although the SC also receives MI inputs from AGl, very few corticocortical neurons were located in the AGm-AGl transition region. Therefore, the AGm-AGl transition region appears to be weakly connected with SC, and this could explain why the border region between AGm and AGl is less effective in evoking EMG responses.

An alternative explanation concerns the intrinsic connections within MI cortex. When tracers are placed in the center of the whisker (AGm) or forelimb (AGl) regions, the labeled connections remain within the injected region (Weiss and Keller, 1994). When tracers are placed at the border between these regions, however, labeled axons project throughout both the whisker and forelimb areas. Hence, when ICMS is applied to the border region between AGm and AGl, it may activate local projections to AGm that, in turn, activate the long-range outputs from AGm. Although the functional role of these local horizontal connections has not been established, they could provide a route by which the sensory processing subregion modulates AGm activity during exploratory whisking behavior.

4.4.3 Functional subregions.

Previous reports indicate that cortical control of whisking is parcellated into distinct subregions, but this view is mainly concerned with rostrocaudal distinctions in the kinematics of whisker movements evoked by ICMS (Haiss and Schwarz, 2005). To our knowledge, no study has demonstrated functionally-distinct subregions in the mediolateral dimension of MI. Even though previous work indicates that SI projections to MI do not terminate at coordinates that are optimal for evoking whisker movements (Brecht et al., 2004), the spatial relationship between sensory inputs and the effects of ICMS were never examined in the same animal. The present
study indicates that SI and SII projections to MI terminate specifically in the AGm-AGl transition region, and this region is less effective than AGm for evoking whisker movements.

Our results suggest that MI whisker cortex contains a sensory-input region that is spatially segregated from the motor-output region. Conceivably, the whiskers and other body part representations in MI are comprised of distinct sensory-input and motor-output regions so that specific motor behaviors are executed by separate parts of MI cortex. For example, PPC inputs may terminate in whisker and neck regions of MI, as defined by ICMS, to facilitate the coordination of the motor elements that comprise orienting behavior. Likewise, SI projections to rostral MI may convey information about the whiskers and the nose representation to help coordinate sniffing and whisking behavior (Hoffer et al, 2003; Welker, 1964). Testing these hypotheses, however, requires the combination of tracing data and physiological recordings in awake behaving animals.
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**Table 4-1.** Summary of Tracer Injections.
Chapter 5

Conclusions and Future Directions
These experiments have demonstrated the existence of previously unknown projections involving the motor cortex and claustrum, and that already known projections from sensory cortex reveal compartmentalization of motor cortex. The first set of experiments verified an interhemispheric circuit via the claustrum that connects regions of the whisker motor cortex, but not the forelimb motor cortex. This suggests that one of the roles of the claustrum is to regulate interhemispheric communication between motor regions involved in bilaterally coordinated behaviors. The second set of experiments showed that the claustral neurons projecting to both wMI and wSI receive input from wMI, but receive no projections from wSI. Thus, the claustrum is not involved in sensorimotor integration, but instead plays a role in sensorimotor coordination. The final study showed that the whisker representations in SI and SII send converging projections to MI, whereas the PPC inputs terminate in a separate, but adjacent region. Furthermore, whisker movements elicited by ICMS originated in a motor-output region separate from the sensory responsive SI/SII input region of MI. These functionally distinct subregions demonstrate parcellation of the whisker representation in rodent motor cortex, as opposed to a homogenous region. These divisions indicate a homologous organizational scheme between rats and primates where multiple motor areas represent the same body part but process different information.

5.1 Functional implications of claustral circuits on sensorimotor processing

Little is known about the claustrum compared to other brain regions, and it remains one of the last brain structures without a clearly defined function (Crick and Koch, 2004). However, given the presence of the claustrum in nearly every mammalian species and its innervation of potentially every cortical area, understanding the claustrum may have a profound impact on our understanding of mammalian cortical processes (Edelstein and Denaro, 2007; Kowianski et al., 1999). Most studies on the claustrum to date have been in rodents and cats, making it hard to
draw conclusions as to whether the claustral circuits described above are present in humans and primates. As a result, the interhemispheric claustral circuit reported here does not yet strengthen the rodent-primate homology argument, but instead identifies testable hypotheses to study in primates to better understand the claustrum. Given that primates do not have whiskers, determining the presence of an interhemispheric claustral circuit involved in a bilaterally coordinated behavior in primates is best tested by tracing the connections of the frontal eye fields and supplementary eye fields. Saccadic movements of the eyes are a bilaterally coordinated behavior that may reflect interhemispheric forebrain circuitry similar to the MI whisker circuitry described in our experiments. This possibility is supported by tracing studies that have shown the frontal eye fields receive dense innervation from the ventral, visual zone of the claustrum in macaque monkeys (Tanne-Gariepy et al., 2002).

Characterizing the complete connections of the claustrum is the first step in identifying its function. Care must be taken in choosing the model species for these studies. As discussed previously, evidence from tracing studies indicates that the primary visual cortex in primates and rodents does not project to the claustrum, though this projection has been shown in cats (Sherk, 1986; Carey and Neal, 1985; Olson and Graybiel, 1980). Our data further expand this principle to the somatosensory system, showing that primary somatosensory cortex does not project to the claustrum in rats. These species differences may reflect phylogenetic proximity. Rats and primates belong to the same superorder, but are separate from cats (Murphy et al., 2004), suggesting that rodents are a more suitable model for understanding primate claustral circuitry.

In addition to its apparent role in interhemispheric communication, the experiments outlined in Chapter 3 suggest a role for the claustrum in sensorimotor coordination. Topography of cortical projections from the claustrum has been demonstrated in rats and primates (see Edelstein and Denaro, 2007 for review). In general, the dorsal claustrum projects to sensorimotor cortex and the ventral claustrum projects to auditory and visual cortices. However, upon closer
inspection of the literature on the claustrocortical projections in primates, the ventral claustrum (the visual region) has been shown to innervate the frontal eye fields (Tanne-Gariepy et al., 2002). This evidence suggests that the organization of claustral connections could be to facilitate sensorimotor coordination by modality, wherein the dorsal claustrum coordinates somatosensory cortex and motor areas of the skeletal muscles and the ventral claustrum coordinates visual cortex with motor areas that control the extraocular muscles. This organization, however, remains to be functionally demonstrated, but may yield strong clues as to the function of the claustrum.

Another surprising finding from these experiments is that the claustrum in rats receives strong input from frontal cortex, but not from sensory cortical areas. The emerging theme from Chapter 3 is that the claustrum is driven by frontal cortex, but then projects to both sensory and motor cortex. In addition to projections from AGm, our retrograde tracer injection into the claustrum revealed labeled neurons in prefrontal cortex (PFC), indicating this structure also provides a major source of input to the claustrum. Given evidence that frontal cortical areas like AGm and PFC are involved in directed attention and goal-oriented behavior (Reep and Corwin 2009; Gabbot et al., 2005), projections to the claustrum from these areas could gain access to vast regions of cortex via the extensive claustrocortical connectivity. Projections from these frontal cortical regions mediating attention could explain the increased neuronal response in the claustrum during auditory and visual stimulation, given the absence of direct projections from sensory cortices (Remedios et al., 2010). However, more tracing studies in rats and primates are needed to verify whether corticoclaustral projections are present from higher order cortical areas, such as PPC, by which sensory information may reach the claustrum. Furthermore, in light of the longitudinal projections throughout the rostrocaudal extent of the claustrum (Smith and Alloway, 2010), an enticing hypothesis emerges wherein the claustrum serves to synchronize multiple modalities across vast regions of cortex and is driven by the executive areas of frontal cortex.
Such connectivity may be crucial in the neural basis of consciousness that Crick and Koch were hoping to find in the claustrum.

5.2 Subdivisions of motor cortex in rodents

5.2.1 Separate sensory-input and motor-output regions

One of the main findings of Chapter 4 is the apparent organization of a sensory-input and a motor-output region in the whisker representation of motor cortex. A summary of these findings is illustrated in Figure 5-1 below. In brief, inputs from the whisker representation of SI and SII terminate in the AGm-AGl transition region (TR), the same region in which neural responses to whisker deflections were observed. In contrast, the region more medially in AGm-proper showed no responses to whisker stimulation and received input from PPC, a higher-order sensory region. Finally, ICMS in AGm elicited robust movements of multiple whiskers and a strong EMG response. ICMS in the sensory input region, however, required higher currents to evoke whisker movements, which occurred at a longer latency and were characterized by a smaller amplitude in the EMG response. The discovery of whisker motor cortex parcellation was quite surprising because no study previously had suggested subregions in the whisker motor cortex, beyond the distinction in motor output between the retraction and rhythmic whisking areas (Haiss and Schwarz, 2005). Furthermore, there was no a priori reason to assume that the sensory and motor functions would be in separate cortical compartments. The functional subdivisions of motor cortex in rodents is exciting due to its implication of a shared trait with primates regarding multiple motor areas.
Figure 5-1. Summary of findings identifying multiple subregions within the whisker representation of motor cortex. Our tracing and physiology data indicate the whisker motor area is divided into sensory-input and motor-output zones. The sensory-input zone (blue region) located in the transition region (TR) between AGm and AGl, displays neural responses to whisker deflections driven by cortical inputs from SI and SII. ICMS in this region requires high threshold currents. The motor-output zone (red region) located in AGm-proper, does not respond to whisker deflections. ICMS in this region easily evokes whisker movements at low stimulation thresholds.

Historically, few studies have been published on somatosensory evoked neuronal responses in the motor cortex of rodents in vivo (Farkas et al., 1999; Chakrabarti et al., 2008). One of the striking observations that led to the experiments in Chapter 4 was an apparent disparity between ICMS maps and sensory responsive sites in MI when comparing previous literature. In studies of motor output, the location of MI neurons which evoked whisker movements at the lowest ICMS thresholds were all located in AGm (Brecht et al., 2004). Conversely, the location of electrode tracks where whisker evoked sensory responses were recorded in MI seemed to be located more lateral, in the AGm-AGl transition zone or even AGl itself (Chakrabarti et al., 2008). This dichotomy between ICMS sites and sensory responsive sites was further supported by tracing studies from SI barrel cortex that also seemed to terminate much
further lateral than the lowest threshold ICMS sites (Hoffer et al., 2003; Alloway et al., 2009; Smith et al., 2010). In light of data showing that the sensory response in MI is derived mainly from SI (Chakrabarti et al., 2008), we expected that the best recordings would be located in the SI projection region. The dual recordings in motor cortex shown in Chapter 4 quite clearly demonstrate that the sensory evoked activity does in fact correspond to the SI/SII input region in the AGm-AGl transition region. However, this region was adjacent to the best sites for eliciting ICMS evoked movements, indicating separated sensory-input and motor-output regions.

This finding however still does not address what type of sensory information is being processed in AGm proper. Our tracing studies show that PPC, a multimodal cortical area, has dense projections to this region. However, retrograde tracing studies also suggest that other cortical areas such as retrosplenial, entorhinal, PV, PR and PFC also project to AGm (Colechio and Alloway, 2009), suggesting that AGm may be a higher order motor cortical area in which spatial, memory, and executive functions converge to mediate the attentional directivity of whisking and orienting. This notion is supported by recent papers recording from this region that report no clear relationship to whisker sensory processing but clearly demonstrate memory guided orienting and value based decision making (Erlich et al., 2011; Sul et al., 2011). Furthermore, our data suggest that the cortical topography changes very abruptly in this region and care must be taken to know precise electrode location both in a mediolateral and rostrocaudal dimension. The projections from SI and SII were found to occupy the entirety of the rostrocaudal extent of the transition region, however, the PPC was only found to target the caudal areas of AGm. This leaves the inputs to rostral AGm as yet not specifically characterized, and may represent another cortical module. Such connectivity is intriguing and could demonstrate more divisions within the whisker representation of motor cortex but require further experimentation to be verified.

The other finding from Chapter 4 is that AGm proper was the site demonstrating the strongest motor output. In the first study characterizing AGm and AGl, Donoghue and Wise
suggest that AGm is the secondary motor cortex due to the much higher IMCS thresholds necessary to evoke movements (Donoghue and Wise, 1982). However, ICMS studies in multiple labs have now demonstrated ICMS in AGm to be very effective at eliciting whisker movements and contend that this area is the face representation of MI (Brecht et al., 2004; Alloway et al., 2008; 2009; 2010; Smith and Alloway, 2010; Tennant et al., 2011). Furthermore, our results now show that AGm is in fact the optimal location for eliciting movements compared to sites in the transition zone closer to AGl; closer to what Donoghue calls MI. This discrepancy in ICMS data has multiple plausible explanations.

Most notably, ICMS is strongly altered by depth of anesthesia (Tandon et al, 2008), and so the lack of evoked responses in AGm by Donoghue may reflect his animals being deeply anesthetized. Oligosynaptic projections like AGm to the facial nucleus likely make ICMS experiments more sensitive to anesthesia, whereas corticospinal projections from the forelimb area may still elicit movements even when the animal is deep. However, when the animal is light, the pathways for eliciting whisker movements may become disinhibited, changing their electrical excitability. Specifically, the use of ketamine as an anesthetic blocks glutamatergic transmission, which is likely diminishing the effectiveness of ICMS until the drug has been metabolized enough that the animal is lightly anesthetized such that microstimulation is maximally effective. This explanation is supported by review of the figures in Donoghue and Wise's paper that reveal almost no whisker evoked sites, similar to the ICMS maps of Tandon et al under deep anesthesia. Anesthetic state and ICMS parameters (such as frequency and duration of ICMS pulses) vary across studies, but are crucial for interpreting ICMS results and thus present a highly plausible explanation for discrepancies (Young et al., 2011). Furthermore, the use ICMS thresholds as a determinant for identifying primary and higher-order motor areas has recently been refuted in light of both the anatomical finding that primary motor, premotor, and supplementary motor areas contain corticospinal neurons and the lowest threshold current for ICMS evoked movements is
the same in each of these motor areas (Dum and Strick, 2002). Thus, ICMS thresholds alone are not a useful criteria for solely defining functional subregions.

Our conclusion that AGm is the motor output area is also supported by multiple anatomical findings. AGm has an expanded and dense layer V compared to AGl and the transition region, owing to the presence of more corticofugal neurons. Furthermore, AGm has a substantially increased amount of myelin compared to AGl and the transition zone, which should increase axonal conductance to facilitate motor output, especially via ICMS. Our tracing studies have also shown the existence of a larger amount of corticotectal neurons in AGm, the main output target of MI in the control of whisking (Alloway et al., 2010). The efficacy of these projections are likely enhanced by the increased myelination. The anatomical efferent argument, however, remains to be further validated by retrograde tracing studies from other brainstem regions mediating motor cortex control of whisking (see Figure 1-3) or neuronal recordings of AGm and the transition zone in awake, behaving animals.

The organization in the rat whisker motor cortex of a sensory input and motor output is not unique to rats. Evidence in the primate frontal eye fields (FEF) indicates a similar organization. Electrophysiological experiments in the primate FEF testing for to visual stimuli induced activation and ability of ICMS to evoke saccades revealed subtypes of neuronal responses (Bruce et al., 1989). This study identified three basic classifications of neuronal responses: visual (increase firing to visual stimulation), visuomovement (increase firing before a saccade), and movement (increase firing during a saccade) responders. Low threshold microstimulation (<50µA) of visuomovement and movement neurons elicited saccadic movement of the eyes. On the contrary, visual responsive neurons required much higher thresholds (>150 µA) to elicit a saccade. This response pattern matches our findings in the whisker system, where the frontal cortex representation is divided into sensory-input and motor-output zones.
5.2.2 Higher order motor regions in rodents.

The existence of multiple (higher-order) motor areas in rats has been addressed by a number of different approaches: cytoarchitecture, connectivity, and physiology. The work by Donoghue used cytoarchitecture and ICMS to suggest that AGm is secondary motor cortex compared to primary motor cortex in AGl, and this has been the most cited work for defining these regions in articles and brain atlases (Donoghue 1982; Paxinos and Watson, 2007; Sul et al., 2011). Other work has suggested AGm is secondary motor cortex based on the presence of a rostral forelimb area in rats (Neafsey and Sievert, 1982; Tandon et al., 2008) and mice (Tennant et al., 2011). This additional forelimb area in rostral AGm (rFA), anterior to the whisker representation, has been characterized by the presence of corticospinal projections that exist in a separate clustering away from the caudal MI forelimb region (cFA) in AGl (Nudo and Masterson, 1990). The rFA has anatomical connections consistent with the whisker representation in AGm, in terms of forebrain projections to the striatum and thalamus. Specifically, rFA has been shown to have dense projections to nucleus VM in the thalamus (the basal ganglia output nucleus) and the contralateral striatum, whereas cFA targets nucleus VL in the thalamus (the cerebro-cerebellar output nucleus) and favors the ipsilateral striatum (Rouiller et al., 1993; Wang and Kurata, 1998). These authors conclude that these findings demonstrate AGm and AGl to be homologous to premotor cortex and primary motor cortex, respectively, in primates.

In addition, rFA and cFA have been differentiated by cortico-cortico connections. Injections into either region has shown that they are reciprocally innervated (Rouiller et al., 1993). Furthermore, retrograde injections in the SI-forelimb region results in labeling immediately adjacent to the injection site, presumably in AGl (cFA), and an additional cluster (rFA) in rostral AGm (Fabri and Burton, 1991). However, no study has traced the connections between the forelimb representation of SI/SII and PPC to motor cortex to see whether these
projections diverge like the projections from whisker SI/SII and PPC do in the whisker representation.

These studies demonstrating two forelimb areas, in addition to our division of the whisker representation, both support the presence of multiple motor areas in rats. However, these regions may not be precisely analogous to primate designations of primary motor, premotor, and/or supplementary motor. One obvious discrepancy is whether or not the different motor whisker fields are akin to the primary motor and premotor organization of the limbs or instead are more analogous to the frontal eye fields. ICMS data in primates shows similar thresholds for MI and PMA of the limbs (Dum and Strick, 2002), but the FEF has been shown to be organized into sensory-input (high ICMS thresholds) and motor-output (low ICMS thresholds) zones within the region itself as discussed above (Bruce et al., 1989). This suggests that the whisker representation in motor cortex may be organized in a manner more similar to the FEF in primates, however, a safer conclusion is to describe these regions according to species specific terminology. Ultimately, the evidence in rodents clearly demonstrate parcellation into multiple functional regions for the forelimb and whiskers in motor cortex, and perhaps that is the extent of the homology. Further studies on the precise input and output connectivity, in addition to neuronal recordings in awake, behaving animals will be necessary to further elucidate differences in the motor processing in each region and determine how similar these areas are to the primate motor areas.

A fundamental question regarding the organization of these different motor areas in rodents is their body topography and the potential for a heuristic regarding the spatial arrangement of the inputs coming from sensory cortex. Our data suggest a different organization than that suggested by Fabri and Burton. These two schemes of cortical topography across sensory and motor regions are demonstrated in Figure 5-2. A general organizational theme of body topography between primary and higher order cortical regions is the observation that they
relate in mirror images. This mirror image flipping of the body can also be found in the organization of corticothalamic projections to primary and higher order thalamic nuclei (Hoover et al., 2003; their Figure 4). As shown in panel A of Figure 5-2, the body representation in cortex flips going from one sensory area to the next. For example, SI and SII are a mirror image along the lateral edge of SI. Fabri and Burton suggest that a similar organization is used in the organization of primary and secondary motor cortex, where in the body map in MI flips along an axis so that MII is located more rostrally. However, our tracing data from the whisker representations in SI, SII, and PPC suggest that all of these sensory areas may have flipped together to create different terminal input zones in the rather large whisker area of motor cortex (Figure 5-2, panel B). Tracing other body representations from these regions, as well as looking at how projections of PV and PR terminate in MI, may provide a heuristic on the organization of mammalian sensorimotor cortico-cortical projections. Genetic and development studies would also be helpful in understanding these mechanisms of cortical organization.

**Figure 5-2.** Illustrations depicting different schemes of cortical map flipping. **A:** A previous theory describing how cortical maps are organized across sensory and motor cortical areas, where MII is represented as a mirror image of the map in MI (Source: Fabri and Burton, 1991). **B:** Depiction of the results from Chapter 4 tracing the projections from the whisker representations in SI, SII, and PPC indicate that these sensory regions have all flipped together to create the motor cortex map.
Our data suggest that the cortico-cortical projections from SI, SII, and PPC are helpful indicators in the divisions of the motor whisker region. Along with the other evidence discussed above, there seems to be multiple subregions within the whisker motor representation. However, care must be taken in labeling these areas as being homologous to primates. Variation in cortical organization correlates with perceptual and behavioral abilities, and have thus evolved based on each species specialized needs (Krubitzer and Hunt, 2007). Ultimately the whisker motor area is unique to rodents (and certain other whisking mammals) that has been specialized for whisking behavior, a behavior not present in primates. Thus, while it may have qualities analogous to the premotor and supplementary motor areas, or the frontal eye fields, these may not be exactly homologous. What may be said to be homologous is the general principle that the motor cortex in both rodents and primates are parcellated into multiple subregions. Ultimately, the whisker region of motor cortex has evolved to meet the demands of a rapid, bilaterally coordinated, exploratory behavior and should be studied and understood under its own evolutionary microscope.
References


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