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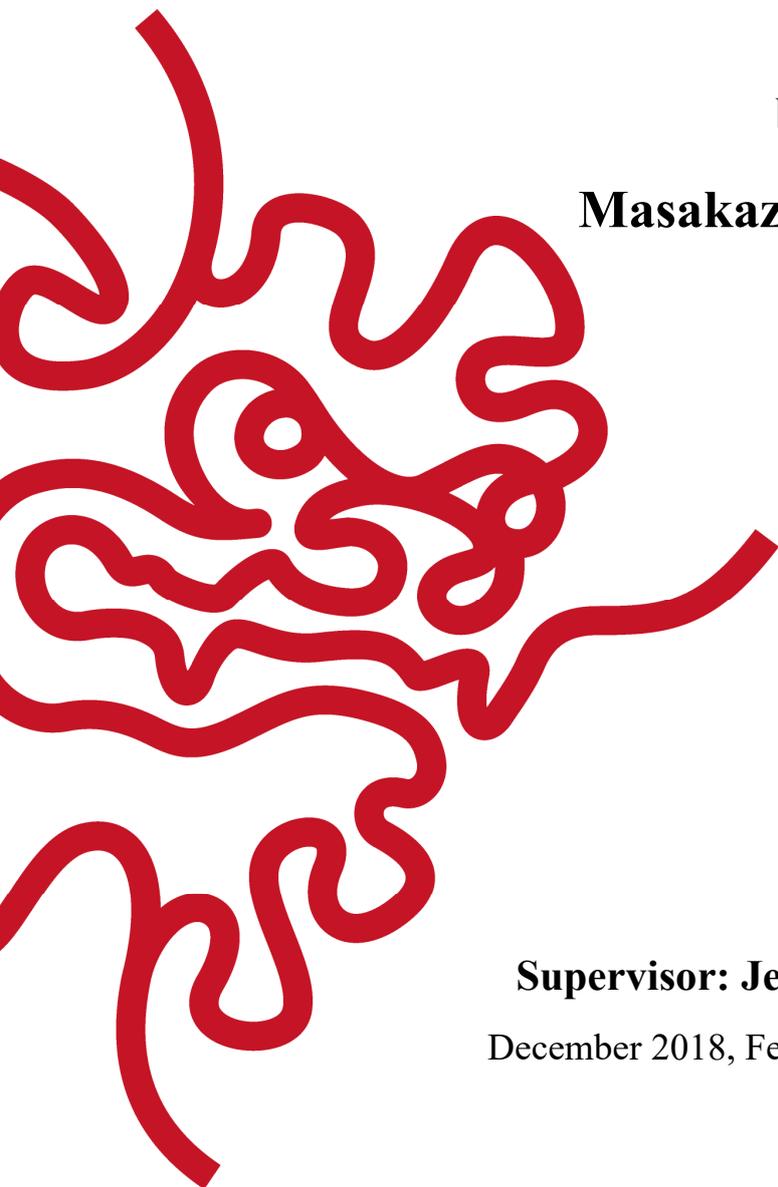
Thesis submitted for the degree

Doctor of Philosophy

**The role of interhemispheric cortico-cortical
connections in bimanual coordination in the rat**

by

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December 2018, February 2019 (revised)

Declaration of Original and Sole Authorship

I, Masakazu Igarashi, declare that this thesis entitled “The role of interhemispheric cortico-cortical connections in bimanual coordination in the rat” and the data presented in it are original and my own work.

I confirm that:

- No part of this work has previously been submitted for a degree at this or any other university.
- References to the work of others have been clearly acknowledged. Quotations from the work of others have been clearly indicated, and attributed to them.
- In cases where others have contributed to part of this work, such contribution has been clearly acknowledged and distinguished from my own work.
- None of this work has been previously published elsewhere, with the exception of the following:

Igarashi, M., and Wickens, J. R. 2018. Kinematic analysis of bimanual movements during food handling by head-fixed rats. *Journal of Neurophysiology*. doi: 10.1152/jn.00295.2018

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Abstract

Title: The role of interhemispheric cortico-cortical connections in bimanual coordination in the rat

Bimanual coordination – in which both hands work together to achieve a goal – is crucial for basic needs of life, such as gathering and feeding. The mammalian body has a left and right side which is often symmetrically shaped, but raises the question of how does the brain organize two sides of our body in a coordinated manner. The overall aim of this thesis is to better-understand neural mechanism of bimanual coordination. Bimanual coordination is highly developed in primates, where it has been most extensively studied. Rodents also exhibit remarkable dexterity and coordination of forelimbs during food handling and consumption. However, rodents have been less commonly used in the study of bimanual coordination because of limited quantitative measuring techniques. To study the neural mechanism of bimanual coordination using rodents, therefore, first requires a method to measure and classify bimanual movements.

In this thesis, I propose a high-resolution tracking system that enables kinematic analysis of rat forelimb movements. The system quantifies forelimb movements bilaterally in head-fixed rats during food handling and consumption. Forelimb movements occurring naturally during feeding were encoded as continuous 3-D trajectories. The trajectories were then automatically segmented and analyzed, using a novel algorithm, according to the laterality of movement speed or the asymmetry of movement direction across the forelimbs. Bilateral forelimb movements were frequently observed during spontaneous food handling. Both symmetry and asymmetry in movement direction were observed, with symmetric bilateral movements quantitatively more common.

Using the proposed method, I further investigated a key hypothesis that the corpus callosum, the thickest commissure connecting two cerebral cortices, mediates bimanual movements. I performed pharmacological blockade of the anterior corpus callosum (aCC) in which commissures from cortical forelimb motor areas are reciprocally connected. The kinematic analysis of bimanual coordination during food handling revealed that the frequency of occurrence of symmetric bimanual movements was reduced by aCC inhibition. In counterpart, asymmetric bimanual movements were increased. Other global scales of motor skills, such as mean food drop rate, and mean consumption time remained unchanged. Bilateral multiunit recordings from corresponding cortical areas showed positively correlated activity patterns in the large majority of interacting pairs. The present study also found that the putative excitatory neurons were also positively correlated with putative inhibitory neurons in the opposite hemisphere, suggesting interhemispheric inhibition via inhibitory neurons. Collectively, these results suggest that the symmetric bimanual movements in rodents are modulated by the anterior corpus callosum via both excitatory and inhibitory connections of two motor cortices.

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LIST OF ABBREVIATIONS

A	anterior
aCC	anterior corpus callosum
AP	anterior-posterior
BG	basal ganglia
CC	corpus callosum
CFA	caudal forelimb area
CSD	current source density
D	dorsal
DV	dorsal-ventral
IT	intratelencephalic
IT-CC	intratelencephalic-cortico-cortical
IT-CS	intratelencephalic-cortico-striatal
L	left
LFP	local field potential
LR	left-right
M1	primary motor cortex
M2	secondary motor cortex
P	posterior
PCM	precentral motor cortex
PM	premotor cortex
R	right
RFA	rostral forelimb area
SMA	supplementary motor area
V	ventral

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Chapter 1. Introduction and Statement of the Problem

1.1. Introduction

Behind a simple movement, such as grasping a coffee mug, the activities of more than 40 muscles, from the hand to the shoulder, are orchestrated into a seamless movement. In bimanual movements, such as threading a needle, there is a need to monitor both hands simultaneously, to issue motor commands each hand based on what the other hand is doing. Since the movement of each hand is represented in a different hemisphere, such bimanual coordination requires information exchange between the two sides of the brain. Studying the neural mechanism of bimanual movements thus has the potential to lead to a better understanding of how the two hemispheres communicate. Although it is obvious that bilateral motor control is computationally more expensive than unimanual control, our daily behavior is full of bilaterally coordinated movements, that are often processed in a subconscious manner. Bimanual movements are twice as frequent as unimanual movement in daily life (Vega-González and Granat 2005). Thus, malfunction of bimanual coordination has a significant impact on our wellbeing. Therefore, understanding the neural mechanism of bimanual coordination is necessary not only for the fundamental understanding of the principles of how information exchange takes place to achieve interlimb coordination, but also for understanding common movement disorders, their neuropathological mechanisms, and treatment.

1.2. Statement of the Problem

The overall aim of this thesis is to better-understand the neural mechanism of bimanual coordination. The mammalian body has a left and right side which raises the question of how the brain organizes the two sides of the body in a coordinated manner. Bimanual coordination has been extensively studied, but most studies have involved human or non-human primates. Relatively few studies have been done in rodents, where more invasive and controlled

experimental manipulations are more readily available. In part, this is because of their small size and rapid movement makes rodent bimanual coordination difficult to observe and measure. There is, therefore, a need to establish methods for precisely and efficiently measuring and classifying bilateral movements in rodents. The specific aims of the thesis research are therefore to develop new methods for observation and analysis of bilateral movements in rodents, and to apply these methods in the experimental study of the neural substrates of bimanual coordination. The research focused on corpus callosum, the thickest commissure connecting the two cerebral cortices, and presumably providing interhemispheric information exchange for bimanual movements.

1.3 Outline of the Thesis

In this thesis, Chapter 2 reviews the conceptual framework for the study of bimanual coordination as developed in humans, and experimental studies in non-human-primates and rodents. The neural substrates of bimanual coordination are then described. Chapter 3 reports my preliminary studies of kinematic recording in free-moving rats, conducted with the aim of establishing a recording system for kinematic analysis and evaluating candidate behavioral tasks for studying bimanual coordination. In Chapter 4, I report kinematic analysis of bimanual coordination in head-fixed rats, applying this to quantifying and classifying bilateral forelimb movements during food handling. In Chapter 5, using the recording system and analytical pipeline of bimanual coordination developed in the thesis research, I report on the role of the corpus callosum in rodents' bimanual coordination. Finally, I report preliminary findings from electrophysiology related to the inter-hemispheric cellular network in bilateral coordination of the limbs.

Chapter 2: Review of the Literature

Impairment in the performance of bimanual tasks has been reported after motor cortical lesions, disconnection of the corpus callosum, and cerebral stroke in human and monkey. Exclusively bimanual movement-related neurons have been found in the supplementary motor area, the primary motor area, and basal ganglia (Donchin et al. 1998; Wannier et al. 2002). These pieces of evidence suggest that multiple brain structures are involved in bimanual movements. Among these, the corpus callosum provides an intratelencephalic network connecting cortical and subcortical structures, suggesting this thick fiber bundle might be a key structure in bimanual coordination. In contrast to the corpus of knowledge obtained from research in primates, there have been relatively few studies in rodents, despite recent advances in the neuroanatomy of rodent and the explosion of powerful new tools to manipulate those circuits.

In this review, I first define a conceptual framework of bimanual coordination investigated by work with humans as experimental subjects, followed by experimental work using non-human primates and rodents. In later sections, the neural substrate of bimanual coordination will be discussed. Finally, I will introduce a possible experimental approach for studying the role of the corpus callosum using the rodent as an animal model.

2.1. The concept of bimanual coordination and its experimental study

Much of the current conceptual understanding of bimanual coordination derives from human studies correlating clinical and pathological evidence. Humans have particularly advanced capabilities for bimanual coordination, which is sensitive to impairment of the CNS. Many theories have been proposed to understand the nature of bimanual coordination and its underlying neurophysiological substrates. Of particular interest, Kelso et al. (1979), reported that bimanual movement does not follow Fitts's law. Fitts's law predicts that the distance and accuracy of the target predict the time of movement completion; however, the bimanual Fitts's

pointing showed that performing different tasks with each limb caused interference. Movement symmetry versus asymmetry is a pivotal and perhaps most of commonly used conceptual framework. The broadest definition of the classification (symmetry vs asymmetry) is based on tasks assigned to each limb simultaneously. Symmetric bimanual coordination means the condition where identical two tasks are assigned to each limb, for instance, drawing circles with each hand. In contrast, asymmetric bimanual coordination requires a different task to be performed with each limb, for example, simultaneously drawing a rectangle with one hand and a circle with the other.

2.1.1. Physiological difference behind bimanual symmetry and asymmetry.

Symmetry versus asymmetry has been classified by patterns of muscle simultaneously activated across two limbs. Symmetric bimanual movement is generated by simultaneous activation of homologous muscle group in the limbs and hands of each side. For instance, rowing a boat with two oars in synchrony involves symmetric bimanual coordination. The simultaneous activation of homologous muscle groups often results in identical movement direction of hands with respect to the sagittal plane such that one arm moves like the mirror image of the other arm if there is no physical obstacle. In contrast, asymmetric bimanual movements are generated by different muscle groups activated simultaneously. Asymmetric movements are seen everywhere in everyday behaviors such as cutting food, when the knife is held in one hand while the food is steadied in the other, or in sporting activity such as serving a ball by swinging a racket with one hand while tossing a ball in the air with the other hand. Such activation of different muscle group often results in different movement directions of the fingers and hands with respect to the sagittal plane. It is well known that symmetric bimanual coordination is intuitive, easy and stable to perform, whereas, instability of asymmetric bimanual coordination has been reported in many experimental paradigms. It has been thought that there is *basic synchronization*

tendency underlying the difference between symmetric and asymmetric bimanual coordination (Swinnen 2002).

2.1.2. Synchronization tendency in many experimental paradigms

Bimanual coordination in symmetric versus asymmetric conditions has distinct features related to stability and difficulty. Symmetric bimanual coordination is more stable and easier to perform than asymmetric action. When two hands are assigned asymmetric motor patterns, the form of hand movements tends to be naturally assimilated toward symmetric forms, especially when higher-speed is required. This assimilation to symmetric forms can be observed in different motor parameters such as timing and space. For example, the synchronization of the timing of action was reported in the Fitt's bimanual pointing task (Kelso et al. 1979). The subjects were required to point at wide and narrow targets placed 24 cm lateral from the center home keys, and move index fingers to the target bilaterally as fast and accurately as possible. The conventional Fitt's law predicts that the wider (easy) target takes a shorter time in preparation, while narrower (difficult) target should take longer time. However, the researchers found that the hand moving to the "easy" target took longer preparation time assimilated to the timing of another hand pointing "difficult" target, showing synchrony in movement velocity and acceleration between two hands. The result suggested that the motor programming of bimanual control is not a linear sum of programming unimanual movements. Another example of timing synchrony can be seen in rhythmic bimanual finger tapping. It has been reported that in-phase mode (simultaneous finger tapping with no phase shift; $\emptyset = 0^\circ$) is more stable than anti-phase mode (tapping with alternation; $\emptyset = 180^\circ$) and out of phase mode (tapping with; $\emptyset = 90^\circ$) (Schoner and Kelso 1988; Yamanishi et al. 1980; Zanone and Kelso 1992). When performing bimanual finger tapping in either in-phase or out-of-phase modes, the shifted phase

of the finger tap shows a natural tendency toward the matched in-phase mode, as tapping speed increases.

The assimilation to symmetry is also evident in space. Kelso et al. (1983) discovered another interesting symmetric assimilation by introducing spatial obstacles into the Fitts' bimanual pointing task. They placed a hurdle in the middle of the movement path of one hand, while the other hand was not hurdled. Interestingly, the *unhurdled* limb showed an upward trajectory as if it was avoiding an obstacle (which did not exist), showing a similar trajectory to the *hurdled* hand. This discovery indicates that the spatial property of a hand movement influences the opposite hand when both hands are performing simultaneously. Spatial assimilation was also found in different discrete bimanual tasks (Franz et al. 1996; Swinnen et al. 1988). Spatial assimilation was also reported in a bimanual circle and line drawing task (Franz et al. 1991; Swinnen et al. 1998). In the case of Franz et al. (1991), when one hand drew lines and the other hand drew circles, line drawing became more circle like and *vice versa*. Swinnen et al. (1998) particularly demonstrated that the preference of performing symmetric action in space is stronger in egocentric constraint where symmetry of form/direction are defined by muscle group than allocentric constraint where symmetry of space is defined by Euclidian space.

2.2 Bimanual coordination in non-human primates

The experimental paradigm of bimanual coordination used for human subject largely relies on complex visual cues and self-pacing actions indicated by a computer screen or experimenter's guidance; therefore, not all subhuman species are able to learn those sophisticated behavioral tasks. There are, however, non-human primates such as monkeys which have been used because of their advanced capability in performing complex bimanual tasks compared to other subhuman mammalian species. More invasive experimental studies such as callosotomy,

cortical lesion, and chronic electrophysiological recordings have been used for non-human primates.

Mark and Sperry (1968) developed a bimanual coordination task to test motor skill of split-brain monkeys. The bimanual coordination task apparatus consists of a transparent acrylic plate with holes horizontally placed in front of the subject (Fig. 2-1). The task is to retrieve food rewards from the holes. Food is put in the holes, and subjects have to coordinate their two hands to retrieve reward. This requires pushing a reward from one side, using an index finger and catching the reward in the opposite hand from underneath the transparent plate. Thus, this task requires asymmetric bimanual coordination. They found that the dissection of forebrain commissure (including optic chiasm, corpus callosum, hippocampal commissure) disturbed task performance, especially when working blind. Interestingly, extensive loss of the midbrain commissure and the cerebellum midline part significantly decreased task performance transiently, but after many training sessions, the coordination slowly improved and became normal even without visual guidance. Brinkman (1981; 1984) used a similar task to test the role of the supplementary motor area (SMA) in bimanual coordination by SMA lesion. They found that the SMA lesion developed mirror movements, which two hands tended to move similar form. Interestingly, the subsequent callosal dissection immediately attenuated this motor symptom, suggesting that the SMA may desynchronize duplicated motor commands conveyed through the corpus callosum. This task has made important contributions to understanding the neural substrates of bimanual coordination.

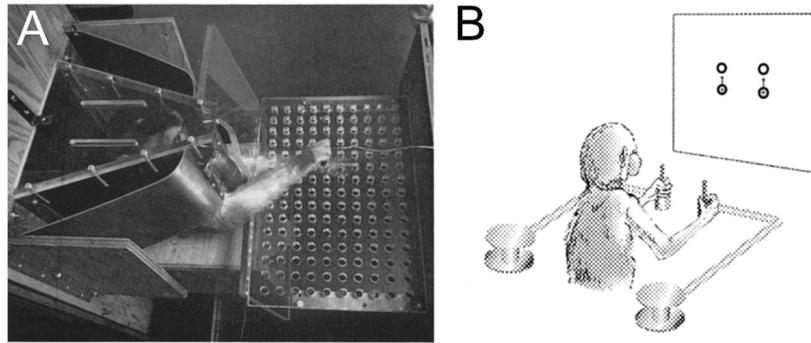


Figure 2-1. Representative bimanual tasks for monkey.

(A) Bimanual coordination task proposed by Mark and Sperry (1968). Right index finger of the monkey pushes food in the hole, while the other hand catches the food. The picture is from Mark and Sperry (1968), reproduced with permission from Elsevier. (B) X-Y levers for bimanual movements. The monkey sits in a chair in front of the display and holds levers to move the cursors on the screen toward eight directions (Cardoso de Oliveira et al. 2001). The figure is reproduced with the permission of John Wiley & Sons.

A bimanual drawer task was proposed by Kazennikov et al. (1998). The goal of this task is to retrieve a reward from a drawer. The drawer remains closed by a spring. The subject should keep the drawer open while the other hand picks the object. The asymmetric contribution is required in the task. They found that the bilateral lesion of mesial frontal cortex (SMA, pre-SMA, and cingulate cortex) significantly delayed movement onset and slowed reaching. The bimanual drawer task was used for isolating neurons that were exclusively modulated during bimanual action, which reportedly exist in SMA, primary motor cortex (PM), and basal ganglia (striatum, GPe, and GPi) (Kazennikov et al. 1999; Wannier et al. 2002).

Bimanual exclusive modulation of neural activity was reported using bimanual key-press task (Tanji et al. 1987). Two independent keys press provided a dissociation between unilateral and bimanual movements. They found neurons which exclusively discharge during bilateral key presses in precentral (PCM), premotor (PM), and supplementary motor cortex (SMC) of the

monkey (Tanji et al. 1988; 1987). Vaadia and his colleagues used 2-D lever pressing to study neural correlates of bimanual coordination (Cardoso de Oliveira et al. 2001; Donchin et al. 1998; Donchin et al. 2002; Steinberg et al. 2002). In this task subjects control two independent cursors on the screen by operating X-Y levers bimanually (Fig. 2-1B). The levers control two cursors and the task is to move them in the direction of markers on the screen. Since the bimanual lever task can test a variety of combination of movement direction bilaterally, study of neural correlates in the common framework of bimanual coordination in human, namely movement symmetry and asymmetry, become possible.

The studies in non-human primates have provided perspective on how brains communicate to coordinate two hands, based on the controlled lesion and electrophysiological recordings. However, the study of bimanual coordination in monkeys has been reported relatively less often in the past decade, although there is still a need for invasive approaches to identify neural substrates and cellular mechanism of bimanual coordination. Other animal models need to be explored, where invasive procedures are more readily and practically available. In the next section, I will review the rodent, the most commonly used mammalian animal model of biological study, as a potential candidate for the mammalian model of bimanual coordination.

2.3 Bimanual tasks in rodents

The neurophysiological mechanisms of motor control have been studied using rodents with well-established behavioral tests for unimanual motor control such as skilled reaching test and rotarod test. However, rodents have not been extensively used as an animal model to study bimanual coordination. In this section, I introduce behavioral tasks which are readily or potentially applicable for studying bimanual coordination of rodents.

Whishaw and Coles (1996) reported food manipulation behavior of rats as a measure of forelimb skill, and demonstrated bimanual coordination in manipulation of several types of food items, such as pasta, peanuts, and grapes. The behavioral test was further developed to the vermicelli handling test (Allred et al. 2008; Tennant et al. 2010; Whishaw et al. 2017b). In the test, vermicelli handling behavior with bilateral hands was scored on several parameters, such as a number of adjustments with hands and time to eat. The unilateral somatosensory lesion, cooling deactivation of secondary motor areas, and striatal dopamine lesion decreases the number of forelimb adjustments contralateral to the lesion (Allred et al. 2008; Brown and Teskey 2014; Whishaw and Coles 1996), suggesting the involvement of these brain areas in motor coordination in feeding behavior. The cortical involvement in these studies suggests that food manipulation behavior has advantages for investigating cortical role in bimanual coordination.

The cylinder test was developed by Schallert and Lindner (1990) and has been widely used for evaluation of motor performance (Gharbawie et al. 2004; Lundblad et al. 2002; Schallert et al. 2000; Schallert and Lindner 1990; Shi et al. 2004). In this test, rodents are placed in a clear acrylic cylinder and spontaneous vertical exploratory behavior is observed. Rats demonstrate a variety of wall touches during exploration in the cylinder, showing one-handed touch and ambidextrous touches with two hands. The test has been used for measuring the motor activity of rodents. Igarashi et al. (2015) suggest that the bilateral forelimb use in cylinder test is not a simple linear sum of unilateral movements, therefore, the test may illuminate bimanually exclusive neural mechanisms. However, it remains unclear how wall touch requires coordination across two forelimbs. Further work is required to develop the cylinder test as a measure of bimanual coordination.

Berridge and his colleague quantified grooming to identify neural substrate of motor sequences in the grooming chain (Berridge 1989). Rodent self-grooming exhibits a variety of stereotypical motor acts including bimanual movements. The self-grooming sequence begins with elliptical bilateral stroke of vibrissae followed by more dynamic bilateral stroke to ears and eyes. The grooming sequence may provide a model of bimanual coordination of rodents because of highly reproducible bilateral forelimb movements. The neural substrates of lateral coordination in grooming chain is still remain unclear. However, decerebration and destriate studies suggest that the grooming motor sequence is encoded in subcortical structures especially striatum (Berridge and Whishaw 1992; Cromwell and Berridge 1996). Therefore, grooming could be a suitable model for bimanual coordination encoded in subcortical structures.

Recently, Soma et al. (2017) demonstrated the two lever-pressing task in head-fixed rats for studying lateralized neural representation across two sides of forelimb motor cortical areas. They studied ipsilateral presentation of neural activity in the caudal forelimb area (primary motor areas of rodents) and rostral forelimb area (secondary motor area of rodents). Although the task did not require pressing two levers simultaneously, rats were required to press the left and right levers alternately, as the rewarded lever changed. Although the current lever-pressing task requires improvements, the approach becomes a powerful tool when rodents learn symmetric and asymmetric bimanual coordination with 2-D levers.

2.4 Neural substrates of bimanual coordination

2.4.1 Motor Cortices

Cortical motor areas have been thought as one of the key neural substrates for bimanual coordination. Several studies suggested that supplementary motor areas and premotor area encode the motor command of bimanual coordination in human and monkey. As described in

foregoing paragraph, Brinkman (1981; 1984) found that monkeys receiving unilateral SMA lesions demonstrated a disability of bimanual task, and mirror movement was observed although the task requires asymmetrical contributions from both hands. In humans, mirror movements have been reported in patients who have had ischemic infarction in anterior cerebral cortices including SMA, medial prefrontal cortex, anterior cingulate gyrus (Chan and Ross 1988; Dick et al. 1986; Laplane et al. 1977). Therefore, it was thought that SMA and medial frontal cortices play an important role for *non-mirror transformation*, discharging symmetric mirror movements transformed into asymmetric (Chan and Ross 1988). In humans, increased SMA and PMd activity was greater during asymmetric bimanual than symmetric bimanual movements (Goerres et al. 1998; Sadato et al. 1997), and more recent study reported that increasing SMA excitability by transcranial direct current stimulation improved performance in anti-phase bimanual supination-pronation movement (Carter et al. 2015), and reinforcing the idea that SMA is involved in asymmetric bimanual movements.

Cellular correlates of bimanual movements have been investigated by *in vivo* electrophysiological recordings in monkeys (Donchin et al. 1998; Kazennikov et al. 1999; Tanji et al. 1987). Bimanual exclusive response of neural firing was reported in M1, PCM, PM, and SMA (Donchin et al. 1998; Tanji et al. 1987) suggesting these motor areas mediate bimanual movement. The population vector (PV) approach was also tested with a two-lever task (Steinberg et al. 2002). They found that the preferred movement directions of most of the recorded cells were well conserved in different movement combinations such as unimanual and bimanual symmetric and asymmetric movements. However, the firing rate was changed depending on the context of movements in some cells leaving preferred direction unchanged. These results suggest that motor cortical areas involve a unique cell population exclusively modulated during bimanual movements, in addition, the neural population encoding unimanual control was also affected when the opposite hand was assigned a task simultaneously.

However, some evidence does not fully support the role of these motor cortical areas in bimanual coordination. Kermadi et al. (1997) and Kazennikov et al. (1998) suggested that the role of SMA is not to modulate a movement pattern of bimanual coordination but is restricted to initiation of bimanual sequences. Kermadi et al. (1997) found that monkeys receiving muscimol microinjection in the SMA showed impaired initiation of bimanual sequential movements, leaving coordination of the two hands intact. Inhibition of human SMA by rTMS caused a temporal delay between two hands in drawer task, but gross noticeable errors were not observed, suggesting that the rostral part of the SMA plays a role in temporal coordination of bimanual movement but not coordination itself (Obhi et al. 2002). Puttemans et al. (2005) followed cortical and subcortical activity during the learning process of bimanual coordination task using fMRI. They found a gradual shift of brain activity from cortical to subcortical areas as subjects overlearned the task. This may explain the contradiction of cortical involvement in bimanual coordination, namely the motor cortical areas presented bimanual specific activity in forgoing literature might be crucial for unfamiliar/learning stage of bimanual coordination.

2.4.2 Basal ganglia and cerebellum

The basal ganglia have been thought to be another neural substrate of bimanual coordination. The idea is based on fMRI studies (Puttemans et al. 2005; Wu et al. 2010) and bimanual coordination deficits in Parkinson's disease (PD) (Johnson et al. 1998; Serrien et al. 2000; Swinnen et al. 1997) and Huntington's disease (Brown et al. 1993; Johnson et al. 2000; Verbessem et al. 2002). Cellular representation of bimanual movement was also discovered in monkeys. Wannier et al. (2002), who recorded unit activity during bimanual drawer task, found neural activities that discharged exclusively to bimanual movements in striatum, internal and external Globus pallidus (Wannier et al. 2002). Therefore, multiple nuclei in the basal ganglia circuit may contribute to bimanual movement. Interestingly, PD patients have difficulty in

performing asymmetric movement (Almeida et al. 2002; Brown et al. 1993; Johnson et al. 1998; Ponsen et al. 2006; Schwab et al. 1954; Serrien et al. 2000; Stelmach and Worringham 1988; van den Berg et al. 2000), and studies suggest that PD patients showed easy reversion from asymmetric anti-phase bimanual movement to symmetric in-phase movement (Johnson et al. 1998; van den Berg et al. 2000). It has been speculated that the deficits of bimanual movement of basal ganglia are due to malfunction of SMA (Cardoso de Oliveira 2002; Wu et al. 2010). Other than that, there is still a possibility that the basal ganglia directly contribute to bimanual movement from an anatomical point of view. The striatum, the input nucleus of the basal ganglia, receives massive input from contralateral cortical projections via the corpus callosum (Reiner et al. 2010; Wilson 1987). At the subcortical level, pedunculopontine nucleus (PPN) has projections to the subthalamic nucleus bilaterally in rat and monkey (Hammond et al. 1983; Jackson and Crossman 1983; Lavoie and Parent 1994). Therefore, the basal ganglia have a substantial lateral connection. The role of the subcortical interhemispheric connection via the basal ganglia is question of future research interest.

The cerebellum has also been a candidate neural substrate for bimanual coordination. fMRI studies suggest that the cerebellum is involved in bimanual coordination especially, vermis and hemisphere of cerebellum and deep cerebellar nucleus (Boisgontier et al. 2018; Puttemans et al. 2005; Soteropoulos and Baker 2007; Tracy et al. 2001). Boisgontier et al. (2018) suggested that the cerebellar lobule VI (vermis and hemispheres) may mediate development and preservation of bimanual coordination skills. Asynchrony in movement onset and termination was discovered in patients with cerebellar dysfunction performing bimanual drawer task (Serrien and Wiesendanger 2000). Diedrichsen et al. (2005) particularly suggest that the cerebellum plays an important for compensating self-produced action, i.e., adjust a force given to a hand to keep isometric posture by predicting load change caused by the contralateral hand.

2.4.3 Corpus callosum

The corpus callosum is the largest reciprocal fiber bundle connecting the two hemispheres. The corpus callosum provides information exchange across the two hemispheres, leading to the idea that the corpus callosum plays an important role in bimanual coordination. In support of this idea, sagittal section of corpus callosum impairs bimanual coordination in human and monkey (Eliassen et al. 1999; Kennerley et al. 2002; Mark and Sperry 1968; Mohn and Russell 1981; Preilowski 1972; Shimada et al. 1997; Sullivan et al. 1993). A preponderance of evidence derived from corpus callosotomy suggests the involvement of corpus callosum in bimanual coordination. However, the corpus callosum has multiple origins and targets, such as basal ganglia, SMA and primary motor cortex. Here, the detailed cellular organization of mammalian corpus callosum will be reviewed which will be followed by the causal role in bimanual coordination.

The source of axons in the corpus callosum is excitatory pyramidal neurons called intratelencephalic (IT) neurons. Although the corpus callosum provides major cortico-cortical connection, neural tracing and electrophysiological studies of IT neurons show a substantial projection to contralateral striatum. The corpus callosum contains two anatomically distinct projections: intratelencephalic cortico-cortical (IT-CC) projection and intratelencephalic corticostriatal (IT-CS) projections (Harris and Shepherd 2015; Shepherd 2013). The cell body of the IT pyramidal neurons are typically found in cortical layer II/III and V (Ferino et al. 1987; Lin et al. 2018; Oswald et al. 2013; Tantirigama et al. 2014; Wise 1975), and terminals of IT-CC projections to contralateral cortex were densely observed in homotopic layer II/III and V in rat and mouse motor cortex (Ferino et al. 1987; Smith and Alloway 2014; Wu et al. 2009; Zhou et al. 2013). Axonal terminals of IT-CS neurons were broadly distributed throughout the entire contralateral striatum (Ferino et al. 1987; Reep et al. 1987; Reep et al. 2008; Wu et al. 2009). It should be noted that the target area of contralateral projection is nicely homotopic to the

ipsilateral projection, although the density is different (Wu et al. 2009). It remains unclear whether IT-CS and IT-CC projections originate from distinct subpopulations of IT neurons. The distribution of IT-CS neurons in cortical laminar is slightly deeper than IT-CC neurons (Wilson 1987, Oswald, Tantirigama et al. 2013). Reconstruction of single cell axonal branches suggests that the single IT neurons projects to both contralateral cortex and striatum (Economo et al. 2016; Lin et al. 2018). Although the recent tracing study revealed the distinct axonal target via corpus callosum, the functional role of this projection still remains unknown. The distinct projection target to contralateral cortex and striatum raises a question of how does the two projection function in motor control. This need to be investigated in the future study.

The causal role of corpus callosum in temporal/spatial synchronization and familiar behaviors have been investigated by corpus callosotomy in human and non-human primates (Eliassen et al. 1999; Kennerley et al. 2002; Mark and Sperry 1968; Mohn and Russell 1981; Preilowski 1972; Shimada et al. 1997; Sullivan et al. 1993). It has reported that timing synchronization was relatively conserved in callosotomy patients, suggesting that the corpus callosum is not involved in the temporal coupling of bimanual movements (Ivry and Hazeltine 1999; Tuller and Kelso 1989; Zaidel and Sperry 1977). Donchin et al. (1999) suggested the involvement of a subcortical structure such as central pattern generator, in timing synchrony of bimanual movements, which explains conserved timing synchrony without corpus callosum. Interestingly, temporal coupling was disrupted when a spatial factor is introduced to the task (e.g., continuously drawing a circle in a 2-D plane). Spatial coupling in a bimanual drawing task (drawing of different forms by two hands) was reduced after corpus callosotomy (Eliassen et al. 1999; Franz et al. 1996). These evidence suggest that the corpus callosum conveys motor command, especially spatial information.

Cardoso de Oliveira et al (2001) further studied interhemispheric interaction in monkey by measuring local field potential in motor cortex bilaterally during bimanual lever-pressing

task (Fig. 2-1B). The LFP showed strongest interhemispheric correlation in symmetric lever press, suggesting the synchronized interhemispheric interaction mediates bimanual symmetric movements. This evidence instantaneously leads to the idea that interhemispheric interaction might be caused by direct excitatory inputs via corpus callosum. However, many pieces of evidence suggest that the corpus callosum plays a role in interhemispheric inhibition (Ferber et al. 1992; Hubers et al. 2008). Hiraoka et al. (2014) reported that interhemispheric inhibition has been observed during the bimanual task. These findings motivate investigating the cellular mechanism of the corpus callosum to understand how inhibition and excitation mediate bimanual coordination.

2.5 Methodological challenges

The current conceptual understanding of bimanual coordination and its neurophysiological mechanism largely derives from human studies, whereas extensive lesion, cellular electrophysiological recordings, and anatomy were studied by non-human primates and other mammalian species. Rodents have not been extensively used to study bimanual coordination, although invasive experimental manipulations, or the use of transgenic approaches to understand the neural mechanisms are readily applied in rats and mice. Given its fundamental role of bimanual coordination in daily behavior in rats and mice such as feeding, there is no surprise that the CNS of rodents also has neural substrates which function similarly to those of primates. Based on the review of the literature, I suggest that the bimanual coordination during feeding behavior has a potential for measurement of bimanual coordination by rodents for the following reasons: (i) a single food object is controlled by two hand, which requires rapid and online information exchange between two hands. (ii) Coordination in feeding behavior is a natural motor behavior, there is, therefore, less estrangement from neural substrate actually used in a natural condition. To implement feeding behavior for the study of bimanual coordination, there are several technical challenges to overcome, which will be addressed in this thesis. Furthermore, another aim of this thesis is to investigate neural substrates of bimanual coordination in feeding behaviors with pharmacological blockade and electrophysiological recordings using developed measurement techniques and analytical pipelines of limbs movements.

2.6 Aims and objectives of the thesis

Based on the foregoing, the overall aim of this thesis is to better-understand neural mechanism of bimanual coordination using rodents as an animal model. In this thesis, I first examine rodents as an animal model to investigate symmetric and asymmetric bimanual coordination. In these preliminary investigations different tasks and measurement systems were explored and

their utility in providing kinematic data for rodents was tested (chapter 3). After these preliminaries, I developed a recording system and apparatus for quantifying bimanual movement in the head-fixed rats, and used this to investigate the organization of forelimb use in feeding behavior using an analytical pipeline developed for this study (chapter 4). Next, I identified the motor corpus callosum using neural tracer and investigated the causal role of the corpus callosum by silencing these with a local anesthetic. Finally, the inter-hemispheric cellular network in bilateral coordination of the limbs will be investigated (chapter 5).

Chapter 3: Preliminary studies in free-moving rats

3.1 Introduction

There have been relatively few studies of bimanual coordination in rodents, which in part is due to the technical challenges of measuring rapid fine movements in smaller animals, and the need to develop anatomically and ethologically appropriate tasks requiring bimanual coordination. The current conceptual understanding of bimanual coordination mostly derives from studies in human and non-human primates, with a substantial focus on contrasting symmetric and asymmetric movements (Swinnen 2002). Symmetric bimanual movements are engendered by simultaneous activation of homologous muscle; in contrast, asymmetric bimanual movements are generated by different muscle groups. Importantly, symmetry and asymmetry are defined by a body-centered coordinate frame in which symmetry indicates mirrored movements with respect to the body longitudinal axis. Therefore, to extend the use of rodents as animal models in the study of bimanual coordination, while including and integrating the rich corpus of knowledge discovered by primate models, there is a need to develop techniques for measuring forelimb movements of rodents in reference to a body-centered coordinate frame. Thus, the purpose of the preliminary work reported in this chapter is to explore systems for measurement of bilateral forelimb movements in rodents, focusing on the ability to measure symmetric and asymmetric bimanual coordination in an egocentric coordinate frame.

Although there are well-established behavioral tests for unimanual motor control such as skilled reaching test and lever pressing task, relatively little has been developed for measuring bimanual coordination in rodents. Considering the previous behavioral paradigm for non-human primates, two-lever pressing might be an interesting approach. However, applying primate-inspired behavioral paradigms to rodents should take into account motor circuits that are actually used for bimanual coordination in natural motor behavior. Wishaw and Coles

(1996) demonstrated that object manipulation involves a variety of forelimb movements showing both symmetric and asymmetric bimanual actions. Object manipulation during feeding behavior is a natural way to observe bimanual coordination, which may be elicited by presenting food objects. Grooming also involves natural action patterns that occur spontaneously, and seen in a variety of species such as rats and cats. In rats, it has been reported that the motor chain in grooming involves both unilateral and bilateral forelimb strokes (Aldridge and Berridge 1998). Therefore, in the present chapter food manipulation behavior and grooming behavior are compared to decide which provides a better model for the current study of bimanual coordination.

The challenge of measuring bimanual coordination in rodents also arises in part from their small size and rapid, small amplitude movement. To study motor control in these smaller animals, high-speed camera and automated tracing have been used, which enables researchers to analyze kinematic variables such as position, velocity, and joint angle with sub-second and sub-millimeter precision. The use of high-speed cameras has become more common in the study of unilateral motor control (Azim et al. 2014; Guo et al. 2015; Kawai et al. 2015; Palmér et al. 2012; Panigrahi et al. 2015). To monitor bilateral forelimb simultaneously, the observation points need to be extended to monitor limbs on both sides of the body. For example, Perrot et al. (2009) used six high-speed cameras to monitor left and right side of the body. However, the generated data volume is proportional to the number of cameras, which drastically increases the cost of computation and storage. Thus, there is a need to minimize the number of cameras to monitor bilateral forelimb for a long duration of recording and efficient data analysis.

In this chapter, I report an imaging system for measuring bilateral forelimb kinematics during feeding and grooming behavior. The system uses two high-speed cameras to monitor the positions in 3-D space of markers placed in the left and right forelimbs. Both markers were recorded simultaneously and separated in off-line analyses. The high-speed cameras were placed underneath a transparent base plate which enabled both limbs to be monitored using a

minimum number of camera positions. Colored markers attached to the wrists were automatically traced, and the marker positions were reconstructed in 3-D space using in-house software assembled in MATLAB. A jacket with a marker provided a body-centered reference for the egocentric coordinate system.

The main findings of the preliminary work reported in this chapter can be summarised as follows. The system for the analysis of bimanual coordination was tested and two different motor behaviors, grooming and object manipulation, were compared. Pairwise cross-correlations between left and right forelimb kinematics were computed. The analysis revealed that symmetric (positively correlated) bilateral forelimb movements were frequently observed during grooming, however, asymmetric (negatively correlated) movements were not observed. In contrast, both symmetric and asymmetric bilateral forelimb movements were observed during object manipulation. These results suggested that object manipulation would be more suitable for studying both symmetric and asymmetric bimanual coordination in rodents. However, there were some drawbacks with the proposed recording system, such as postural artifacts caused by the movements of the jacket supporting the egocentric reference mark. Further work was required to overcome these limitations of the system for measuring bimanual coordination of rodents efficiently and precisely, which will be reported in Chapter 4.

3.2 Materials and Methods

Animals. A total of two Male Long Evans rats were used in this study. Rats were kept in a clear plastic cage with free access to food and water until food restriction commences. During food restriction, rats received 17-18 g of food pellets per day, after each session, to maintain their body weight. Sugar tablets were used as rewards for motivating the animal to perform the behavioral tasks. All rats were placed in the experimental chamber with free access to a small number of sugar reward tablets for 15 min in order to habituate to the experimental chamber. All procedures were approved by the Okinawa Institute of Science and Technology Graduate University Animal Care and Use Committee.

Behavioral apparatus and recording system. Motor behavior in freely moving animals was monitored by high-speed cameras. The design of the recording apparatus is shown in figure 3-1. Rats were placed in a white narrow acrylic box (13 cm width, 30 cm depth, 30 cm height) with a transparent floor. Two high-speed cameras (HAS-L1, DITECT, Tokyo, Japan) were placed below the behavioral box with both sides of the body in the field of view of each camera. Prior to behavioral experiments, two green markers were attached to the lower side of the wrists under light isoflurane anesthesia (1.5 - 2%). To analyze forelimb movements in body centered egocentric coordinate axes, a custom-made reference jacket was used. The reference jacket embedded three blue markers on the lower side of body, providing the reference points from which the software determined the caudal-rostral, the left-right, the dorsal-ventral axes. This provided 3-D coordinates for both markers. The jacket was put on the animal under light anesthesia (1.5 – 2.0% of isoflurane). After the recovery from anesthesia, rats were placed in the experimental chamber, and monitored during two types of motor behavior: grooming and object manipulation. Forelimb motor behavior was recorded at 300 frames per second by the high-speed cameras, and stored to hard disk. The marker positions were automatically traced

by color filtering and point detection by MATLAB computer vision toolbox and image processing toolbox 2014b (Mathworks, MA, USA).

Grooming test and object manipulation task. Grooming sequences were monitored to determine whether bilateral motor behavior could be observed. Water mist was sprayed to elicit grooming if the rat did not spontaneously initiate grooming behavior (Jaeger et al. 1995). Recording continued for 30 min, allowing observation of 5 to 10 grooming bouts. After 30 minutes of recording of grooming behavior, the object manipulation task was conducted. Rats were given a small wooden sphere (2.5 mm of diameter) on which six sugar reward tablets were embedded in holes on the surface. To consume the sugar tablets, rats were required to manipulate (e.g. rotate and hold) the spherical object by their forelimbs. The motor behavior of the forelimbs during grooming and object manipulation was recorded by the two high-speed cameras and stored to hard disk.

Data analysis of bimanual movement of rat. The positions of the reflective markers in the camera coordinates $[x, y, z]^T$, where $x = [x_1, x_2, \dots, x_t]$, $y = [y_1, y_2, \dots, y_t]$, $z = [z_1, z_2, \dots, z_t]$, were transformed into the egocentric coordinate system using the reference markers on the jacket (Fig. 3-1B). The marker position of right forelimb were then represented as $[lr, ap, dv]^T$, where lr, ap, dv corresponds to time series data of marker position in left-right (lr) axis $[lr_1, lr_2, \dots, lr_t]$, caudal-rostral (cr) axis $[cr_1, cr_2, \dots, cr_t]$, and dorsal-ventral (dv) axis $[dv_1, dv_2, \dots, dv_t]$ respectively. Finally, the time series data of forelimb position in the right forelimb $[lr, cr, dv]_R^T$ and the left forelimb $[lr, cr, dv]_L^T$ were obtained.

Movement symmetry and asymmetry by cross-correlation. To visualize movement synchrony of forelimb movements, cross-correlation of velocities was computed. Symmetric movements are in other words, mirror movements. Physiologically, symmetric bimanual movements are

generated by synchronized activation of homologous muscles, in contrast, asymmetric bimanual movements are generated by activation of non-homologous muscles. Positive correlation between two forelimbs displacements suggests that the movements are symmetric where two forelimbs move toward similar direction, whereas, negative correlation indicates forelimb movements are asymmetric when the two forelimbs move against each other. Prior to the computation of the cross-correlation, a mirror transformation was applied to the left trajectories, where the mirrored left forelimb trajectory is $[-l_r, c_r, d_v]^T$. This is to apply the concept of symmetrical and asymmetrical bimanual movement in the egocentric coordinate system, where movement symmetry is regarded as mirror movement with respect to the sagittal plane, which is discussed by Swinnen et al. (Swinnen et al. 1998). Transient periods of symmetric or asymmetric bilateral forelimb movements were determined by cross-correlation of velocities within moving window of 120 ms duration (Gruber et al. 2009; Laurent and Davidowitz 1994).

3.3 Results

Measuring two forelimbs by two high-speed cameras. Prior studies used more than three high-speed cameras to monitor each side of the body from a horizontal perspective. To reduce computation and storage cost, the present study employed only two cameras which is the minimum number required for depth reconstruction. This required reconsideration of the camera configuration. The tested camera configuration is shown in Figure 3-1A. Instead of facing cameras horizontally toward the subject, cameras were placed under the transparent base plate. This configuration enabled simultaneous monitoring of both forelimbs from each angle, satisfying the requirement for depth reconstruction of forelimb position. The 3-D position of both forelimbs was thus successfully reconstructed by *stereo camera calibrator package* (MATLAB computer vision toolbox 2014, The Mathworks, MA, USA, Fig. 3-1C).

Reconstruction of bilateral forelimb trajectories in body-centered egocentric coordinates. A technical challenge of studying bimanual coordination by rodents is to follow forelimb kinematics in a controlled posture. Controlling an animal's posture is difficult especially when the animal is moving freely, and this causes significant artifacts in relation to orientation, locomotion, and rearing. Therefore, in the current approach, the reference jacket was used to subtract the postural coordinates. The reference jacket has three points blue markers which indicating egocentric axis. The original camera centered allocentric coordinate system (Fig. 3-1C) was transformed into an animal-centered egocentric coordinate system (Fig. 3-1D) using the obtained egocentric axes derived from the markers on the reference jacket. With the imaging setup and reconstruction process, grooming and object manipulation behaviors were monitored to compute kinematics of forelimbs.

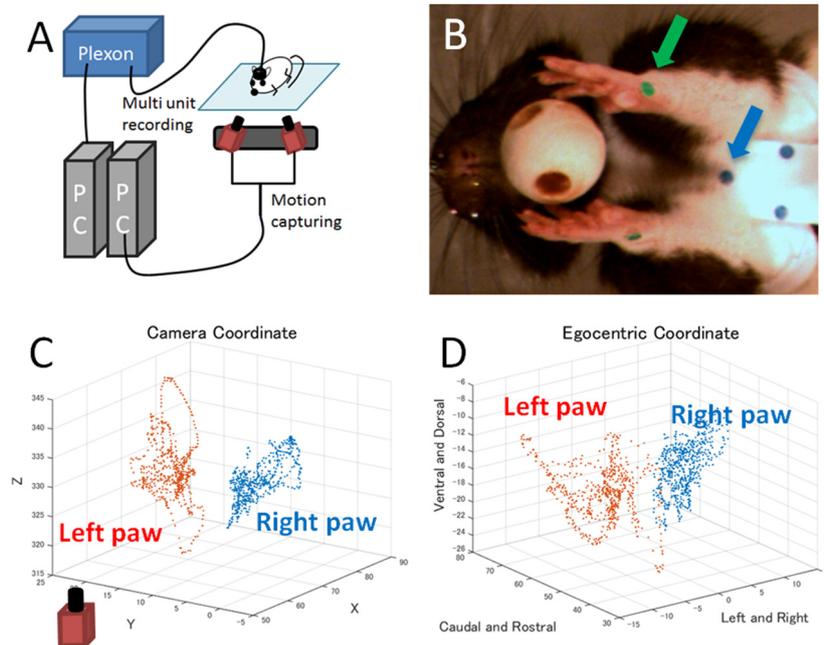


Figure 3-1. Tested recording system for analysis of bilateral forelimb movements in freely moving rats.

(A) Schematic diagram of recording apparatus. Rats were positioned on the transparent acrylic base. Forelimb motor behaviors were monitored and recorded by two high-speed cameras placed under the transparent base. The frames of image data were stored on the HDD with a time stamp synchronized with other recording systems, in this case, a multiunit recording system. (B) representative image (single frame) taken by a high-speed camera. The colored markers were used to track forelimb motion by post-hoc color filtering and point detection. The green markers (green arrow) are attached lower side of wrists for tracking forelimb position. The blue markers (blue arrow) were used to provide reference points for the body-centered (egocentric) coordinate system of rats. (C-D) Transformation to egocentric coordinate system from camera coordinate. (C) Reconstructed 3D trajectories of forelimb position in the camera centered system. (D) Transformed forelimb trajectories in the egocentric coordinate system using the blue reference markers on the jacket.

Bilateral forelimb movements in grooming and object manipulation. To determine the motor behavior suited for study symmetric and asymmetric bimanual movements, bimanual grooming sequences and object manipulation behavior were compared. Grooming is an innate motor sequence which begins with elliptical bilateral stroke. The elliptical bilateral stroke continues two to three times in a second. During the movement, rats stroke their nose sides and vibrissae. Observing animals during these movements suggested that the elliptical bimanual strokes were typical symmetric bimanual movements. As it has been reported by Jaeger et al. (1995) the elliptical strokes are often followed by unimanual and bimanual ear strokes and licking. My initial observation suggested that grooming behavior would be useful for the study of symmetric bilateral forelimb movements.

Forelimb motor behavior during an object manipulation task was recorded in the same rat that had performed the grooming behavior. In the object manipulation task, the rat holds a sphere with both hands and starts retrieving the rewards embedded in the cavities. Once the animals have completed retrieving a reward in one hole, they usually started to change the position and angle of the sphere, presumably in search of another reward. Unimanual and bimanual movements could be observed during the manipulation. Unimanual movements were often seen during a tripod stance, allowing the rats to manipulate the sphere with a single hand and sometimes with their nose. The bimanual movements were different depending on the motion of the sphere. For example, when translating the sphere, animals usually used both hands symmetrically, whereas, when rotating the sphere, two types of forelimb movements could be observed: alternate bimanual rotation, in which the rat used both hands alternately but not at the same time to rotate the sphere; simultaneous bimanual rotation, in which the rat moved its forelimbs in opposite direction. This is a typical asymmetrical bimanual movement. From the general observation of these bimanual movement, it is likely that the elliptic bimanual stroke might be used to test their symmetrical bimanual movement; whereas, bimanual object

manipulation might be used to test asymmetric bimanual movements. In the next section, I will report a pilot analysis of bimanual movement using kinematic data.

A pilot analysis of bimanual movement using cross-correlation. In order to analyze detailed kinematics of grooming and object manipulation, positions of the wrists were reconstructed in an egocentric coordinate system. Two seconds of typical bimanual movement of grooming and object manipulation were selected arbitrarily (Fig. 3-2). Three-dimensional trajectories of bilateral wrist position were reconstructed (Fig. 3-2A). The rat showed larger strokes during grooming in ventral-dorsal axis (Fig. 3-2A), and elliptic trajectories could be observed as their name suggests. Bimanual object manipulation was less continuous (Fig. 3-2B), and several groups of dense scatter could be observed where wrists did not move. With the same time-series data, movement synchronization was visualized by plotting relative velocity in three body-centered axes.

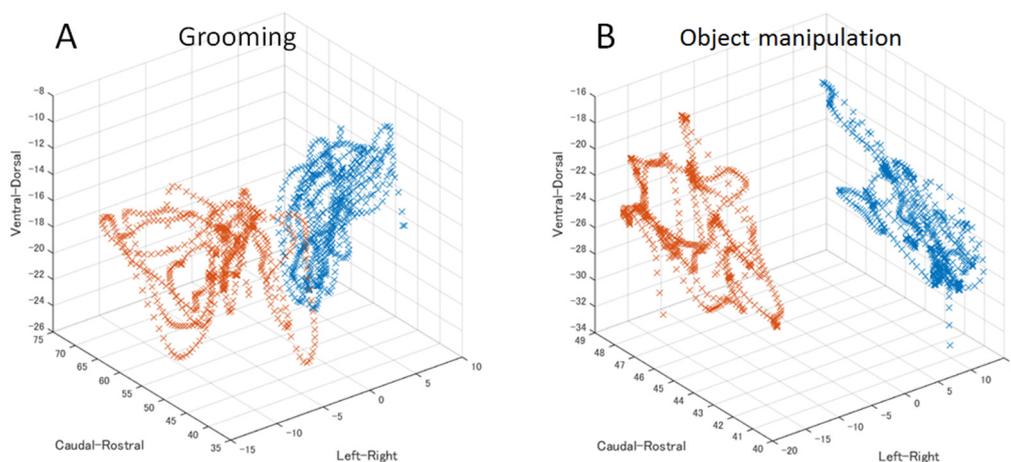


Figure 3-2. Representative trajectories of forelimbs during grooming and object manipulation.

(A-B) 3-D forelimb position during grooming and object manipulation. The scales in each axis are presented in millimeters. (A) Trajectories from grooming behavior in egocentric coordinate axes. x markers indicate the position of right (orange) and left (blue) forelimbs. (B) Trajectories during object manipulation behavior.

The velocity of forelimb movements during grooming and object manipulation behavior relative to each other were plotted (Fig. 3-3). In grooming behavior, there appears to be a strong positive linear relationship between left and right velocity in the caudal-rostral axis and the ventral-dorsal axis (Fig. 3-3A). This might represent symmetric bilateral strokes in those axes. In object manipulation, there is no clear tendency like that seen in grooming behavior (Fig. 3-3B). In the ventral-dorsal axis, it may be that there is a positive linear relationship. However, it appears that there is also a small group of points that have a transient tendency toward a negative linear relationship. In the bimanual object manipulation, symmetrical and asymmetrical appeared transiently. To measure temporal symmetry or asymmetry in the sequence of object manipulations, a smaller time window would be more suitable for characterizing such transient actions.

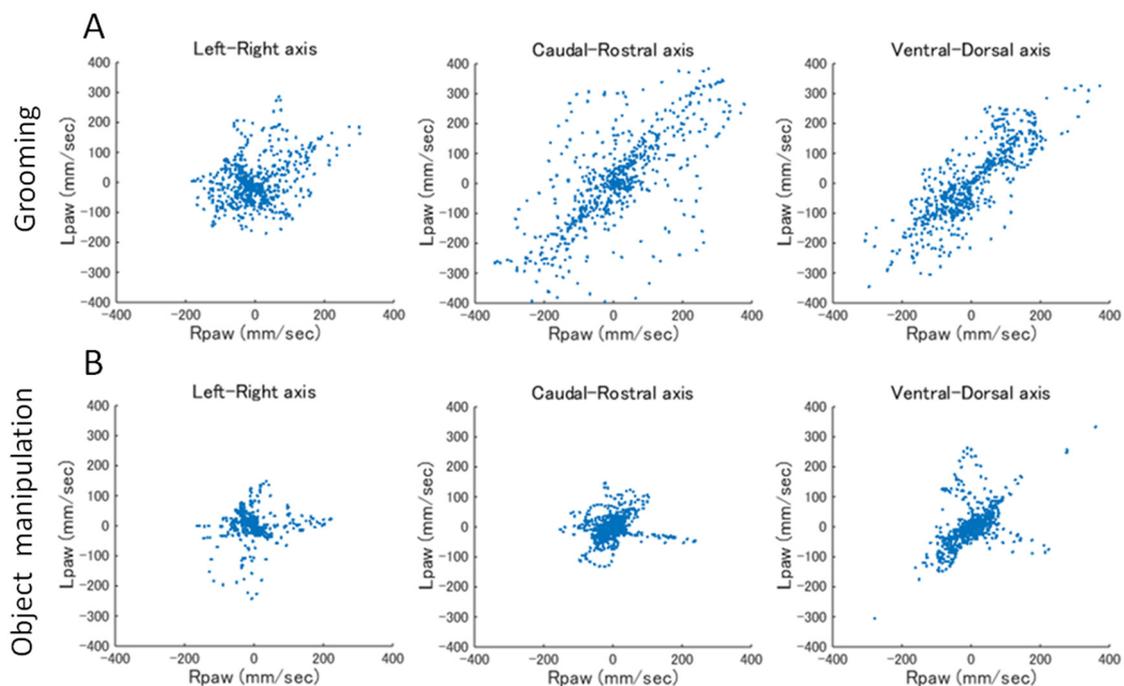


Figure 3-3. Movement symmetry and asymmetry represented by relative velocity in three body-centered axes.

(A-B) Relative velocity between right and left forelimbs in grooming (A) and

object manipulation (B) across three different axes. Relative velocity of left and right forelimbs in left-right (left panel), caudal-rostral (center panel), ventral-dorsal axes (right panel) were plotted to observe positive and negative relation. Strong positive correlations were observed in grooming behavior especially in caudal-rostral and ventral-dorsal axes (A). In contrast, negative correlation was seen in object manipulation in ventral-dorsal axis (B).

Transient movement symmetry and asymmetry. Finally, in order to characterize transient shift between symmetric and asymmetric mode during grooming and object manipulation behavior, sliding window cross-correlation was computed (Gruber et al. 2009; Laurent and Davidowitz 1994). Instead of computing cross-correlation of velocity of the entire time, the cross-correlation was computed in 120 ms time windows, and the windows were shifted every 1/300 seconds. In grooming behavior, several peaks of correlation were observed in the caudal-rostral and the dorsal-ventral axis (Fig. 3-4C). Those peaks suggest that the rat showed symmetrical bimanual movement at these moments (Fig. 3-4A). In contrast, during object manipulation, in addition to transient symmetric bilateral forelimb movements, the rat uses asymmetrical movements (Fig. 3-4B), which shows a negative correlation (Fig. 3-4D). The results suggest that grooming sequences are suitable for measuring symmetric bilateral movements but not for asymmetric movements. In contrast, object manipulation involves both symmetric and asymmetric bilateral forelimb uses. Therefore, the object manipulation task can be used to study both symmetric and asymmetric bimanual coordination in rats.

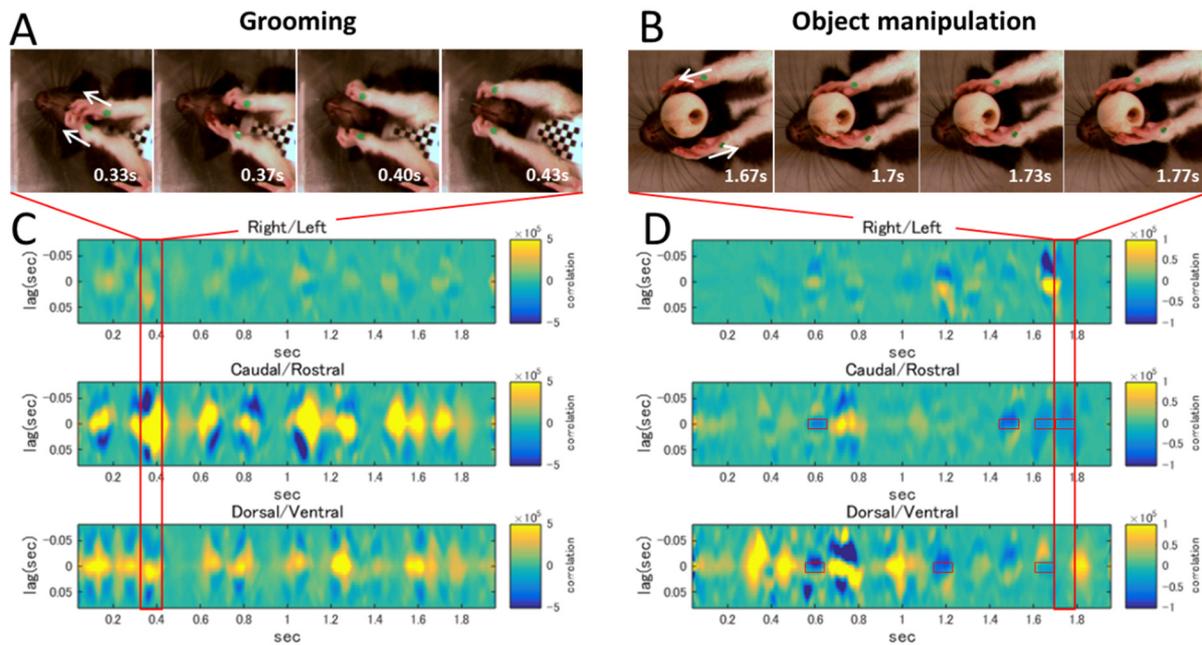


Figure 3-4. Temporal movement symmetry and asymmetry were visualized by sliding window cross-correlation.

(A-B) The sequences of representative bilateral forelimb movements in grooming (A) and object manipulation (B) were shown. (A) Bilateral stroke in the forward direction. (B) Rotation of object by moving hands in the opposite direction. (C-D) The sliding window cross-correlation of velocities in right-left axis (top panel), caudal-rostral axis (middle panel), and dorsal-ventral axis (bottom panel). Vertical axes in each plot indicate time lag of cross-correlation. The color scale indicates cross-correlation between velocities of right and left forelimb movements in each axis. (C) Window-shift cross-correlation in grooming behavior. Strong positive correlation (symmetric movements) were colored in yellow. Red lines indicate a representative symmetric bilateral stroke shown in (A). (D) Window-shift cross-correlation in object manipulation. Small red rectangles indicate the areas where correlation was negative suggesting asymmetric movements in these axes. Red lines indicate the representative asymmetric rotating movement shown in (B).

3.4 Discussion

Here, I demonstrated preliminary kinematic recording system and its application for studying bimanual coordination in rats. The proposed recording apparatus with two high-speed cameras reconstructed depth of forelimb position. The forelimb position was projected in body-centered coordinate by reference marker embedded on the jacket. To determine the optimal behavioral task for symmetric and asymmetric bimanual coordination, grooming behavior and object manipulation behavior were compared. The window shifts cross-correlation analyses revealed that symmetric forelimb movements dominate in grooming behavior. In object manipulation, the symmetric forelimb movements are also frequently seen, but asymmetric bilateral forelimb movements are also observed.

The proposed 3D recording pipeline for bilateral forelimb movements in freely moving environment provided fine kinematic information about forelimb movements during grooming and object manipulation behaviors. The present study especially attempted to subtract postural artifact by using reference jacket. The reference coordinate obtained from the jacket successfully transformed the camera allocentric coordinate to egocentric coordinate space. However, there were several drawbacks in this approach. First, the reference jacket was not comfortable for the animal and evoked shaking of the body to remove it, causing an additional artifact for measurement. Second, the markers attached to the jacket were frequently disturbed by postural changes and forelimb movements, which resulted in several moments when the program was unable to detect markers. Therefore, I conclude measuring forelimb kinematics in freely moving animal is possible, however, not suitable for the aims of the current study. To overcome the limitations, fixing a frame of reference is possible by fixing the body orientation using head-fixation devices that have recently become available for rodents (Guo et al. 2014; Hira et al. 2015; Isomura et al. 2009; Schwarz et al. 2010).

Grooming sequence and object manipulation were compared as the model of bimanual coordination in rodents. Grooming is inherent motor behavior in rodents and has used for the study of motor function (Aldridge and Berridge 1998; Cromwell and Berridge 1996). Dynamic and repetitive bilateral forelimb strokes were observed during grooming sequence in the recording apparatus. Using the tracking system, 3-D forelimb position during the actions was reconstructed. The window shift cross-correlation analysis revealed that the repetitive bilateral movements were positively correlated suggesting symmetric bilateral forelimb movements. Therefore, grooming sequence may be a model of symmetric bilateral forelimb movements to study its neurophysiological mechanism. Object manipulation behavior was also tested. Object manipulation using both hands is essential for daily behavior such as feeding. The object manipulation in the present study used a wooden sphere in which were embedded reward sucrose tablets. The animal consumed the reward sucrose tablet by holding and rotating the object. The window shift cross-correlation analysis revealed transient symmetric bilateral forelimb movements and asymmetric bilateral forelimb movements. Therefore, the object manipulation has greater potential for the study of bimanual coordination in rodent with respect to both symmetric and asymmetric coordination.

3.5 Conclusion and Future Directions

The preliminary experiments reported in this chapter evaluated the recording apparatus and tasks for monitoring bilateral forelimb with a minimum number of high-speed cameras. Kinematic analysis of bimanual coordination based on cross-correlation suggests that object manipulation can be used to quantify both symmetric and asymmetric bimanual coordination. However, the recording system needed to be improved for two reasons: (1) the jacket with reference marker is an extra stressor that induces additional postural artifacts; and, (2) markers on the jacket are frequently hidden by forelimb movements. To solve these issues, the recording

system needed to be improved. In the next chapter, an improved recording system and analytical pipeline of bimanual coordination will be presented.

Chapter 4: Kinematic analysis of bimanual movements during food handling by rats

4.1 Introduction

The ability to execute bimanual actions –involving the coordinated interplay of both limbs – is crucial not only for the most basic needs of daily life, such as gathering and feeding, but also for the heights of human creative achievement exhibited in art and music. Despite being computationally expensive, the ability to coordinate the limbs bilaterally has been advantageous and selected for in evolution. The neural mechanisms underlying bimanual movements have long been a focus of research in primates because of their significance for behavioral neuroscience, for the pathophysiology of movement disorders, and as a basis for rehabilitation or diagnosis (Ponsen et al. 2006; Reinkensmeyer et al. 2016; Swinnen 2002; Swinnen and Wenderoth 2004; van Delden et al. 2012; Wu et al. 2010). Many important advances toward understanding bimanual coordination have been made using human and non-human primates as the experimental subject (Swinnen 2002). Primates have many advantages because of their advanced capabilities and the availability of sophisticated analytical apparatus. However, primates are less well suited to invasive experimental manipulations, or the use of transgenic approaches to understand the neural mechanisms. In contrast, such manipulations are readily applied in rodent models, which are therefore advantageous for addressing neural mechanisms of the mammalian brain. Like primates, rodents also exhibit dexterous coordination of forelimbs to handle food objects when eating (Whishaw and Coles 1996). There is, therefore, much to be gained from further developing quantitative and qualitative measuring techniques suitable for use with rodent models in the study of bimanual coordination. The aim of the present investigation was to develop a method for quantifying bimanual movement in the rat.

Quantification of bimanual coordination during spontaneous food handling behavior has been reported in freely moving (Allred et al. 2008; Tennant et al. 2010; Whishaw and Coles 1996) and head-fixed rodents (Whishaw et al. 2017b). Evaluation of behavior in these reports

has been based on investigator observation of action and postures of hands by off-line video analysis. In recent years, the emergence of kinematic analysis with 2-D lever and 3-D motion capture has enabled the documentation of qualitative measures such as tortuosity, oscillations, and variability in unimanual motor control (Azim et al. 2014; Guo et al. 2015; Kawai et al. 2015; Palmér et al. 2012; Panigrahi et al. 2015). Implementation of these analytical techniques has furthered our fundamental understanding of rodent motor behavior. However, it is important to extend these methods to the problems of classification of bimanual movements during natural action sequences.

Here I report an imaging system for measuring bimanual coordination in rats. The system uses a pair of high-speed cameras to capture 3-D forelimb position during bimanual food handling. In the system, rats are head-fixed in order to provide a reference frame for recording. A semi-automated tracking program generates trajectories of forelimb position in animal egocentric 3-D space. Trajectories are transformed into kinematic parameters such as speed, velocity, or movement direction. To show potential uses of kinematic data obtained with the system, I demonstrate segmentation and mathematical analysis of rat forelimb movements to measure laterality of movement speed and asymmetry of movement direction during food handling. Finally, using a classification algorithm, I demonstrate high-throughput of large amount of kinematic data from multiple rats to quantify spontaneous food handling behavior.

4.2 Materials and Methods

Animals. Male Long Evan rats (10 – 12 weeks old) weighing 350-450 g were used in this study. The rats were kept under a reversed 12 hrs light/dark cycle (10:00 am to 10:00 pm), constant temperature (25°C) and humidity. Rats were housed with *ad libitum* access to water and food before weight restriction. Animals were habituated to the experimenter for more than three days before the start of behavioral recording. All experiments were approved by the Committee for Care and Use of Animals at the Okinawa Institute of Science and Technology.

Surgery for head-fixation. Carprofen (Rimadyl, Pfizer, 50 mg ml⁻¹, sc) was administered immediately before surgery. Rats were anesthetized with isoflurane (3 - 4% induction, 1.5 - 2.5 % for maintenance), and placed on a stereotaxic frame for chronic experiments (SR-10R-HT, Narishige, Japan). Body temperature was monitored and maintained at 36.5 - 37.5°C with a heating pad. The skull was exposed and carefully cleaned with saline and cotton swabs. Super-Bond Green Activator (Sun Medical Inc., Japan) was judiciously applied to the skull, left for 20 sec, and then removed by saline. After the surface preparation, eight anchor screws (M 1 × 2) were drilled into the skull. The screws were then covered with a layer of dental cement (Super-Bond, Sun Medical Inc., Japan). A chamber frame (CFR-1, Narishige, Japan) was positioned above the skull and secured by additional layers of dental cement. Antibiotic was intraperitoneally administered after the surgery. A dietary supplement with Carprofen (Medigel CPF; Clear H₂O, ME., US.) was given during post-op recovery for 5 days.

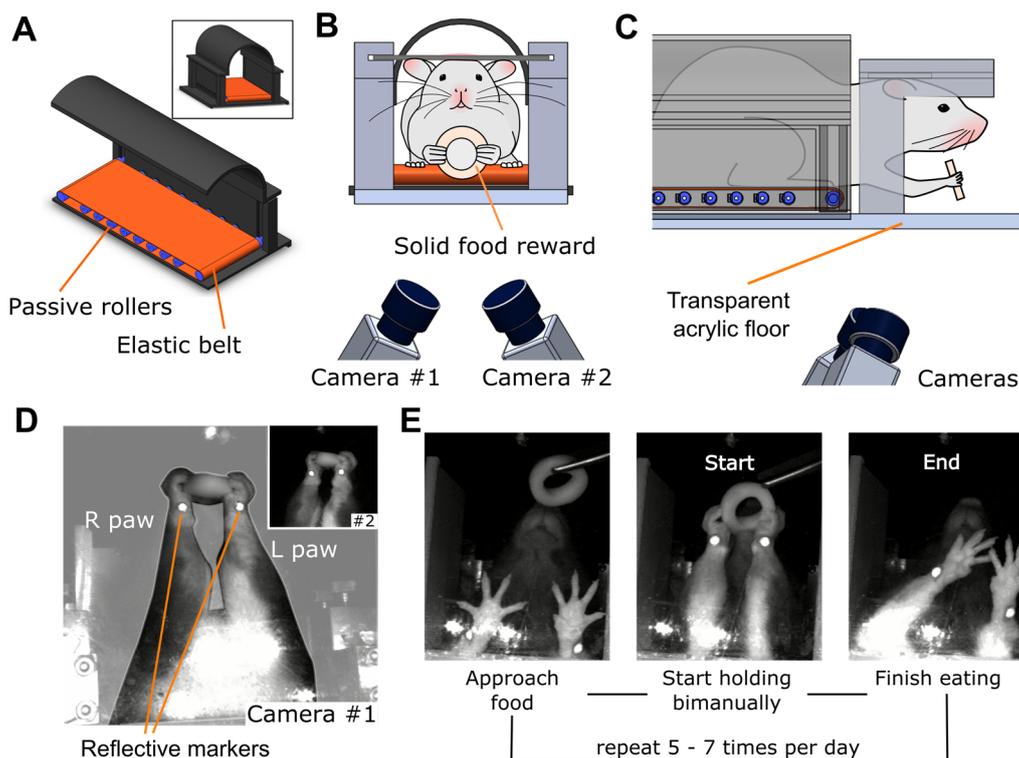


Figure 4-1. Schematics of apparatus and imaging setup.

(A) Illustration of the passive treadmill for the head-fixed behavioral device. Inset shows the assembled apparatus. (B and C) Schematic diagrams of the configuration of the rat positioned in the apparatus, from the front (B) and the side (C). The rat is shown on the passive treadmill holding a retrieved donut shaped food item. Two high-speed cameras are placed 45 cm below the transparent floor to monitor the reflective markers on the wrists. (D) View from camera 1 and camera 2 (inset). Note reflective markers attached to rat's wrists for semi-automatic tracing. (E) Timeline of the sequence of a trial of food handling and consumption.

Behavioral apparatus and recording setup. A custom made stereotaxic frame for chronic experiments (SR-10R-HT, Narishige, Japan) was used for head-fixed behavioral experiments. A 3-D printed passive linear treadmill (80 mm wide and 130 mm long; Fig. 4-1A) was used to minimize animals' stress by allowing hindlimb movement. The treadmill was placed above a transparent acrylic base plate, and two high-speed cameras (HAS-L1, $f = 6\text{mm}$, DITECT,

Japan) with infrared LEDs were positioned 45 cm below the base to monitor forelimbs (Fig. 4-1 B and C). The two cameras were placed 130 mm apart and directed at an angle of 30° to each other. The accuracy of depth reconstruction was confirmed by using the MATLAB Stereo Camera Calibrator application. The mean reprojection error (the mean distance between the detected marker position and the reprojected points in the reconstructed model space) was 0.77 pixels (Hartley and Zisserman 2003).

Habituation to head-fixation and pre-training. Rats were food restricted prior to behavioral training. Body weight was maintained between 80 % and 90 % of the original weight. Animals were then habituated to the head-fixed apparatus. Habituation was based on the procedures previously reported (Isomura et al. 2009; Ollerenshaw et al. 2012; Schwarz et al. 2010), but modified for food restriction. Briefly, rats underwent the following steps: (1) Rats were placed in the behavioral chamber with *ad libitum* access to food for 20 min for 2 days. (2) The experimenter guided the rats into the half-cylindrical tunnel by providing a sweet jelly reward (Purin mix, House foods, Japan) using a stainless reward spout connected to a 50 mL syringe. The experimenter controlled the position of reward spout to induce animals to slide the chamber frame into the head attachment clamp. 10 – 20 ml of reward was provided in a day. (3) The experimenter held the rat's chamber with gentle force while providing reward. Initially, some rats tried to escape, and it took 2 - 3 attempts for the rats to retrieve 10 - 20 ml of sweet jelly reward. (4) Pre-training. Immediately after head-fixation, the experimenter gave a food reward cut into an annular shape (20 mm outer diameter, 10 mm inner diameter, 5 mm thickness, Fish Sausage, Marudai Food Co., Ltd, Japan) instead of the jelly reward. The pre-training continued until rats could retrieve, without dropping, five rewards for three consecutive days.

Behavioral task and recording. The reflective markers were handmade by covering a 3 mm diameter plastic half sphere with reflective tape (DITECT, Japan). On the day of the behavioral

task, the experimenter gently held the forelimbs while the half-spherical markers were attached to the lower side of the wrists using double-sided tape. The marker could be removed easily after behavioral testing. Rats did not try to remove the marker during the behavioral task. Trials started with bimanual grasping of food offered by the experimenter (Fig. 4-1E), and ended when the last piece of food was brought to the mouth. Rats underwent 16 - 21 trials in three days (5 - 7 trials per day). Cases where rats showed unusual behavior, such as crossing two forelimbs or adopting a tripod stance during eating, were excluded from further analysis. In the present study, only two cases out of 79 recorded sessions across five rats were excluded. All trials were recorded at 200 frames per second (1/500s exposure time and 600x800 pixel) and stored to hard disk.

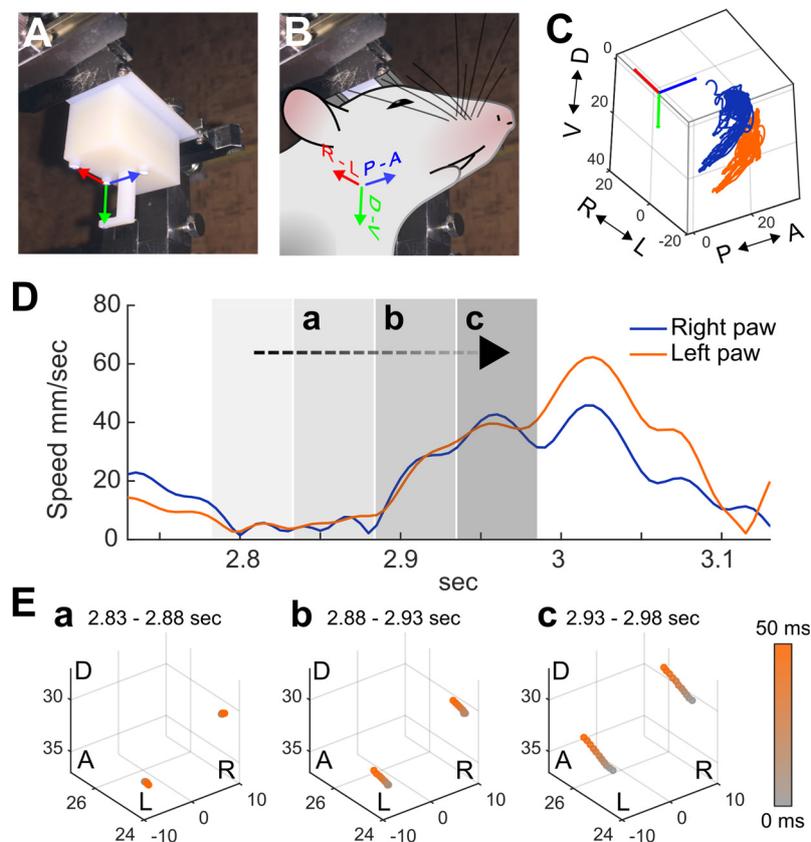


Figure 4-2. Egocentric coordinate reference frame in the recording frame, and segmentation of forelimb trajectory.

(A-C) Forelimb position was projected on the egocentric coordinate system based on the reference marker. (A) Example of 3D printed reference frame of egocentric coordinates. Four triangularly placed reflective markers indicate the origin, posterior to anterior axis (P- A), left to right axis (L - R) and dorsal to ventral (D - V) axis of the rat (B). (C) Example of 3-D forelimb trajectories projected on the egocentric coordinate space. (D) Example 50 ms time window for data segmentation. Note the speed of the right and the left forelimb increased over time indicating bilateral movement initiation. (E) Representative segments of forelimb trajectories in each time window in (D). D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

High-speed cameras and 3-D reconstruction. The positions of reflective markers were traced using an in-house program assembled from a MATLAB toolbox (Computer Vision System Toolbox release 2016b, The MathWorks, Inc., MA., USA). Tracking was automatic except for adjustments to tracking parameters such as threshold which were required in response to the changes in reflection caused by marker angle. The program produced x and y coordinates of the marker position from 2-D video frames of Camera #1 and Camera #2. Depth reconstruction of the marker point was estimated by triangulation of the paired points on the 2D plane from Camera #1 and Camera #2 and the camera geometries. The resulting 3-D positions of the reflective marker in the camera coordinate system were represented as a time series data (x_t, y_t, z_t) . Depth reconstruction of marker position and calibration of camera position were conducted using the stereo camera calibrator package of the *MATLAB Computer Vision System Toolbox*. The reference frame defining the egocentric coordinate axes was included in the field of view of the cameras (Fig. 4-2A, B). Using this reference, the 3-D position data (x_t, y_t, z_t) was transformed into the egocentric coordinate system (lr_t, ap_t, dv_t) which represented time series data of forelimb position in left-right (lr) axis, anterior-posterior (ap) axis, and dorsal-ventral (dv) axis (Fig. 4-2 C). All data were filtered through a 20-Hz low-pass finite impulse response filter.

Discretization of time series data. Continuous position data were discretized into 50 ms duration segments, s_t (Equation 1), with 5 ms shifts (Fig. 4-2D, E). The segment s_t was defined as the array of 3-D position data of right and left forelimbs in a 50 ms time window.

$$s_t = \begin{bmatrix} lr_t & \cdots & lr_{t+50ms} \\ ap_t & \cdots & ap_{t+50ms} \\ dv_t & \cdots & dv_{t+50ms} \end{bmatrix} \quad (1)$$

All segments of the right and left forelimb, s_{Rt} and s_{Lt} , were stored as the set $S \ni \begin{bmatrix} s_{Rt} \\ s_{Lt} \end{bmatrix}_{1,2,\dots,N}$, where s_{Rt} and s_{Lt} are vertically stacked. The sets, S , from different trials were horizontally concatenated. Therefore, the total number of segments N depend on the time of each trial and number of trials used for analysis. All segments in the set S were evaluated by classification scoring methods.

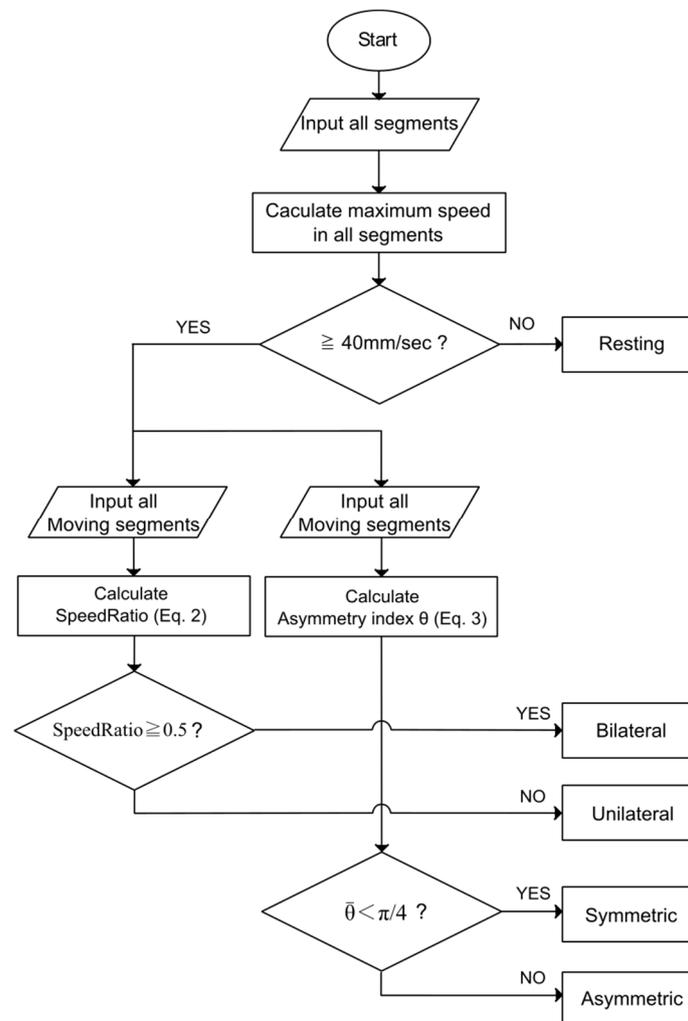


Figure 4-3. Decision tree for classification of segments.

Qualitative measure of forelimb movements and classification. All segments of behavioral data were analyzed by the following three steps (Fig. 4-3):

(1) Extraction of moving segments. I first defined the maximum speed function $\max(\bar{V}_R, \bar{V}_L)$, where \bar{V}_R and \bar{V}_L are mean speed in a segment s_R and s_L . The function returns the value of maximum speed among right or left forelimb in a segment. I defined the moving segment as $\max(\bar{V}_R, \bar{V}_L) \geq 40$ (mm/sec). That is, if mean speed of either left or right forelimb exceeded a threshold, the segment was classified as a moving segment. Conversely, resting segments were defined as $\max(\bar{V}_R, \bar{V}_L) < 40$ (mm/sec). The all moving segments were then analyzed by following two metrics: (2) Speed Ratio, and (3) Asymmetry index.

(2) Speed Ratio Function. Bilateral forelimb movement was considered to occur when movement amplitude across limbs remained uniform within a set limit; conversely, unilateral forelimb movement was considered to occur when there was significantly biased movement amplitude across limbs. To formalize the definition of bilateral and unilateral forelimb movement, the speed ratio of forelimbs was used (Equation 2).

$$SpeedRatio = \frac{\min\{\bar{V}_R, \bar{V}_L\}}{\max\{\bar{V}_R, \bar{V}_L\}} \quad (2)$$

The *SpeedRatio* function is a measure of laterality of speed across both forelimbs, where 1 indicates equal movement amplitude across the two forelimbs.

Movements were classified as bilateral or unilateral. A criterion of $SpeedRatio \geq 0.5$ was used to isolate bilateral movements across two forelimbs, indicating when one forelimb was moving at no more than twice the speed of the other forelimb. Conversely,

SpeedRatio < 0.5 was used to define unilateral movements, indicating when one forelimb moved at least twice as fast as the other forelimb in that segment. The boundary value was set to the half-maximum of SpeedRatio which is 0.5.

(3) Asymmetry index. Symmetric movements are also called mirror movements in cases where one limb moves as a mirrored copy of the contralateral limb. Physiologically, symmetry implies synchronized activation of homologous muscle groups, and asymmetry implies activation of non-homologous muscle groups. This definition is embedded in the egocentric framework discussed by Swinnen et al. (1998, 2001), in which movement is related to the longitudinal axis of the body and the coordination of corresponding limbs. The alternative, allocentric framework was not used in the present study because the limb movements were referenced to the body rather than the surrounding space. I defined symmetric movements as movement in the similar movement direction by both forelimb. Conversely, asymmetry index θ (Equation 3), is the angle between movement direction of the velocity vector of the left forelimb v_L (Equation 4) and the mirror transformed velocity vector of right forelimb v_{R_M} (Equation 5), estimated by the inverse cosine similarity function.

$$\theta = \cos^{-1} \left(\frac{v_{R_M} \cdot v_L}{|v_{R_M}| |v_L|} \right) \quad (3)$$

$$v_L = \left(\frac{\Delta l r_L}{\Delta t}, \frac{\Delta a p_L}{\Delta t}, \frac{\Delta d v_L}{\Delta t} \right) \quad (4)$$

$$v_{R_M} = \left(-\frac{\Delta l r_R}{\Delta t}, \frac{\Delta a p_R}{\Delta t}, \frac{\Delta d v_R}{\Delta t} \right) \quad (5)$$

The mean asymmetry index of a segment $\bar{\theta}$ was calculated from the mean of θ in a 50 ms time window.

With the estimated $\bar{\theta}$, symmetric and asymmetric movements were classified. A segment was defined as symmetric movement if $\bar{\theta} < \pi/4$ where the angle of movement direction of the left forelimb and mirrored right forelimb remained less than 45 degrees. Conversely, a segment was defined as asymmetric movement if $\bar{\theta} \geq \pi/4$, indicating that the angle between the two velocity vectors was greater than or equal to 45 degrees (Fig.3). The boundary value was based on the previous literature in which orthogonal lever press of two hands was defined as asymmetric bimanual movement (Cardoso de Oliveira et al. 2001). The present study used the value $\pi/4$ which is intermediate between perfect symmetric movement (0 degrees) and orthogonal asymmetric movements (90 degrees).

4.3 Results

Behavior in the apparatus during training. After habituation to head-fixation, all rats spontaneously entered the treadmill. Some rats were able to slide the chamber frame into the head attachment clamp without the experimenter's guidance. All rats were able to perform food handling while in the head-fixed position. The rats rarely dropped food during bimanual food handling, showing a mean success rate (food consumption without dropping) of $97.89 \pm 2.90\%$, and a mean consumption time of 27.92 ± 2.77 sec per trial ($n = 5$). Overall, head-fixation did not impede spontaneous food manipulation.

During food consumption, rats made periodic transitions between resting and moving. In the resting state, rats held the food item in a low position, and chewed on it. During movement, rats brought the food item to a higher position and dynamically manipulated it, changing the holding position and rotating the object. Frequently observed behaviors are shown in single video frames in Fig. 4-4A-D. Some of these behaviors have been reported by Whishaw and colleagues (Whishaw and Coles 1996; Whishaw et al. 2017b). Rats exhibited bimanual downward and upward reaching behaviors at different times. On first exposure to the food item, reaching down, grasping, and upward movement of the forelimbs occurred to bring the food item toward and against the mouth. These movements often punctuated the transition between resting and active states. In some cases, rats used the downward reaching behaviour to break the food item by tearing with the teeth. The bimanual and unimanual displacements involved in releasing and holding the food item were usually seen when rats changed their grasping position (Fig. 4-4C, D).

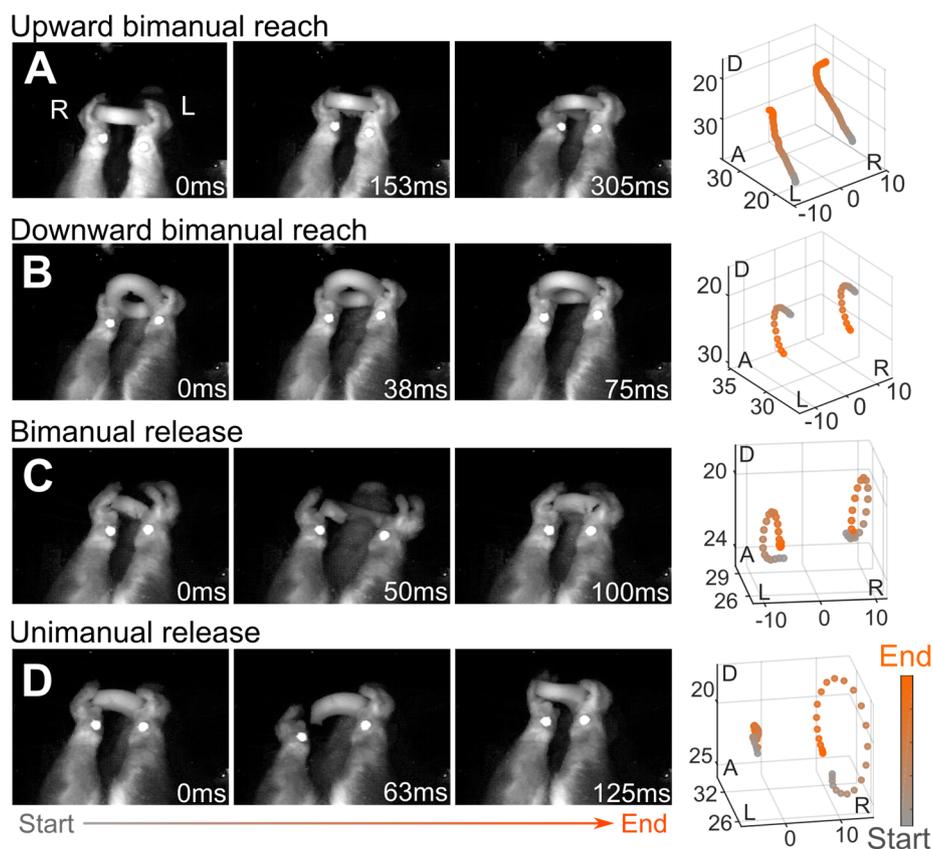


Figure 4-4. Representative behavior under head-fixed conditions.

Frames in the left three columns show four different behaviors. Scatter plots in the right column illustrate corresponding 3-D forelimb trajectory. The color scale indicates the normalized time. Note that the duration of each behavior is variable. (A) Bimanual upward reach. Both forelimb simultaneously move toward the anterodorsal side. (B) Bimanual downward reach. Both forelimb simultaneously move toward the posteroventral side. (C) Bimanual release. Both hand simultaneously release the food item and regrasp it to change the position of the hands. (D) Unimanual release. One hand release and regrasp of the food object with support of other hand. Abbreviations: D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

To interpret the 3-D trajectories of typical forelimb actions, I compared the video frames of representative manually identified behaviors with the corresponding 3-D scatter plots of wrist position marker coordinates (Fig. 4-4, right column). I found that the sequence of wrist positions as represented in the scatter plots clearly illustrated bimanual and unimanual behaviors. For example, upward bimanual reach was evident in the sequence of points indicating the position of each wrist as they shifted towards the ventral side (Fig. 4-4A). In unimanual movements the separation between the points corresponding to the moving wrist indicated larger displacements, contrasting with the closely spaced points corresponding to the other, relatively stationary forelimb (Fig. 4-4D). These observations illustrate the potential of analyzing the transition of wrist positions for quantifying several types of active forelimb states.

Full 3-D reconstruction of position of wrists. To provide a basic data set of the entire action sequence of an eating behavior, time series data of wrist positions in egocentric coordinates were generated from 77 trials across 5 rats. Representative trajectories of both wrists in the egocentric coordinate space are shown in Fig. 4-5. On the ventral side, forelimbs followed an arc-shaped relatively convergent trajectory, whereas on the dorsal side the trajectories diverged and became intermingled. The intermingled structure suggests that food manipulation consists of highly variable action patterns, such as symmetric/asymmetric bimanual movements and unimanual movements. To categorize forelimb use during continuous action sequences, I segmented the continuous time series data of wrist positions using a 50 ms sliding time window (Fig. 4-2D, E). The segments were generated from all data sets across 5 rats, and subjected to analysis in order to score and classify them into subtypes of unimanual and bimanual movements.

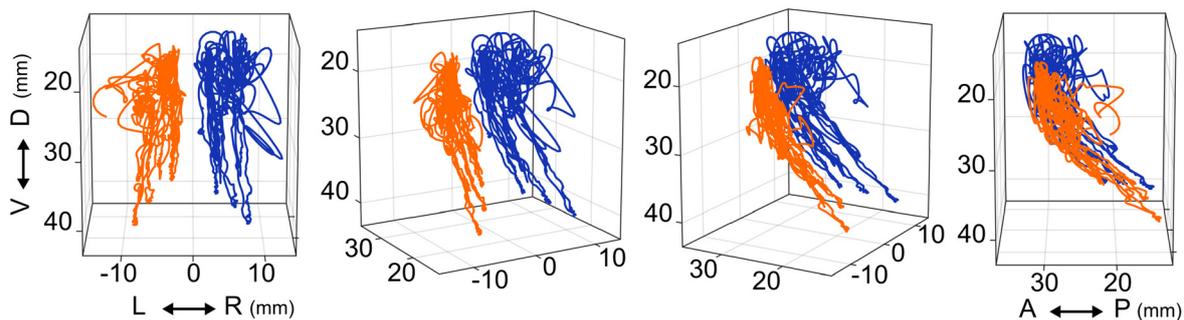


Figure 4-5. Reconstruction of the whole sequence of forelimb movement during spontaneous food handling behavior.

Positions of the right (blue) and the left (orange) forelimbs were captured by camera. Frames are shown rotated in 30° steps. Abbreviations: D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left.

Extraction of Moving segments. I considered movement to be occurring whenever the speed of one limb exceeded a threshold of 40 mm/sec (Fig. 4-6A-E). This non-zero criterion for “movement” was chosen because, even in the resting state, some physiological activity such as chewing, breathing, or sniffing, causes jittering of the forelimb position. The probability distribution of forelimbs speed showed a natural dip around 40mm/sec (Fig. 4-6A). The natural dip was also seen in the probability distribution of the processed speed variable which is maximum speed function (Fig. 4-6B). Thus, the thresholding process removed physiological movement artifacts. According to this criterion the portion of time spent moving was 0.29 ± 0.05 (Fig. 4-6C, $n = 5$).

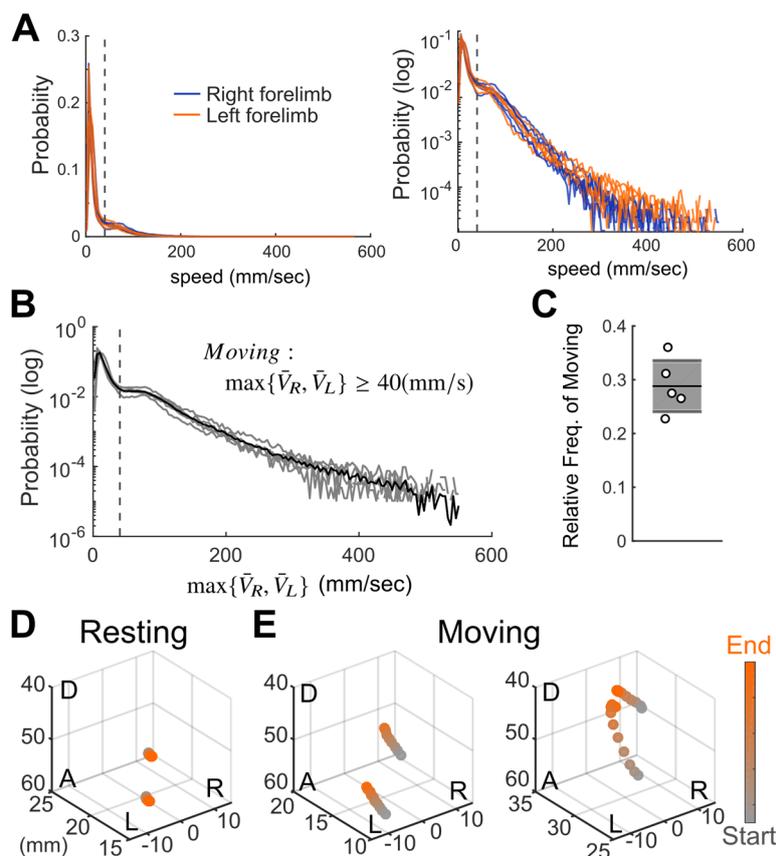


Figure 4-6. Moving segments were exclusively selected by maximum speed function.

(A) Probability distribution of speed of right and left forelimb movements across 5 rats. Left panel, linear scale. Right panel, logarithmic scale. Note the dip of probability density at the threshold indicated by the dotted line (40 mm/sec). (B) Mean probability distribution of maximum speed function across 5 rats. (C) Mean proportion of moving segments in all segments. (D-E) Example trajectories of the segments in the resting state (D) and during movement (E). Abbreviations: D, dorsal; A, anterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

Bilateral movements vs unilateral movements. The extracted moving segments described above included both bilateral and unilateral forelimb movements. I analyzed these movements, based on the laterality of movement speed across two forelimbs by applying the speed ratio function (Fig. 4-7). The speed ratio was defined as the ratio of mean speeds between two forelimbs in a

segment, based on the idea that both forelimbs move at similar amplitude for bilateral movements, while one forelimb moves faster than the other in unilateral movements (Fig. 4-7A-B). I found that the mean probability distribution of the speed ratio was biased towards 1, suggesting that the majority of forelimb movements during food handling were bilateral; conversely, unilateral forelimb movements were less frequent (Fig. 4-7B). Some representative segments of bilateral or unilateral forelimb movements based on the boundary value of 0.5 are shown in Fig. 4-7C, D.

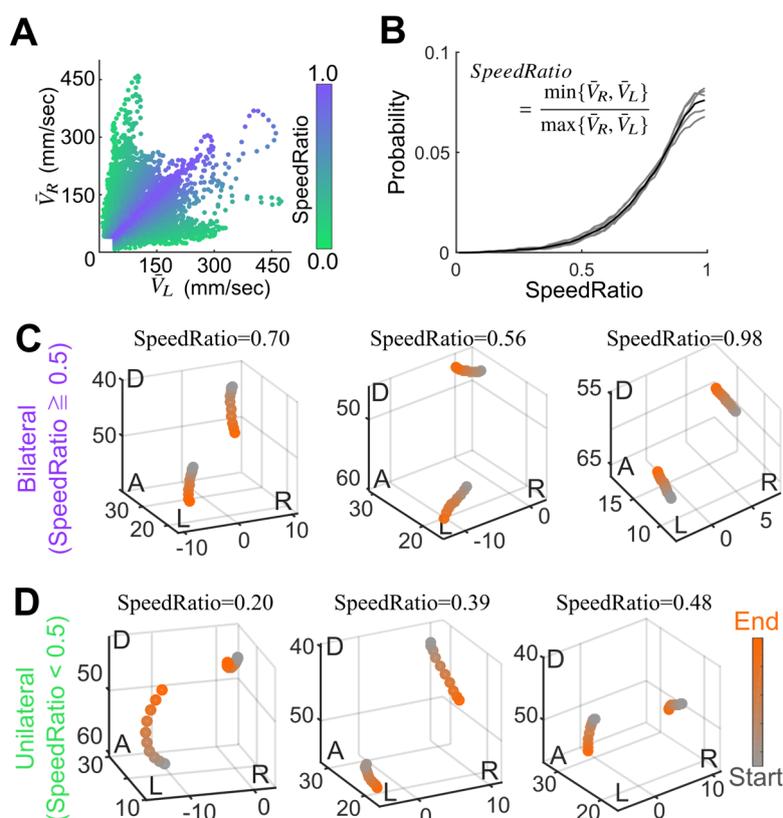


Figure 4-7. Bilateral and unilateral forelimb movements during food handling.

(A-C) Laterality of movement speed was quantified by speed ratio. (A) Graphical representation of speed ratio as a measure of laterality of left and right forelimb in speed. Each dot represents the mean speed of the right \bar{V}_R and left forelimb \bar{V}_L in a segment. The empty space at the left-bottom corner represents resting segments not included in the analysis. (B) Mean probability distribution of the speed ratio across 5 rats. (C-D) Example segments of bilateral and unilateral forelimb movements (C) and unimanual movements (D). Abbreviations: D, dorsal; A, anterior; R, right; L, left. Numbers in 3-D plot are

expressed in millimeters.

Symmetric vs asymmetric movement. One of the main purposes of the kinematic analysis of forelimb movements is to determine the relative amounts of symmetric and asymmetric movement during the natural sequence of food handling behavior (Fig. 4-8A-D). Symmetric bimanual movements are a subset of bimanual movements generated by the activation of homologous muscle groups across two limbs. Conversely, an asymmetric bimanual movement is caused by different (non-homologous) muscle groups. In the present study, symmetric bimanual movements were defined as those in which the movement direction of a forelimb mirrors the other forelimb with respect to the sagittal plane of the body (Fig. 4-8A). The asymmetry index θ is found by subtracting the angle of movement direction of a forelimb from the mirrored angle of the contralateral forelimb movement.

I assigned the asymmetry index θ to each segment, and calculated the probability density function of the asymmetry index. The probability distribution was significantly biased in the less asymmetric direction suggesting that the symmetric state predominates (Fig. 4-8B). Representative symmetric or asymmetric movements based on the boundary value of $\pi/4$ are shown in Fig. 4-8C, D.

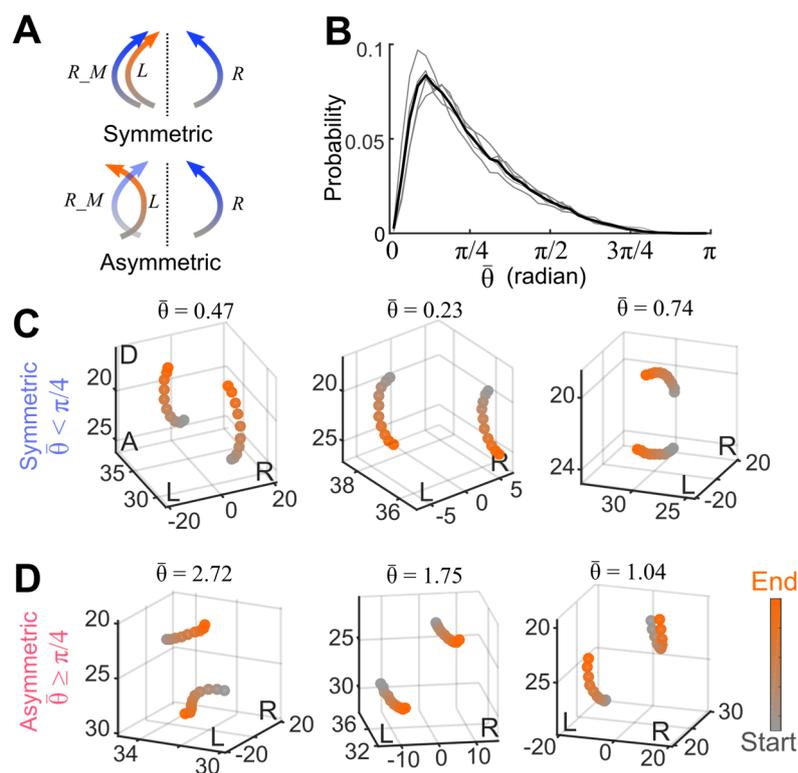


Figure 4-8. Symmetric and asymmetry forelimb movements during food handling.

(A-B) Asymmetry in movement direction was analyzed in terms of the error of movement vector direction between two forelimbs. (A) Graphical representation of mean angle of vector direction $\bar{\theta}$. Arrows indicates the example trajectory of left forelimb L and the mirrored right forelimb R_M . The asymmetry index $\bar{\theta}$ was calculated based on the error of movement vector direction between L and R_M . The dotted line illustrates the midline for the mirror transformation. (B) The probability distribution of the mean similarity of vector direction $\bar{\theta}$. (C-D) Examples of symmetric movement (C) and asymmetric movement (D). Abbreviations: D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

High-throughput analysis of kinematic data for quantification of forelimb movements. Finally, many (more than 430,000) segments from 77 trials across 5 rats were subjected to automatic analysis and classification. The speed ratio and asymmetry index of all moving segments were measured (Fig. 4-9). The classification algorithm (Fig. 4-3) was applied to all segments to illustrate the time series of the following motor behaviors: bilateral movement, unilateral movement, symmetric movement, or asymmetric movement. The time series data of those categories of motor behavior revealed the frequent transition of movement mode during feeding behavior (Fig. 4-9A and B). The transition of movement mode was visualized by overlaying the colored movement categories on the continuous 3D trajectories of forelimb position (Fig. 4-9C). The time fraction of unilateral versus bilateral and symmetric versus asymmetric movements were quantified. Relative frequency of each mode of forelimb movements revealed the organization of bimanual motor behavior during the natural sequence of eating (Fig. 4-9D). The mean percentages of forelimb use in respect of movement amplitude were 89% of bilateral movements and 11% of unilateral movements. The mean percentages of forelimb use in respect of movement direction were 41% of asymmetric movement and 59% of symmetric movement.

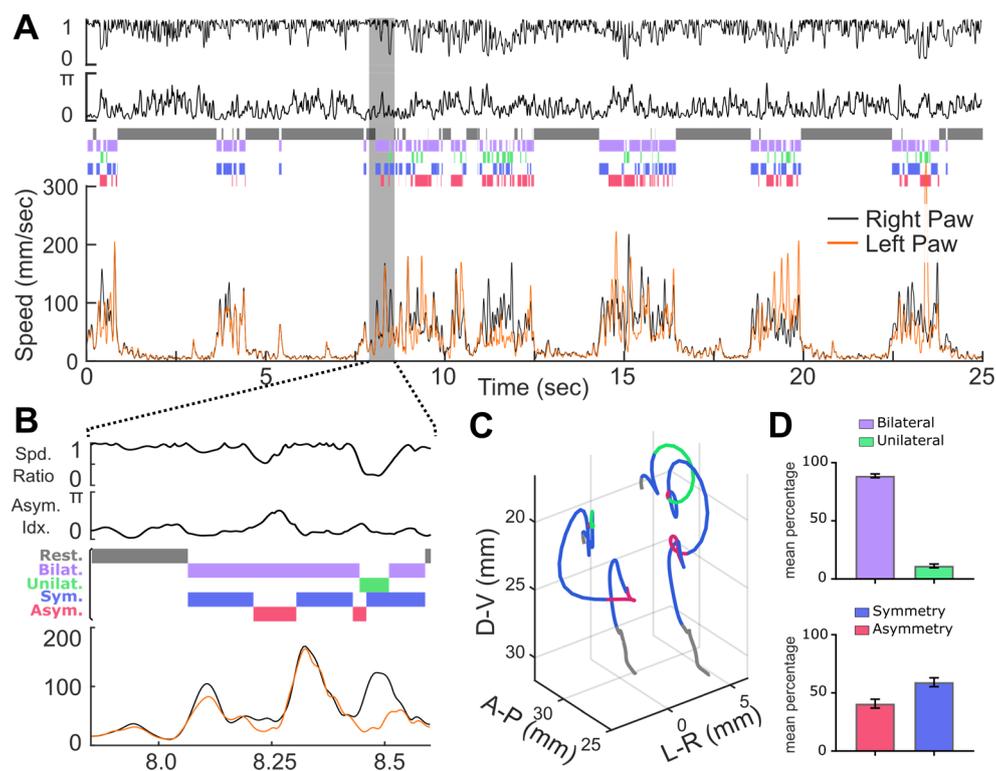


Figure 4-9. Analytical pipeline enabling high-throughput of kinematic data for quantification of bilateral forelimb movements.

(A) Pipeline of classification characterizes time course of behavioral states of spontaneous food handling behavior. The top two black traces indicate speed ratio and asymmetry indices respectively. The colored bars indicate the time of occurrence of each motor behavior defined by thresholding. The color code of each behavioral mode is shown in (B). Line graph indicates the speed of the right and left forelimb. (B) Magnified view of the shaded area in (A). (C) Corresponding actual trajectories of forelimbs. The color indicates the behavioral type shown in (B). Note that for clarity the color of unilateral movement overrides other categorizations when speed ratio exceeds the predefined threshold. (D) Quantitative analysis of behavioral types. Mean percentage of the behavioral type of movement classified in accordance with speed ratio (top) and asymmetry index (bottom). Abbreviations: Rest, resting; Bilat, bilateral forelimb movement; Unilat, unilateral forelimb movement; Sym, symmetric movement; Asym, asymmetric movement; D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left.

4.4 Discussion

I report on the development of a high-resolution tracking system for kinematic analysis of rat forelimb movements and its application to the study of bimanual coordination. The system uses optical motion tracking to obtain 3-D bimanual wrist movement trajectories from natural action sequences. The 3-D trajectories were used in the kinematic analysis of coordination of forelimb movements in head-fixed rats during food handling and consumption. Movement laterality and asymmetry across forelimbs was quantified in movement segments automatically extracted from the continuous action sequence. Results showed that the speed of forelimb movement during eating behavior were highly balanced bilaterally. Symmetry in movement direction was more frequently observed than asymmetry. However, a considerable amount of asymmetry in movement direction was also observed. To our knowledge, this is the first application of this method to visualizing bilateral forelimb trajectories during spontaneous food handling behavior in rodents, extending previous studies of food handling behavior.

Limb use in spontaneous food handling behavior was first reported as a method of motor assessment in rats by Whishaw and Coles (1996). Since then, this quantitative method has been widely used to assess motor function in research on movement disorders and motor control (Allred et al. 2008; Brown and Teskey 2014; Manfré et al. 2017; Tennant et al. 2010; Wang et al. 2017; Xu et al. 2009). In these earlier studies, assessment of forelimb motor skills relied on manual identification. Grasping pattern, position of forelimb, timing of adjustment as well as global scores such as consumption time and drop rate required manual observation of video frames. The present study extends this method of assessment of motor skill in food handling behavior by using a kinematic tracking system. The sub-second kinematic information obtained from this system enables detection of subtle changes in behavior, such as changes in the ratio of symmetric to asymmetric activity during bimanual movement. Such measurements are difficult to obtain by manual observation of video frames.

Quantifying the incidence of specific motor patterns during natural action sequences is challenging. To make quantitative analysis feasible, the study of motor control often focuses on trained, repetitive, uniform action sequences such as skilled reaching, and lever pressing tasks (Guo et al. 2015; Hira et al. 2013; Isomura et al. 2009; Kawai et al. 2015; Palmér et al. 2012). Measures of such motor patterns are low dimensional, not requiring extensive data processing, unlike more natural sequences. In contrast to these more uniform action sequences, food handling behavior involves highly variable action sequences of forelimb movements. While the analysis of such sequences is more demanding, they provide good examples of naturally occurring bimanual coordination.

To quantify natural action sequences, it is necessary to identify specific behaviors when they occur. Segmentation with a sliding window, as used in the present study, is commonly used to detect behavioral events in time series data sets such as moving pictures and multi-point body kinematic information, in human as well as animal behavior (Burgos-Artizzu et al. 2012; Liu et al. 2009). Once the analytical criteria for a specific behavior – the “*detector*” – has been defined, the behavior can be identified in the continuous sequence dataset. Our mathematical definitions of bimanual movements were used to detect bilateral versus unilateral, and symmetric versus asymmetric forelimb movements within the natural action sequence data (Fig.9).

In the present study, I demonstrated the use of a movement asymmetry index and speed ratio for quantifying asymmetry of movement direction and laterality of movement speed. A limitation of this strategy is that each class of movement includes actions which may be mediated by different neuromotor channels (Whishaw et al. 2017a). For example, movements classified as symmetric bilateral forelimb movements in the present study involve movement symmetry with bimanual holding (Fig.4A) and bimanual release (Fig.4C). Specific motor

behaviors such as movement of hand to mouth, or reaching, may require a distinct movement detection algorithm. For instance, the distance between mouth position and forelimb position could be useful in defining hand to mouth movements. Another limitation is that the present movement detection has the threshold of 40mm/sec. The threshold would not permit the detection of slow bilateral forelimb movements such as the moment during transition from resting to upward reach. To study, in particular, slow upward motion arising from rest, another definition of movement onset based on position, such departure from a delineated area defined as resting position, might be useful. Recently, dimensionality reduction algorithms and machine learning approaches have captured action repertoires from natural action sequences (Berman et al. 2014; Robie et al. 2017). Further development is needed for exploring bimanual action patterns from bimanual food handling behavior.

The proposed mathematical definitions of symmetric and asymmetric bimanual movement were based on movements of the forelimb markers. Physiologically, however, symmetric versus asymmetric bimanual movements are distinguished by the pattern of activated muscle groups across limbs. For instance, simultaneous activation of homologous muscle groups generates symmetric bimanual movements. In contrast, activating different muscle groups with the same timing causes asymmetric bimanual movements. The present definition of the asymmetry, index θ , is the directional error between movement vectors of forelimbs calculated by the cosine similarity function. It is based on the idea that the activation of identical muscle groups across forelimbs results in mirror-image endpoint trajectories. This implicitly assumes that movement and muscle activity are measures of the same thing. However, it should be noted that significant physical perturbations may occur and cause, in response, changed muscle activation patterns even though the trajectory of the forelimb marker is unchanged. Thus, in the proposed system, perturbations such as bumping a part of the head-fixing device, should be excluded from the analysis.

Using the proposed system in the present study led to the finding that both asymmetric and symmetric bilateral movements occur in food handling behavior, with symmetric bilateral forelimb movements quantitatively more commonly observed. In rodents, previous studies of forelimb use have observed laterality in grasping (a release to re-grasp movements against food object) and holding position asymmetry in food handling (Allred et al. 2008; Whishaw and Coles 1996; Whishaw et al. 2017b). The present study further extended the previous results by adding that asymmetry was observed in dynamic bilateral forelimb use in rats. Our results suggest that symmetric bilateral forelimb movements were more frequently observed than asymmetric bilateral forelimb movements during handling of donut-shaped food reward. The shape of the food may have been a factor in the symmetry of the hand to mouth movements in feeding, functionally linking the forelimbs together when they were used to bring the food item to the mouth (Fig. 4A). Another possible interpretation is that the animal has a natural tendency towards symmetric movements, which has been reported in various experimental conditions in humans (Swinnen 2002). Symmetric forelimb movements might be a fundamental mode of bilateral forelimb movements in rats, however, this idea needed to be investigated further.

The use of awake head-fixed rats under food restriction is less frequently reported than their use with water restriction, with the most recent report more than 10 years ago (Heck et al. 2007). Technical aspects of shaping behavior by food reward may be a factor in the less frequent use of food restriction. The training of head-fixation in rats used graded exposure methods (Schwarz et al. 2010), based on compensating restraint anxiety with reward. In many experimental paradigms, water reward is easy to provide with a spout while animals remain restrained. In contrast, providing solid food items prompts rats to return from the restrainer by backward locomotion, making it difficult for the animal to associate the reward and environment. We, therefore, delivered jelly reward via stainless spout to guide rats to the head-

fixed position, instead of providing pieces of solid food. In addition, a linear passive treadmill that I implemented significantly buffered backward locomotion reducing restraint stress on the animal. The two approaches synergistically improved training efficiency.

Bimanual coordination deficits are observed in neurodegenerative disorders such as Parkinson's disease (Almeida et al. 2002; Johnson et al. 1998; Vercruyssen et al. 2014), Huntington's disease (Brown et al. 1993; Johnson et al. 2000; Verbessem et al. 2002), Alzheimer's disease (Martin et al. 2017), and traumatic brain injury (Caeyenberghs et al. 2011; Gooijers et al. 2016). Parkinsonian patients have difficulty in asymmetric bimanual coordination (Almeida et al. 2002; Ponsen et al. 2006; Stelmach and Worringham 1988). Recent evidence suggests that recovery from hypokinesia in Parkinson's disease is not necessarily correlated with improvement in coordinated bimanual movements (Almeida and Brown 2013; Daneault et al. 2016; Igarashi et al. 2015). The decline in bimanual motor performance is also seen in healthy aging (Serbruyns et al. 2015). The unique mechanisms of bimanually coordinated movement need to be further studied to advance understanding of physiological mechanisms of neurodegenerative disorders and aging. I suggest that the presented measurement will illuminate bimanual coordination as a target of investigation by new – but almost exclusively rodent-based – research tools such as optogenetics, chemogenetics, *in vivo* electrophysiology, and multi-photon imaging.

4.5 Acknowledgements

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Chapter 5: The role of anterior corpus callosum in bilateral coordination.**5.1 Introduction**

Bilateral coordination of the upper limbs is crucial for many functions of daily life. Humans exhibit advanced capabilities for inter-limb coordination. For example, controlling a steering wheel while turning a corner requires both hands and forearms. Similarly, bilateral coordination is necessary for the most basic needs, such as feeding, which are also important for non-human primates and rodents. To achieve bilateral coordination, information exchange between the two sides of the body is required. Given that the primary motor cortical representations are lateralized, ie, the sides of the body are represented in separate hemispheres, an interconnecting neural structure is crucial for coordinating the right and the left side. The corpus callosum is a direct commissure between cerebral hemispheres, which may provide information exchange between contralateral cortices. In this chapter, I investigate the role of the corpus callosum and the inter-hemispheric cellular network in bilateral coordination of the limbs.

The corpus callosum is thought to be important for bimanual coordination because it provides neural connections between homologous cortical regions across two cerebral cortices. Such connectivity seems to favor symmetrical movement, by supporting correlated activity in homologous representations bilaterally. In support of this idea, a spontaneous transition from asymmetric movements to symmetric movements has been reported in many experimental conditions, such as rhythmical bimanual finger tapping/drawing, bimanual circular drawing (Kennerley et al. 2002; Swinnen et al. 1998). Studies of spatial synchronization in patients who have had callosotomy suggest that the corpus callosum is a cause of this spatial synchronization tendency (Eliassen et al. 1999; Franz et al. 1996). However, callosotomy patients had less difficulty in familiar bimanual actions such as tying shoes and opening drawer raises a question the role of corpus callosum in general motor behavior (Franz et al. 2000; Serrien et al. 2001). Moreover, it has been unclear whether the corpus callosum causes excitation of opposite cortex; and there is some evidence that callosal inputs cause inhibition of contralateral cortex via

inhibitory interneurons (Hiraoka et al. 2014; Hubers et al. 2008; Palmer et al. 2012; Pluto et al. 2005).

Experimental studies of the motor functions of the corpus callosum in rodents have been relatively limited to date (Li et al. 2016; Mohn and Russell 1981; Noonan and Axelrod 1992; Sullivan et al. 1993). Rodents exhibit dexterous bimanual coordination when feeding, holding, manipulating, and bringing the food object to mouth suggesting the efficient information exchange across two hemispheres. The previous chapter developed measurement technique of bilateral forelimb movements during spontaneously handling food object using high-speed cameras and head-fixing apparatus. The system captured fine kinematic parameter of forelimb movements bilaterally. In this chapter used the developed measurement system to quantify symmetric and asymmetric bimanual movements in rats, and measure the effects of temporary and reversible lesions of the corpus callosum.

In this chapter, the contribution of the corpus callosum was investigated by measuring food handling behavior in head-fixed rats. Our working hypothesis was that the motor parts of the corpus callosum are important for symmetry in movements. To address this question, I studied the topological organization of the motor corpus callosum connections between the two forelimb motor areas using a neural tracer. I found that the motor cortical interhemispheric connections ran through the anterior part of the corpus callosum in rats, and confirmed that this motor cortex to motor cortex callosal connection was silenced by a sodium channel blocker. I then examined the organization of forelimb movements during eating using 3-D kinematic analysis. Kinematic analysis was used to test two specific hypothesis: i) motor corpus callosum mediate synchronization of forelimb movement speed, ii) motor corpus callosum mediates symmetry of movement direction. I found that suppressing the motor corpus callosum connections altered the ratio of movement symmetry toward more asymmetry in movement

direction of forelimbs. However, laterality of movement speed between left and right was unchanged. Similarly, other aspects of skilled forelimb use, such as time of consumption, were unchanged. Bilateral multiunit recordings from corresponding cortical areas showed positively correlated activity patterns in the majority of interacting pairs, and a substantial portion of negative correlation. Our analyses suggest that the anterior corpus callosum modulates the degree of symmetry of forelimb bimanual movements via both excitatory and inhibitory connections of two motor cortices.

5.2 Materials and methods

Animals. Male Long Evan (LE) rats weighing 350-450 g were used in the study. Rats were kept under a reversed 12 hrs light/dark cycle, constant temperature and humidity with ad libitum access to water and food before food restriction. Animals were habituated to the experimenter for more than three days before the start of behavioral training. All experiments in the present study were approved by the Committee for Care and Use of Animals at the Okinawa Institute of Science and Technology.

Tracing callosal fibers from forelimb motor areas. 7-9 weeks old male LE rats were anesthetized with isoflurane (4.0% induction, 1.5-2.0% maintenance). To visualize the axonal fiber bundle, 1,1'-Diiododecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI, Invitrogen, USA) was used as a non-viral neural tracer. 2% DiI solution was prepared by dissolving DiI crystals in 100% ethanol. 100 nL of the 2% DiI solution was then slowly injected into either rostral forelimb motor area (RFA) or caudal forelimb motor area (CFA) at 1 nl/sec (Nanoject III, Drummond Scientific). The coordinates for the RFA and CFA in male Long Evans rats were based on previous reports (Brown and Teskey 2014; Neafsey and Sievert 1982) (RFA: 3.2 mm anterior and 2.3 mm lateral from bregma, 0.8 mm deep from cortical pia; CFA: 1.0 mm anterior and 2.5 mm lateral from bregma, 0.8 mm deep from cortical pia). Animals were kept alive for two weeks to allow for the diffusion of DiI injections. Subsequently, rats were then euthanized with pentobarbital and perfused with 100 mL of heparin saline solution followed by 100 mL of 4% PFA in Phosphate Buffer (PB). Brains were removed and post-fixed with 4% PFA for 2 hours followed by two days of saturation in 30% sucrose PB solution. The brains were sectioned at 100 μ m and the slices were then rinsed in PB and nuclear DNA was stained with NucBlue™ Fixed Cell Stain using the manufacturer's protocol (Life Technologies,

Grand Island, NY). The fluorescence of DiI and NucBlue™ were observed under a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan) using DAPI and mCherry filters.

In vivo anesthetized LFP recordings. Eight male LE rats were used for LFP recording. Animals were placed on a stereotaxic frame under isoflurane anesthesia (3.0% induction, 1.5% maintenance and recording). Two millimeters square craniotomies centered on 0.5 mm anterior, 1.5mm lateral to the bregma were made on both right and left sides of the skull. This location corresponds to the central region of CFA in Long Evans rats (Brown and Teskey 2014; Neafsey and Sievert 1982). A 16-ch array silicone probe (A1x16-5mm-150-413, NeuroNexus, USA) was inserted to the depth of 2.2 mm from the pia to record from the entire cortical laminae. A concentric bipolar stimulation electrode (CBARC_75, FHC Inc., ME, USA) was slowly inserted 1.0 mm from the cortical pia contralateral to the silicone probe. Lidocaine was injected into the anterior corpus callosum using a glass pipette connected to a microinjector (Nanoject III, Drummond Scientific, PA, USA, 0.5 mm anterior and 0.8 mm lateral from the bregma, 2.9 mm deep from the cortical pia). A wideband signal was recorded using *OmniPlex D multichannel recording system* (Plexon, TX, USA). The signal was filtered with a 200 Hz lowpass cutoff Bessel.

Recording of stimulus-evoked field potentials. Intracortical microstimulation (ICMS) was applied to measure cortico-cortical connections. Cathode-lead biphasic current pulses (± 150 μ A, 1 ms) were applied every 10 seconds using a programmable stimulus generator (STG4004, Multichannel Systems, Germany). Stimulus-evoked LFPs were continuously recorded for 40 minutes. The first 10 minutes were recorded and used as baseline. Immediately after, 500nL of 2% Lidocaine solution was injected at 1nL/sec and stimulus-evoked LFPs were recorded for 30 min. The recorded 16-ch LFP data set was overlaid by the onset of current stimulation, and the mean of the stimulus-evoked LFP over multiple trials was computed using Neuro Explorer

(Nex Technologies, MA, USA). The *mean peri-event LFP* traces were then exported to MATLAB for current source density (CSD) analysis. Inverted current source density (iCSD) plots were generated using iCSD plot toolbox for MATLAB distributed by Pettersen et al. (2006). After completion of LFP recordings, a 10 μ A current was applied for 10 sec to identify the location of the electrode tip. Rats were then perfused with 4% PFA for histology, the brains were sectioned with, a vibratome and stained with NucBlue™. Fluorogold and NucBlue™ were imaged under a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan) using DAPI and GFP filters.

Surgery for head-fixation. Ten to twelve week old male LE rats weighing 350-450g were used for behavioral experiments. Rats were anesthetized with isoflurane (3 - 4% induction, 1.5 - 2.5 % maintenance), and placed on a stereotaxic frame (SR-10R-HT, Narishige, Japan). The detailed procedures of implantation of head-plate has been previously described (Igarashi and Wickens, 2018). Briefly, the skull was exposed and carefully cleaned with saline and cotton swabs. The eight anchor screws drilled to the skull were then covered with a layer of Super Bond (Sun Medical Inc., Japan). A chamber frame (CFR-1, Narishige, Japan) was positioned and secured by additional dental cement. Dietary supplement with Carprofen (Medigel CPF; Clear H₂O, ME., US.) was given during post-op recovery for 5 days.

Spontaneous food handling under head-fixation. Rats were placed on the food deprivation protocol 1 week before habituation. Behavioral testing was conducted during the middle hours of the dark cycle (10am – 4 pm, reversed light cycle). At the time of testing, the last feed had been given to the animals on the previous day. Testing was conducted on one animal at a time in a quiet room. Rats were habituated to the experimental chamber for three days, and gradually guided to the head attachment clamp by the experimenter using a sweet jelly reward (Igarashi and Wickens, 2018). To elicit bimanual motor behavior, modified version of spontaneous food

handling task, originally proposed by Whishaw and Coles (1996), was used. The annular shaped food reward (20 mm outer diameter, 10 mm inner diameter, 5 mm thickness, Fish Sausage, Marudai Food Co., Ltd, Japan) was used instead of vermicelli or pasta. Trials started with bimanual grasping of the food reward offered by the experimenter, and use of forelimbs during consumption were recorded. A successful trial was defined as a complete consumption of a single food reward without dropping. Rats underwent 6 trials in a day and continued for 6 days (Fig.5-3). Cases where rats showed unusual behavior, such as adopting a tripedal stance during eating, were excluded from further analysis.

Intracallosal drug infusion. After training of behavioral test, rats were anesthetized with 2% isoflurane, placed in a stereotaxic frame, and implanted with a stainless guide cannula (26G, 7mm, PlasticsOne, VA, USA) into the anterior part of the corpus callosum (1.0 mm anterior posterior and 0.8 mm lateral from bregma; 2.0 mm ventral from cortical pia). Due to the blood vessels along the sagittal sinus, the cannula was placed 0.8 mm lateral from the midline. The cannula was fixed by dental cement (Super-Bond, Sun Medical Inc., Japan) and secured by a dummy cannula (7mm, PlasticsOne, VA, USA). A dietary supplement of Carprofen (Medigel CPF; Clear H₂O, ME, USA) was given during post-op recovery for 5 days. 15 min prior to behavioral test sessions, 500nl of 2% Lidocaine (dissolved in saline) was injected via an internal cannula (1.0 mm exposure from guide cannula) using a 10 µl gas tight syringe loaded on a syringe pump (KDS-101, KD Scientific Inc., MA, USA). Lidocaine was slowly injected at a rate of 1.67nl/sec (5 min total) and the internal cannula was kept in position for 5 min to allow diffusion. After completing six sessions of behavioral experiments, rats were perfused. The brains were sectioned and the loci of the tip of cannula was confirmed by histological analyses (Nissl staining).

Recording of forelimb motor behavior and 3-D reconstruction. On the day of the behavioral recording, 3 mm diameter half-spherical reflective markers were attached to the lower side of the wrists with double-sided tape. Rats were head-fixed in the custom-made apparatus (SR-10R-HT, Narishige, Japan; Fig.). The reflective markers attached on the forelimb were tracked during food handling by two high-speed cameras (HAS-L1, $f = 6\text{mm}$, DITECT, Tokyo, Japan) positioned below the transparent acrylic floor. All trials were recorded at 200 frames (1/500s exposure time and 600x800 pixel) per second and stored in hard disk. The positions of reflective markers were semi-automatically traced and reconstructed using MATLAB programs (The MathWorks, Inc., MA., USA). The positions of the reflective markers were represented as a time series data in the camera coordinate $[x, y, z]^T$, where $x = [x_1, x_2, \dots, x_t]$, $y = [y_1, y_2, \dots, y_t]$, $z = [z_1, z_2, \dots, z_t]$. The data $[x, y, z]^T$ were transformed into the egocentric coordinate system $[lr, ap, dv]^T$ using a reference frame based on the head-fixed apparatus (Igarashi and Wickens, 2018), where lr, ap, dv corresponds to time series data of marker position in left-right (lr) axis, anterior-posterior (ap) axis, and dorsal-ventral (dv) respectively.

Analysis of kinematic data. To analyze organization of bilateral forelimb coordination, laterality of movement speed and asymmetry in movement direction were computed. The procedures were detailed by Igarashi and Wickens (2018). The kinematic data was analyzed by following three steps:

- (1) *Detection of forelimb movements.* Rats demonstrated frequent transition between the resting states and the active use of forelimbs during food consumption. The active use of two forelimbs was detected by the maximum speed function $\max(\bar{V}_R, \bar{V}_L)$, where \bar{V}_R and \bar{V}_L are mean speed computed from $[lr, ap, dv]^T$ by each 50 ms sliding time window. The threshold of moving detection was set to $\max(\bar{V}_R, \bar{V}_L) \geq 40$ (mm/sec), that is, if mean speed of either left or right forelimb exceeded 40 mm/sec the time frame was defined as

movement. The value of threshold was previously validated (Igarashi and Wickens, 2018). Conversely, the resting state $\max(\bar{V}_R, \bar{V}_L) < 40$ (*mm/sec*) was excluded from further analysis.

(2) *Speed ratio*. The extracted active movements were further analyzed by speed ratio function $\frac{\min\{\bar{V}_R, \bar{V}_L\}}{\max\{\bar{V}_R, \bar{V}_L\}}$. The speed ratio function was used to compute synchronization of movement speed across two forelimbs disregarding movement direction. The speed ratio function receive the speed of left and right forelimbs \bar{V}_R and \bar{V}_L , and returns the ratio between the larger value and the smaller value (e.g. \bar{V}_R/\bar{V}_L in the case of $\bar{V}_R < \bar{V}_L$). SpeedRatio = 1 indicates the speed of two forelimb are identical (bilateral movements), whereas the SpeedRatio = 0 suggests the speed of one forelimb is zero (unilateral). The bilateral and unilateral forelimb movements were detected by threshold set to 0.5.

(3) *Asymmetry index*. Asymmetry in movement direction was computed using inversed cosine similarity function $\theta = \cos^{-1}\left(\frac{v_{R_M} \cdot v_L}{|v_{R_M}| |v_L|}\right)$, where theta is a measure of angle between movement vector of left forelimb v_L and the mirrored right forelimb v_{R_M} . The v_{R_M} was obtained by mirror transformation of movement vector of the right forelimb v_R with respect to sagittal plane. The mean asymmetry index $\bar{\theta}$ was computed in each 50 ms sliding time window. For quantitative analysis of the asymmetry index during behavioral test, threshold value of $\pi/4$ (45 degree) was used to classify into two categories: i) symmetric movements, ii) asymmetric movements. Orthogonal lever press of two hands has been used as asymmetric bimanual movements, which is neurophysiologically significantly different from perfect symmetry (0 degree) (Cardoso de Oliveira et al. 2001). The present study used the value $\pi/4$ which is intermediate between perfect symmetric movement (0 degree) and orthogonal asymmetric movements (90 degree).

Global scores of spontaneous food handling behavior were computed, including mean speed of forelimbs, rate of successful food consumption, mean time of completion of food intake, and cross correlation between forelimb.

Electrophysiological recordings. Multiunit activity of bilateral motor cortices was recorded from awake, head-fixed rats. Two well-trained rats were subject to electrophysiological recording. Craniotomy was performed with the same procedure of the implantation of chamber frame. 64 channel custom made flex-drive was implanted to the motor cortex. Eight tungsten tetrodes array (12.5 μ m, California fine wire) were inserted in each hemisphere around the coordinate of caudal forelimb area (+1.0 mm from the bregma, 2.5 mm lateral from the midline, 1.0 mm deep from the cortical surface). The flex drive was fixed with dental cement. Continuous spike data was recorded after a 40 kHz digital bandpass filter from wideband signal using OmniPlex D Data Acquisition System (Plexon, TX, USA). To reduce chewing artifact, either common average filter or common mean filter was applied during recording. After the data acquisition, spike sorting was performed using the Plexon Offline Sorter and the spike data were analyzed by the MATLAB. Marker lesions (10 μ A, 10 sec) were made to identify the location of electrode tip. The rats were perfused with 4% PFA for histology after the completion of all recording sessions. The brain were sectioned with vibratome and Nissle-stained to localize recording site.

RS and FS neuron isolation. The isolated units from CFA were clustered into regular spike (RS) units and fast-spike (FS) units (Soma et al. 2017). RS units are putative excitatory neurons, whereas FS units are putative inhibitory neurons (Bruno and Simons 2002; Kim et al. 2016). RS unit exhibit wider waveform compared to FS units (Fig. 5-5 C) showing bimodal distribution of spike duration (Fig. 5-5 D), therefore, the recorded units were clustered into RS

and FS units based on spike duration (Fig. 5-5 D). The histogram of spike duration was fitted by two-components Gaussian Mixture Model (GMM) using MATLAB curve fitting tool box. The two-component GMM was defined by $\sum_{i=[RS,FS]} \phi_i \mathcal{N}(d|\mu_i, \sigma_i)$ where d is duration of spike waveform, and $\mathcal{N}(d|\mu_i, \sigma_i) = \frac{1}{\sigma_i \sqrt{2\pi}} \exp\left(-\frac{(d-\mu_i)^2}{2\sigma_i^2}\right)$. Based on the border of first and second component of GMM, units were clustered into RS units and FS units (0.484 ms; Fig. 5-5 D).

Cross-correlation matrix. Pairwise cross-correlation of spike trains was computed for all combination of recorded pairs. Spike data were transformed to continuous spike density function using Gaussian kernel (Wallisch et al. 2009). The spike binary data were convoluted by Gaussian kernel ($\sigma = 35$ ms) to generate continuous spike firing density of all units in layer V of cortex. Spearman's correlation matrix of the spiking activity was then computed for the spike firing density data to generate similarity matrix of correlation coefficient. The computed correlation coefficient of all pairs of units was then used for empirical cumulative probability distribution function (ecdf). To quantitatively show the proportion of positive and negative correlation of all pairs of units, correlation coefficient r was classified based on Evans's definitions, "weak" $0.39 > |r| > 0.2$, "moderate" $0.59 > |r| > 0.4$, "strong" or "very strong" $|r| > 0.6$ (Evans 1995).

5.3 Results

Callosal projection topography of forelimb motor areas. The corpus callosum (CC) provides interhemispheric cortico-cortical connection. In human, the CC is functionally organized along anteroposterior axis with respect to the origin of cortical areas (Doron and Gazzaniga 2008). It has reported that the pre motor and motor cortical projection to the contralateral hemisphere were bundled at the posterior body and isthmus of the corpus callosum in human (Hofer and Frahm 2006; Wahl et al. 2007). However, it was unclear whether the topography of the motor corpus callosum in human is conserved in rodents. To identify the callosal bundle from motor forelimb areas in rats, the neural tracer DiI was injected into two identified forelimb motor areas: the rostral forelimb area (RFA) and the caudal forelimb area (CFA) (Fig. 5-1A). The DiI positive axonal fibers were clearly identified in their course through the corpus callosum (Fig. 5-1B). Contrary to the motor corpus callosum in human, DiI positive fiber bundles from both CFA and RFA coursed mainly in the anterior part (genu) of the corpus callosum (Fig. 5-1C). The DiI positive fiber bundle of RFA were found more anteriorly than CFA (Fig. 5-1D). Most of the callosal fibers from RFA and CFA were observed anterior to bregma. No DiI positive axonal fiber bundles were found in the posterior part of the corpus callosum (data not shown). These results suggest that the motor components of the corpus callosum in rodents propagate mainly through the anterior part.

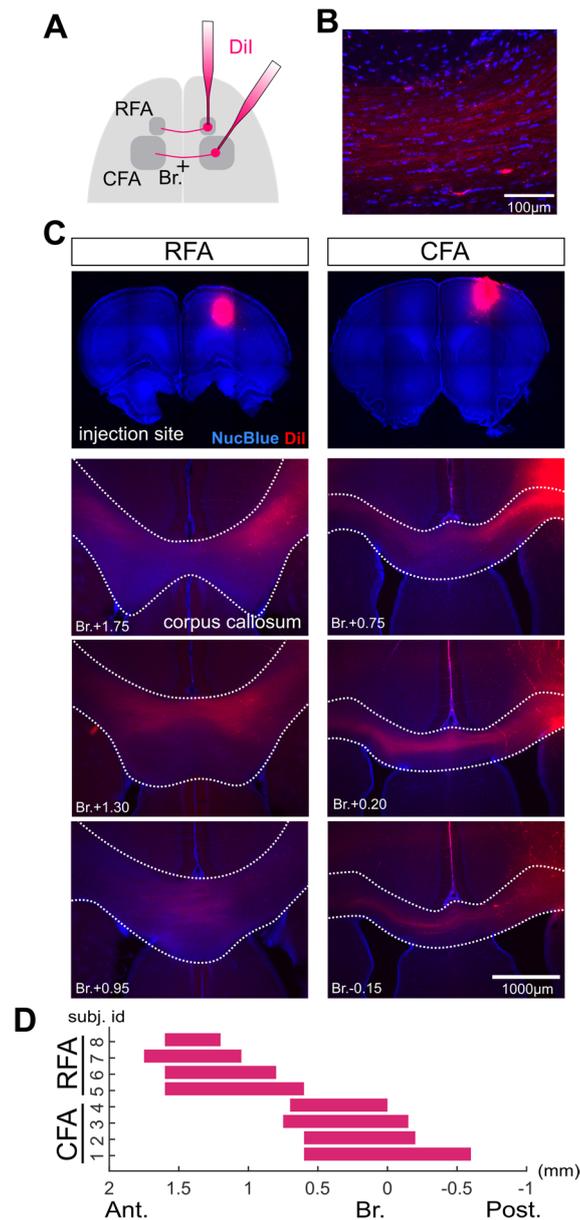


Figure 5-1. Location of motor corpus callosum in LE rats.

(A) Schematic diagram of callosal axon fiber bundle labeling by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). Axonal projections from two forelimb areas were labelled by an injection of DiI in either the rostral forelimb area or the caudal forelimb area. (B) Representative DiI positive callosal fibers from CFA. (C) Anteroposterior profile of motor callosal path. The top two panels show the injection site. The following six panels below show the DiI positive callosal projection from RFA and CFA though anterior part of corpus callosum. (D) Summary of the anteroposterior location of axonal fiber bundle projection from CFA (n = 4, subject id 1, 2, 3, 4) and RFA (n = 4, subject id 5, 6, 7, 8). Scale bars, 100 µm (B), 1000 µm (C).

Pharmacological inactivation of corpus callosum. To study the causal role of aCC, pharmacological blockade of the corpus callosum was used. Lidocaine was used to attenuate axonal conductance on the corpus callosum by blockade of the sodium channel. To validate the efficacy of attenuation *in vivo*, the strength of cortico-cortical connection was monitored by stimulus-evoked LFP response. Intra-cortical current stimulation (ICMS) were delivered to 1.0mm deep from cortical pia while silicone probe recording electrode was placed in the contralateral side of electrical stimulation (Fig. 5-2A). The ICMS evoked population excitatory postsynaptic potential (pEPSP) in the contralateral cortex (Fig. 5-2B). The amplitude of pEPSP indicates the strength of cortico-cortical connectivity. To discriminate the monosynaptic event from the disynaptic event, the time delay of LFP response followed by electrical stimulation was computed. The delay of LFP sink in the present study was 13.13 ms (Std. Deviation = 2.031 ms), which is consistent with previous reports measured by antidromic spike (Soma et al. 2017; Wilson 1987), suggesting the LFP sink is excitatory monosynaptic event. Next, the current source density (CSD) profile was computed from these LFP traces. The cationic current flux from the extracellular to intracellular (sink) was colored by red (Fig. 5-2D, E). The CSD profile of the LFPs showed a significant sink (positive currents leaving extracellular medium) response at 1.05 mm from cortical pia (Std. Deviation = 0.30 mm). This shows the presence of an excitatory input from the contralateral cortex to cortical layers at a depth corresponding to layer 5. The ICMS-evoked sink response was maintained 30 min after saline injection (Fig. 5-2D). In contrast, 500nL of 2% Lidocaine injection significantly suppressed the sink amplitude (Fig. 5-2E). The suppression was observed from 10 min after injection and sustained for 25 minutes (Fig. 5-2F). These result suggest that the 2% Lidocaine injection efficiently suppresses the cortico-cortical synaptic transmission. Thus, the 2% Lidocaine was used to investigate the role of aCC in bimanual coordination in awake behaving animal.

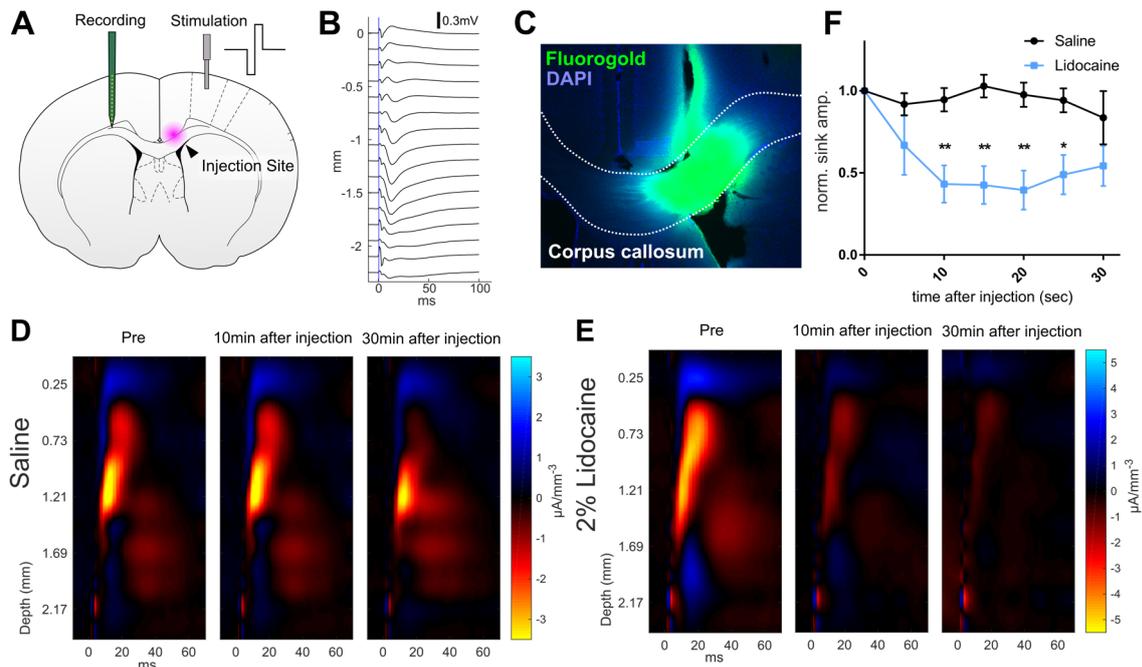


Figure 5-2. Lidocaine suppresses cortico-cortical signal transmission.

(A) Validation of pharmacological suppression of cortico-cortical signal transmission. Stimulation electrode in motor cortex, recording electrode in the contralateral homotopic motor cortex, and focal microinjection of Lidocaine in the anterior corpus callosum. (B) Mean evoked local field potentials (LFP) across different cortical depth triggered by electrical stimulation of contralateral homotopic cortex (vertical blue line). (C) Post-hoc examination of the injection site of Lidocaine by FluoroGold (green) on the background stained by NucBlueTM (blue). (D-F) Current source density (CSD) profiles corresponding to the recorded LFP across cortical depths. Negative current source was generated immediately after the onset of electrical stimulation (0 ms). (D) Stimuli evoked CSD after saline injection to the corpus callosum. (E) Significant attenuation of stimulus evoked CSD response after Lidocaine injection. (F) Time course of normalized peak LFP sink response for 30 min. Significant attenuation of evoked LFP by Lidocaine injection after 10min to 25min (two-way ANOVA ($F(1,6) = 22.57$; $p=0.0032$) followed by Bonferroni's multiple comparisons test (** $p < 0.01$, * $p < 0.05$). The error bars are representing SEM.

Obtaining kinematic parameters of bimanual motor acts. To study bilateral forelimb motor behavior during a natural motor act, male LE rats were trained to be head-fixed under food restricted condition (Fig. 5-3A) as reported by Igarashi and Wickens (2018). Rats were given an annularly shaped food reward and consume the reward by manipulating it bimanually (Fig. 5-3B). All rats were able to consume annular shaped food reward under head fixation without dropping. The bilateral forelimb motor behaviors were recorded with two high-speed cameras placed underneath the apparatus (Fig.5-3A). The reflective markers attached to the lower side of wrists were used to track position of forelimb in 3D (Fig. 5-3C), and the tracked position were reconstructed in 3-D egocentric coordinate space (Fig. 5-3D).

Pharmacological inactivation of aCC in awake animal. The trained 11 LE rats were received 2% of Lidocaine solution into the anterior part of the corpus callosum, where motor corpus callosum (Fig. 5-1). The rats underwent test sessions consisting of three repeated daily cycles of conditions under Saline, and Lidocaine injection to the aCC (Fig. 5-3E). The forelimb motor behavior was recorded for all sessions. After completion of all behavioral session, the loci of injection cannula were confirmed by post histological verification using Nissle staining (Fig. 5-3F, G, H). The nine of eleven rats have successfully received Lidocaine injection into the targeted region and were therefore used in the data analysis (Table 1).

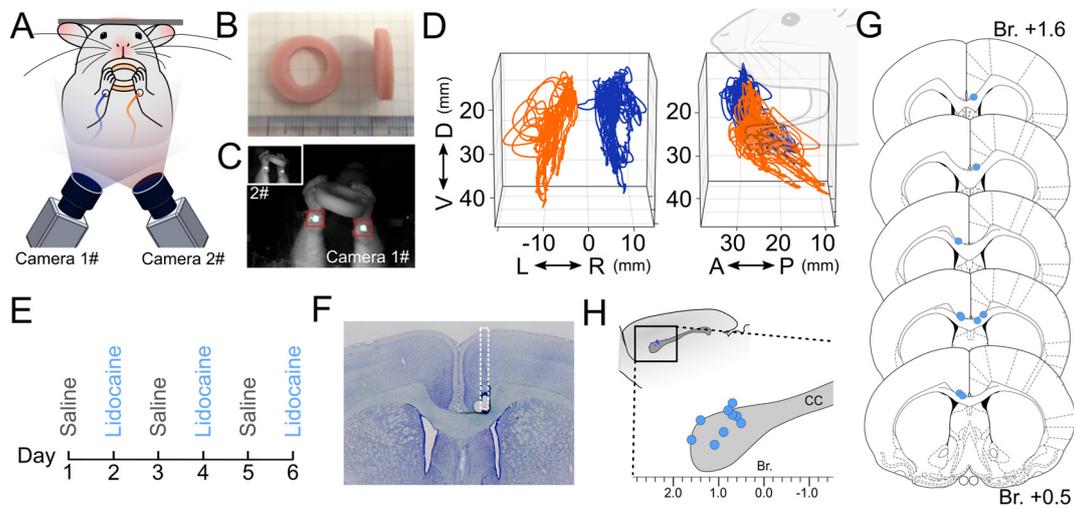


Figure 5-3. Pharmacological suppression of aCC by Lidocaine in awake rats.

(A) Schematic diagram of recording system of forelimb motor behavior. Rats were placed in a head-fixing apparatus with transparent floor and consumed food reward (B). The forelimb motor behaviors during food consumption were monitored by two high-speed cameras placed below via reflective markers. (C) Tracing forelimb trajectories by automatic detection of reflective markers attached to the lower side of wrists. (D) post-hoc 3-D reconstruction of the marker position. The positions of left (orange) and right (blue) forelimbs during food consumption view from back (left panel) and side (right panel). Note the 3D trajectories were projected in body centered (egocentric) coordinate. (E) Experiment schedule of behavioral recordings. Test sessions consisted of three repeated day cycles of conditions under baseline (Saline), and CC inhibition (Lidocaine) with more than 24 hrs interval. (F) Verification of cannula implantation. The location of the tip of cannula was confirmed by Nissle staining. (G) Schematic of injection sites shown in coronal view, and the sagittal view (H). D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left.

Table 5-1 Coordinate of injection site

Subject ID	Anterior from Bregma	Left from Bregma	Ventral from pia matter
Subj_38	+0.8mm	+0.9mm	+3.0mm
Subj_39	+0.9mm	+0.4mm	+3.5mm
Subj_40	+1.1mm	-0.9mm	+3.5mm
Subj_41	+1.8mm*	+0.5mm	+3.0mm
Subj_42	+1.6mm	+0.6mm	+3.0mm
Subj_43	+1.8mm*	+0.7mm	+3.0mm
Subj_47	+0.7mm	-0.9mm	+2.8mm
Subj_49	+0.5mm	-0.8mm	+3.2mm
Subj_50	+0.7mm	-0.8mm	+3.2mm
Subj_51	+1.4mm	+0.5mm	+3.0mm
Subj_52	-0.4mm*	+0.8mm	+3.0mm
Subj_53	+0.6mm	-1.0mm	+3.2mm

*missed target

Effect of aCC blockade on movement speed synchrony. I first examined whether the blockade of the aCC modulates synchronization of movement speed across two forelimbs during food handling behavior. To compute synchronization, a speed ratio function was used. The speed ratio computed the ratio between faster forelimb and slower forelimb (Fig. 5-4A). Synchronized movements (bilateral movements) were represented by balanced movement speed, exceeding half-maximum in the speed ratio ($SR \geq 0.5$, Fig. 5-4B), and unbalanced movements (unilateral movements) were represented as lesser than the half-maximum ($SR < 0.5$, Fig. 5-4C). The speed ratio was computed along with trajectories of forelimbs for all recorded trials of two conditions (Saline and Lidocaine, Fig.5-4D). As clearly seen in the colored trajectories, the majority of time was bilaterally synchronized (Fig. 5-4D). Quantitative analysis of speed ratio revealed 89.37 % of time fractions were spent on synchronized mode in the saline control group (Fig. 3-4E), and the rest of 10.63% were unilateral movements (Fig. 5-4F). After injection of Lidocaine into aCC, neither reduction nor increase was observed (Fig. 5-4E,F) suggesting the aCC was not mediating the movement synchronization in forelimb speed during natural food handling behavior.

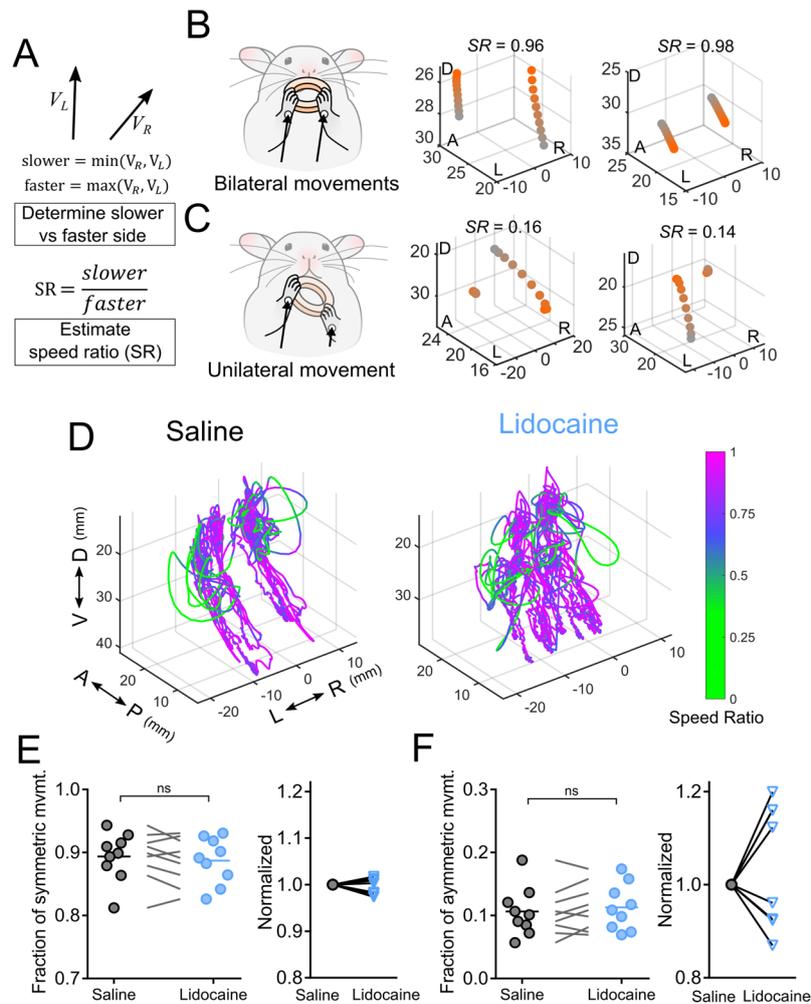


Figure 5-4. No consistent changes were observed in laterality in movement speed by blockade of aCC.

(A-C) Kinematic data were analyzed by speed ratio function to calculate laterality in movement speed across left and right forelimb. (A) Speed ratio computed the ratio of speed between the faster and slower forelimb. The higher speed ratio indicate the speed closer in two forelimb. (B) Schematic drawing of bilateral forelimb movements (left panel). The displacements of forelimb are not significantly different across two forelimbs. Representative fraction of forelimb trajectories in bilateral mode showing $SR \geq 0.5$ (middle and right panel). (C) Unilateral forelimb movements (left panel). The displacement of one side is significantly greater than the contralateral forelimb. Two representative fraction of forelimb trajectories in unilateral mode showing $SR < 0.5$ (middle and right panel). (D) Forelimb trajectories of saline (left) and Lidocaine (right) injected group. The speed ratio was overlaid on the 3D trajectories in color scale. (E-F) Time fraction on unilateral and bilateral forelimb movements were unchanged.

(E) Quantitative analysis of the fraction of time spent on bilateral movements ($SR \geq 0.5$) during food consumption (left), and the normalized change (right) (paired t-test, $p = 0.2326$, $n = 9$). (F) Quantitative analysis of the fraction of time spent on unilateral movements ($SR < 0.5$) during food consumption (left), and the normalized change (right) (paired t-test, $p = 0.2326$, $n = 9$). D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

Movement symmetry was shifted towards asymmetry. Next, I tested the hypothesis that the aCC mediates symmetry in movement direction during bilateral forelimb movements. To measure similarity in movement direction, the asymmetry index was used. The asymmetry index measures the degree of similarity in movement direction (Fig. 5-5A). The velocity of left forelimb and the mirrored right forelimb was used to compute the asymmetry index (Fig. 5-5A). The asymmetry index was presented as θ where it is represented as radian of two movement vector of forelimbs. The value of the asymmetry index was computed for all recorded trials. Representative trajectories with the asymmetry index were visualized as colored trajectories in figure 5-5D. Quantitative analysis revealed that 57.17 % of the time was spent in the symmetric mode ($\bar{\theta} \leq \pi/4$, Fig. 5-4E), and the remaining 42.83% in the asymmetric mode ($\bar{\theta} > \pi/4$, Fig. 5-4F) in saline control group. After injection of Lidocaine into aCC, a reduction of symmetric movements was observed in all nine subjects showing the time fraction of the symmetric mode of 53.87 % (Fig. 5-4E), and in turn, asymmetric movements increased (46.13 %, Fig. 5-4E). The statistical significance was consistently observed with different time window of segmentation (supplemental figure 5-2); and there was no reduction of symmetric movements in Naïve control (supplemental figure 5-3). These results suggest that the aCC mediates symmetric bilateral forelimb movements during natural food handling behavior.

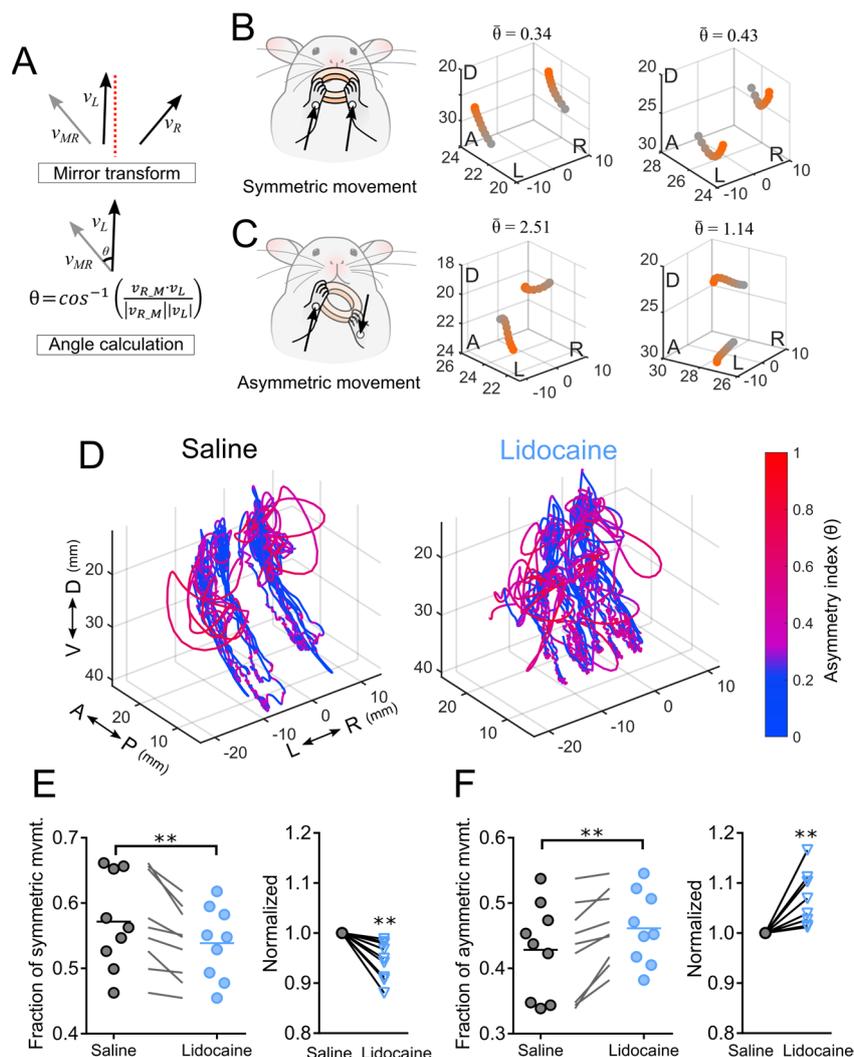


Figure 5-5. Blockade of aCC modulates the ratio of symmetry and asymmetry in movement direction.

(A) Asymmetry index computed the difference of movement direction between the left forelimb velocity v_L and the mirrored right forelimb velocity v_{MR} obtained by mirror transformation. The higher asymmetry index value indicates diverging movement direction (asymmetric movements). (B) Schematic drawing of symmetric forelimb movements (left panel). The movement directions are not significantly different across forelimbs. Representative fraction of forelimb trajectories in symmetric mode showing $\bar{\theta} \leq \pi/4$ (middle and right panel). (C) Asymmetric forelimb movements (left panel). The movement direction diverge across forelimbs. Two representative fraction of forelimb trajectories in asymmetric mode showing $\bar{\theta} > \pi/4$ (middle and right panel). (D) Colored forelimb trajectories of saline (left) and Lidocaine injected group (right). The asymmetry index was indicated as color scale. (E-F)

Consistent shift of time fraction from symmetric movements towards asymmetric movements. (E) Quantitative analysis of the fraction of time on symmetric movements $\bar{\theta} \leq \pi/4$ during food consumption (left), and the normalized change (right) (paired t-test, $p = 0.0043$, $n = 9$). (F) Quantitative analysis of the fraction of time on asymmetric movements $\bar{\theta} > \pi/4$ during food consumption (left), and the normalized change (right) (paired t-test, $p = 0.0043$, $n = 9$). Note the fraction of time of asymmetry counterparts symmetric movements. D, dorsal; V, ventral; A, anterior; P, posterior; R, right; L, left. Numbers in 3-D plots are expressed in millimeters.

Effect of Lidocaine injection to global motor function. Although the suppression of aCC reduced movement symmetry during bilateral food handling, there was no significant disturbance of task performance (supplemental figure 5-1). There was no significant difference in mean consumption time. The success rate was unchanged (task completion without dripping). The mean movement speed of forelimb was computed with respect to injection location, and found there is no consistent reduction of movements speed, suggesting that the Lidocaine injection did not cause motor disability of unilateral forelimb by the spread of lidocaine to motor cortex.

It is of interest how interhemispheric interaction occurs at the cellular level during bimanual feeding behavior. To address the organization of interhemispheric network, multiunit activity was recorded bilaterally from caudal forelimb areas in awake head-fixed rats (Fig. 5-6A). The recording depth was aimed to cortical layer V (Fig. 5-6B), where the axons densely terminate from the contralateral cortex (Ferino et al. 1987; Smith and Alloway 2014; Wu et al. 2009; Zhou et al. 2013). Three recording sessions were conducted (1 session per day), and total of 315 units were isolated from two subjects (rat_1 and rat_2). The isolated units were then clustered into regularly spiking units (RS, putative excitatory units) and fast-spiking units (FS, putative inhibitory neurons) by using two-component Gaussian mixture model (GMM) determining the boundary between the two cell types. Given the boundary value (0.484 ms) obtained from GMM, the bimodal distribution of the histogram of spike duration was split and RS and FS neurons were isolated (Fig. 5-6 D). The parameters used for two-components GMM were as follows: $\mu_{RS}=0.597\text{ms}$, $\sigma_{RS}=0.0455\text{ms}$, $\phi_{RS}=4.83$, $\mu_{FS}=0.3496\text{ms}$, $\sigma_{FS}=0.0686\text{ms}$, and $\phi_{FS}=2.3317$. The location of the recording electrode was validated by histology, and the RS and FS units recorded from layer V were selected and subject to further analysis (169 units from Rat_1 and 47 units from Rat_2).

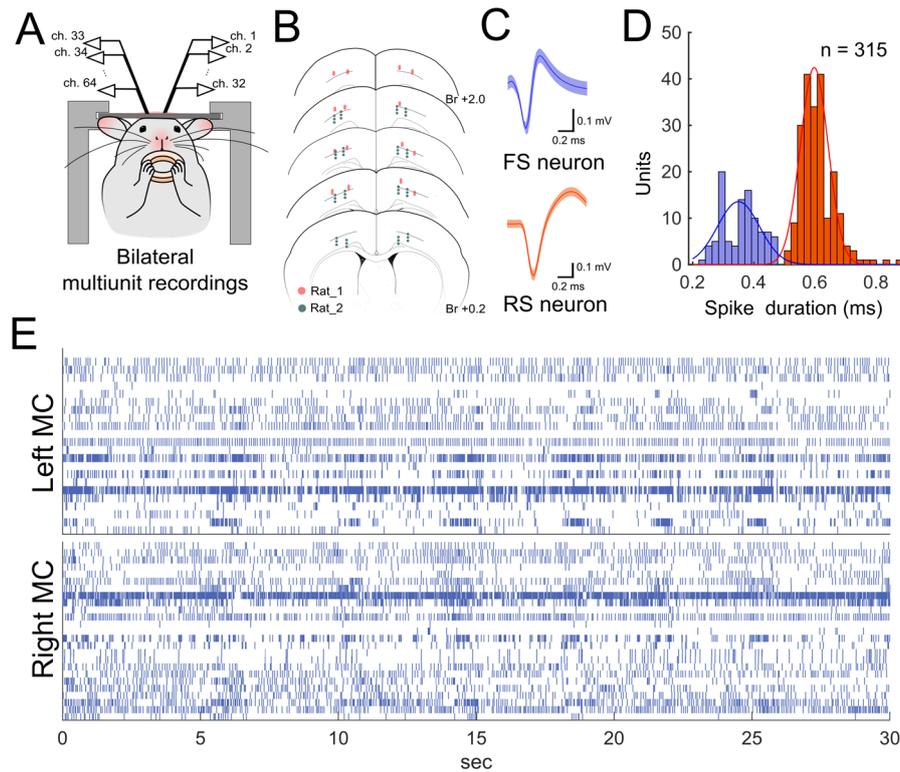


Figure 5-6. Bilateral multiunit recording of motor cortex in awake rats.

(A) Schematic diagram of multiunit recording under awake animal. Multiunit activity from caudal forelimb area were recorded during feeding behavior via chronically implanted tetrode bundles in each hemisphere. (B) Location of recording site from rat_1 (red dot), and rat_2 (green dot). The dotted line in cortex indicate the border of layer 5. (C) Representative waveform of isolated regularly spiking (RS) neuron and fast-spiking (FS) neuron. (D) Recorded neurons were classified based on the duration of spike waveform. Two distinct Gaussian distribution was observed and fitted by one-dimensional Gaussian mixture model (GMM), and thereby classified based on the border of the first and second distribution (RS neurons: > 0.484 ms, FS neurons: < 0.484 ms). (E) Representative multiunit spiking activity of rat_2. Multiunit activity from left and right motor cortices were simultaneously rerecorded in awake.

To study the organization of interhemispheric interaction using the recorded population of units, pairwise cross-correlation were computed within hemisphere and inter-hemisphere (Fig. 5-6A). Both positive and negative correlation was observed within left hemispheres (Left MC vs Left MC; Fig. 5-6 A lower left block), and within right hemisphere (Right MC vs Right MC; Fig. 5-6A upper right block). Positive and negative correlation was also broadly observed inter-hemispheric neural pairs (Left MC vs Right MC; Fig. 5-6A upper left block or lower right block). Representative positively and negatively correlated interhemispheric neural pairs were shown in figure 5-6 Aa and Ab. To quantify the balance of positively correlated pairs and negatively correlated pairs, all correlation coefficients of inter-hemispheric neural pairs were computed and pooled. Overall tendency of inter-hemispheric neural pairs was biased to the positive correlation (Fig. 5-6B-D left panels). The positive correlation predominates among RS inter-hemispheric pairs (Fig. 5-6 B), while a substantial portion of negatively correlated pairs was also observed. The excitatory inputs may cause the positively correlated pairs at both monosynaptic and disynaptic (or polysynaptic) level, whereas, the negative correlation possibly requires inhibitory interneurons via disynaptic (or polysynaptic) events (Fig. B right panel). A significantly biased positive correlation coefficient was observed among FS interhemispheric neural pairs (Fig. 5-6B). This may reflect restricted possible cellular architecture, where synchronization of FS neurons is dependent on interhemispheric synchronization between RS pairs (Fig. 5-6B right panel). Interestingly, there was a substantial proportion of positive interhemispheric correlation between FS and RS interhemispheric pairs (Fig. 5-6D). This may be caused by excitatory inputs to contralateral FS neurons or inhibition of FS units by inhibitory interneurons received excitatory inputs from the contralateral cortex (Fig. 5-6D right panel). To conclude, positive correlation predominates in interhemispheric interaction. Positive correlation of interhemispheric RS neurons indicates the excitatory transcortical neural population. The positive correlation was also observed between RS and FS neurons, suggesting that the RS neurons may inhibit contralateral hemisphere via putative inhibitory interneurons.

Substantial amount of negative correlation was observed, the negative correlation probably caused by polysynaptic events via inhibitory interneurons. However, these still remain hypothetical cellular organization based on correlation network.

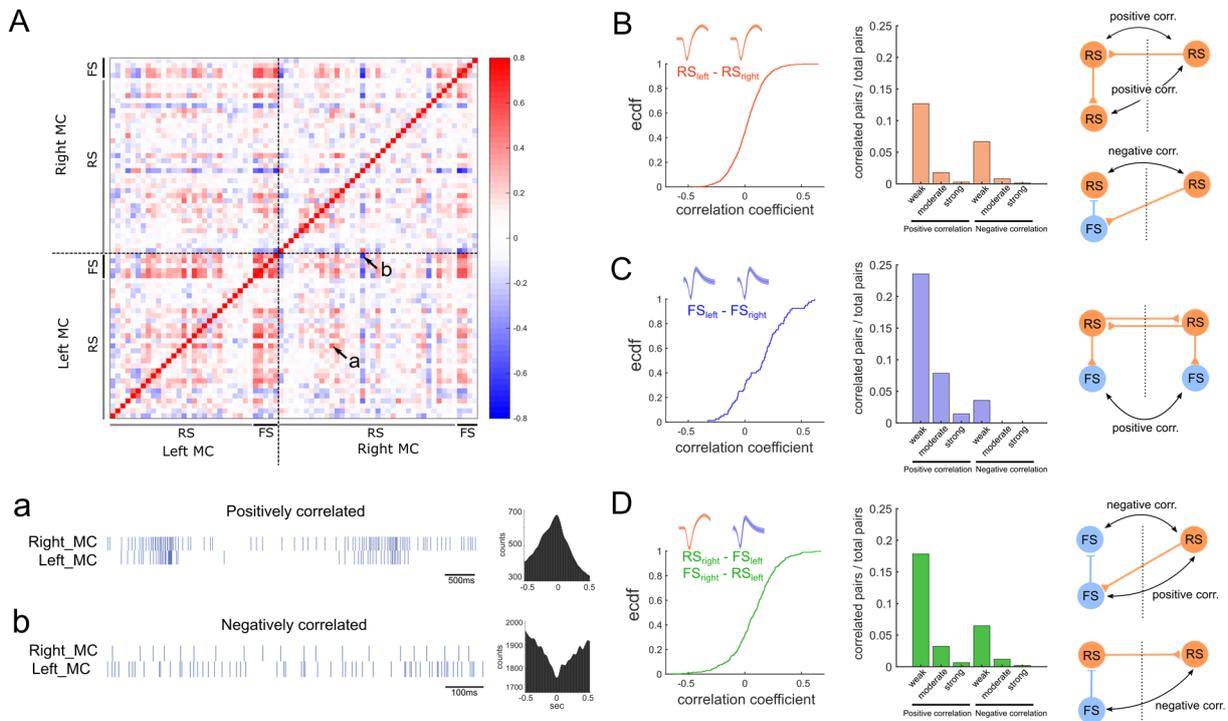


Figure 5-7. Correlated neural pairs across two hemispheres.

(A) Similarity matrix of recorded spike trains from left motor cortex (Left MC) and right motor cortex (Right MC). Regularly-spiking (RS; gray lines) units and fast-spiking (FS; black lines) units were ordered separately. The color scale indicates correlation coefficient computed by Spearman's correlation. Representative spike trains of positively correlated pairs (a) and negatively correlated pairs (a) are shown. Note the negatively correlated pair fires alternately, avoiding simultaneous firing. (B-D) Positive interhemispheric correlation predominates. (Left panels) Empirical cumulative distribution function (ecdf) of correlation coefficients among RS neurons in left hemisphere (RS_left) and RS neurons in right hemisphere (RS_right) pairs (B), FS neurons in left hemisphere (FS_left) and FS neurons in right hemisphere (FS_right) pairs (C), and RS_left - FS_right / FS_left - RS_right pairs (D). (Center panels) Proportion of positive and negative correlation coefficients by three levels of strength: “weak”, “moderate”, and “strong (and very strong)” (see Methods for

the definition). (Right panels) Possible monosynaptic and disynaptic interhemispheric interactions. Schematic diagram illustrates positive and negative correlation caused by monosynaptic and disynaptic neural circuit.

5.4 Discussion

The role of corpus callosum in bimanual motor control was examined by analyzing forelimb kinematics during object manipulation, before and after pharmacological suppression of the anterior corpus callosum. The neural tracing study showed that the fiber bundle from the forelimb motor areas in rats passes through the anterior portion of the corpus callosum. I then confirmed with electrophysiology that neural transmission in this transcortical motor pathway was attenuated by injections of local anesthetic, which reduced the LFP response evoked by ICMS of the contralateral hemisphere. Kinematics of forelimb movements with and without suppression of the anterior corpus callosum were then compared during bimanual food handling. Suppression of the anterior corpus callosum decreased the fraction of forelimb movements that were bilaterally symmetric, whereas synchrony of movement timing and other global scores were unchanged. Bilateral multiunit recordings from corresponding cortical areas showed positively correlated activity patterns in the majority of interacting pairs, and a substantial portion of negative correlation. The present study also found that the putative excitatory neurons were also positively correlated with putative inhibitory neurons in the opposite hemisphere, suggesting interhemispheric inhibition via inhibitory neurons. Taken together, these results suggest that the anterior corpus callosum contributes to symmetry in the form of bilateral forelimb movements, possibly by both direct excitatory and indirect inhibitory connections. To our knowledge, this is the first study to investigate the role of the rodent motor corpus callosum in bimanual coordination, extending previous knowledge of the role of corpus callosum in bimanual coordination in natural feeding behavior.

Studies of the location of motor fibers in the corpus callosum of human, using diffusion tensor imaging, have shown that the callosal motor fibers are found in the posterior body and the isthmus of the corpus callosum (de Lacoste et al. 1985; Hofer and Frahm 2006; Wahl et al.

2007). In the present study, the location of callosal motor fibers of rats was studied using a neural tracer. The results suggest, in contrary to the human motor corpus callosum, that the callosal motor fibers of rats are most dense in the anterior part (or the genu) of the corpus callosum. The RFA callosal motor fibers ran more anteriorly than the CFA callosal motor fibers. This result is consistent with the projection map of the *Allen Mouse Brain Connectivity Atlas* (<http://connectivity.brain-map.org>, Oh et al., 2014), which shows that mouse primary and secondary motor areas are connected by the anterior part of corpus callosum. Therefore, the anterior corpus callosum was targeted in the present study.

In order to reversibly block axonal conduction of the corpus callosum, without altering the neurons of origin, lidocaine was injected into the anterior corpus callosum. The blockade of axonal conduction was confirmed by ICMS and LFP recordings. Control records showed that a significant sink response was evoked across cortical laminar in the homotopic contralateral cortex, with a maximum at a level corresponding to layer 5 in sensorimotor cortex in rats (Chapman et al. 1998). Since 99% of the axonal fibers of the corpus callosum are excitatory fibers which originate from glutamatergic intratelencephalic neurons (Harris and Shepherd 2015; Shepherd 2013) the sink responses in the present study almost certainly reflects excitatory inputs from the contralateral cortex to these layers (Pietrasanta et al. 2012). Injecting lidocaine into the anterior corpus callosum significantly suppressed the sink of LFP, confirming that axonal conduction was blocked in the direct excitatory interhemispheric connection between homotopic motor cortical areas. There remains, however, a theoretical possibility that the evoked sink might be caused by polysynaptic events such as peripheral inhibitory inputs mediated by the callosal-interneuron pathway, or other components of the genu, such as connections between prelimbic, anterior cingulate or insular cortices (*Allen Mouse Brain Connectivity Atlas*, <http://connectivity.brain-map.org>, Oh et al., 2014). However, the latency of the response is consistent with previous reports measured by antidromic spike (Soma et al.

2017; Wilson 1987), suggesting that the sink observed in the present study is indeed caused by monosynaptic excitatory synaptic inputs (Einevoll et al. 2013). Thus, the lidocaine lesion effectively blocked the excitatory interhemispheric connections.

In the present study, pharmacological blockade of axonal conduction was used instead of corpus callosotomy, which has been a widely in previous studies of the role of interhemispheric communication in rodents (Li et al. 2016; Mohn and Russell 1981; Noonan and Axelrod 1992; Sullivan et al. 1993) and non-human primates (Mark and Sperry 1968). In human, patients received callosotomy as treatment of epilepsy have been subject to test the role of corpus callosum in visual perception (Gazzaniga et al. 1962), and motor control (Ivry and Hazeltine 1999). The pharmacological blockade has the advantage of being reversible. In the present study 2 of 4 rats showed recovery from suppression approximately 30 min after the Lidocaine injection, which provided rapid reversibility of the pharmacological suppression of the corpus callosum. For studies requiring longer duration of suppression, longer-acting sodium channel blockers, such as QX-314, are available (Binshtok et al. 2009).

In the present study, inhibition of aCC did not alter the ratio of bilateral to unilateral movement during food handling, suggesting the timing of movement between two forelimbs was not significantly changed. During food handling, forelimb movements were well-synchronized in speed, with bilateral forelimb movements predominating, as previously reported (Igarashi and Wickens 2018). The predomination of bilateral synchronization has been also reported in the previous studies. For example, in human, synchrony of movement timing can be observed in rhythmic bimanual finger tapping; and the in-phase mode (simultaneous finger tapping with no phase shift; $\phi = 0^\circ$) is more stable than anti-phase mode (tapping with alternation; $\phi = 180^\circ$) (Schoner and Kelso 1988; Yamanishi et al. 1980). Interestingly, the bilateral synchrony of temporal coupling was well-preserved in callosotomy patients (Ivry and Hazeltine 1999; Tuller

and Kelso 1989). Donchin et al. (1999) proposed the involvement of a subcortical structure, a central pattern generator (CPG), in synchronizing bimanual movements. This proposal might explain how conserved timing synchrony is possible without corpus callosum, because this would leave a subcortical CPG intact. Therefore, it is possible that the synchronized movement speed in feeding behavior is mediated by subcortical areas in rodents.

In contrast, inhibition of aCC by Lidocaine reduced the symmetry of bilateral forelimb movements. A possible interpretation of this reduction of movement symmetry is that the corpus callosum is necessary for the symmetry of bimanual movements. Humans exhibit a tendency toward spatially symmetric movements during bimanual tasks (Franz 1997; Swinnen et al. 1998; Walter et al. 2001). There is evidence that this depends on the corpus callosum. Spatial coupling in a bimanual drawing task (drawing of different forms by two hands) was reduced in split-brain patients (Franz et al. 1996). In addition, disruption of temporal synchrony in split-brain patient became evident when the task involved spatial requirements in addition to timing (e.g., continuously drawing circle in a 2-D plane) (Kennerley et al. 2002), suggesting that the symmetric form of bilateral forelimb movements is mediated by corpus callosum in humans, consistent with the present results obtained in rats during natural eating behavior. In rats, the present study suggests that the frequent symmetrical upward and downward bimanual reaching action that occurs in feeding may be mediated by the anterior corpus callosum. However, it still remains unclear whether the symmetric movements mediated by the corpus callosum are responsible for the specific motor pattern of bimanual acts, such as upward bimanual reaching during food-to-mouth behavior, and downward bimanual reaching during tearing of food. Further work is needed to address this issue.

The mechanism underlying the contribution of the corpus callosum to symmetric bimanual movements is not well understood at the cellular level. It is unclear whether the connection is

functionally excitatory or inhibitory. The multiunit recordings obtained in the present study showed that the great majority of interhemispheric neural pairs exhibited positively correlated firing patterns. A number of other findings support excitatory role by corpus callosum. For example, the attenuation of the spread of seizure and loss of information integration in split-brain patients suggests that the corpus callosum is used for excitatory interhemispheric signal transmission. The purpose of such a connection is not yet known. One possibility is suggested by the proposal of Li et al. (2016), in the form of a modular attractor model comprised of independent modules encoding particular actions. In the model, callosal excitatory connections link homotopic modules in each hemisphere. Given the existence of topographical maps related to forelimb movement form (Brown and Teskey 2014; Young et al. 2011) and direction (Hira et al. 2015), linking these across hemispheres might contribute to spatially symmetric movement. Interestingly, repeated stimulation to corpus callosum (kindling stimulation) forms bilateral representation in rat forelimb motor area (Teskey et al. 2002). The effect of the lesion on the extent of symmetric movement might then make sense if these excitatory callosal projections are connected to functionally homotopic areas across motor cortices.

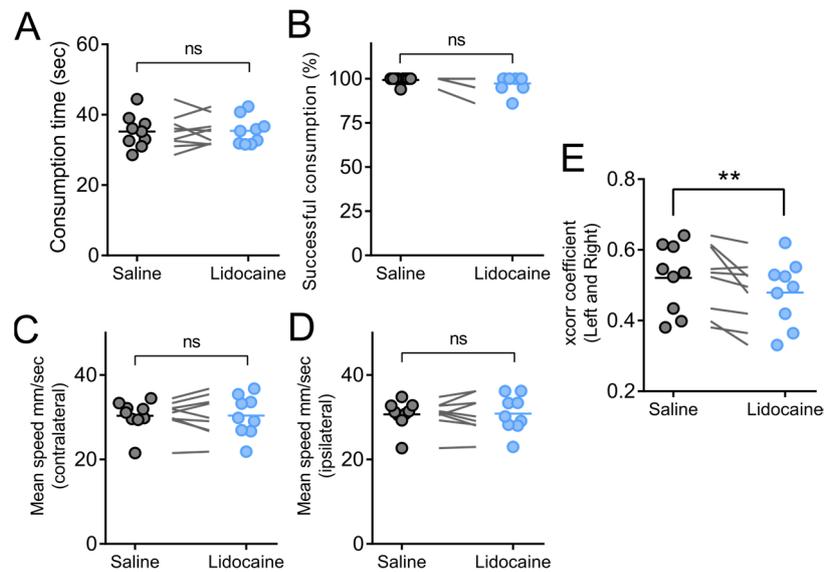
On the other hands, it has been postulated that the corpus callosum inhibits neurons in the contralateral hemisphere (interhemispheric inhibition, IHI) (Ferber et al. 1992; Hubers et al. 2008). In the present study, a percentage of pairs of RS neurons (putative excitatory neurons) from corresponding cortical areas exhibited negatively correlated firing patterns. At the cellular level, IHI would result from dysynaptic connections involving inhibitory interneuron activated by excitatory inputs of the corpus callosum (Kokinovic and Medini 2018; Palmer et al. 2012). The electrical stimulation of corpus callosum causes EPSP in the sensorimotor cortex followed by IPSP (Chapman et al. 1998; Teskey et al. 1999). The present study also found that the RS neurons positively correlate with FS (putative inhibitory) neurons. The functional significance of such inhibition is suggested by experiments in which cooling of contralateral somatosensory

cortex unmasked larger receptive field, therefore, less selective to sensory inputs (Clarey et al. (1996). A similar report has been reported in rats (Pluto et al. 2005). These are consistent with the idea that the callosal connection provides a source of inhibition for shaping the finer receptive field. It should be noted, however, that the excitatory model and inhibitory model are not mutually exclusive. Rather both excitatory and inhibitory connections might play important roles in activating the contralateral motor cortex to perform finer bilateral forelimb movements accurately by inactivating unnecessary movements.

The present kinematic analysis revealed a reduction of symmetric bimanual movements by blockade of aCC, however, the rats were still able to perform the food handling under head-fixation without significant reduction in mean consumption time and successful completion rate. This raises the question of why the blockage of the aCC did not significantly modify bimanual eating behavior. As described above, prior studies in primates demonstrated that the corpus callosum plays an important role in bimanual coordination in a variety of tasks. However, it has been reported that callosotomy patients had less difficulty in familiar bimanual actions such as tying a shoe and opening drawer (Franz et al. 2000; Serrien et al. 2001). Therefore, it is possible that the corpus callosum is not crucial for the execution of familiar bimanual actions such as food handling. Nevertheless, information exchange between the two sides of the body is necessary for highly synchronized movement direction, and the present study suggests that this is mediated by the aCC.

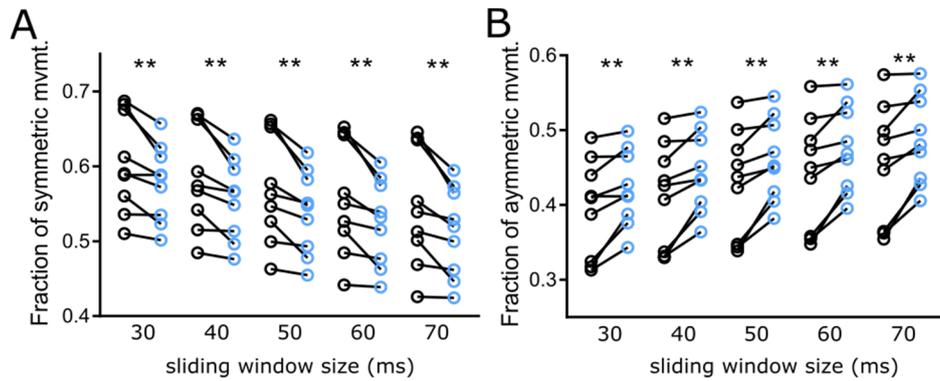
5.5 Acknowledgment

This work was supported by Japan Society for the Propotion of Science Reserch Fellow Grant-in-Aid 16J05329.

Supplemental figure 5-1

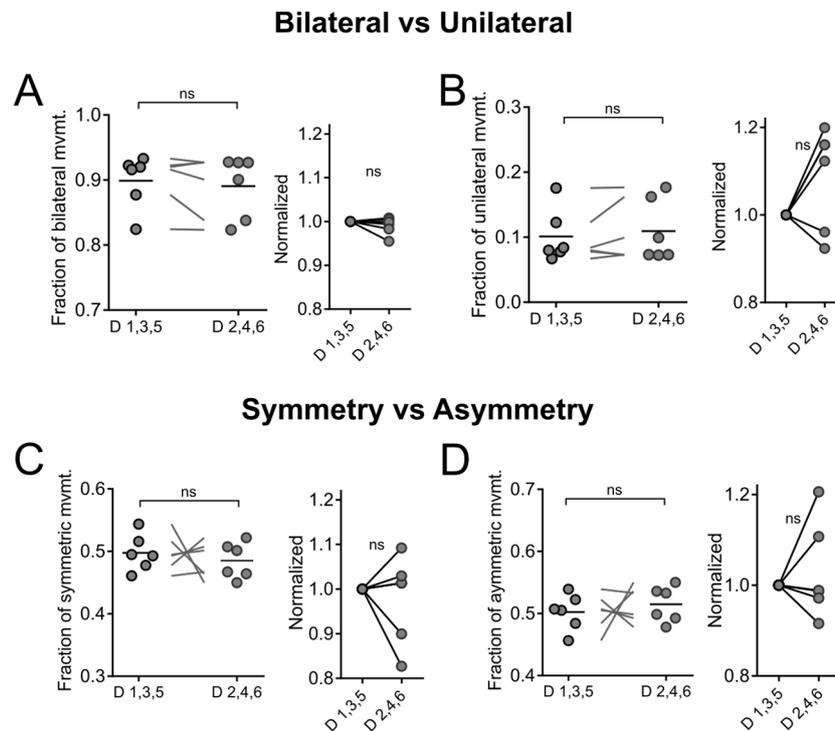
Supplemental figure 5-1. Inhibition of aCC did not alter global motor/task performances but cross correlation.

(A) Mean consumption time (ns, $p=0.8635$). (B) Successful rate was unchanged (ns, $p=0.0909$). (C) The mean speed of forelimb contralateral to the Lidocaine infusion (ns, $p=0.9825$), and the ipsilateral (D, ns, $p=0.8361$). (E) cross-correlation between velocity of left and mirrored-right forelimb (significant, $p=0.0255$). All statistical significance were validated by paired t-test ($n=9$).

Supplemental figure 5-2**Supplemental figure 5-2. Reduction of symmetric movements with different sliding window sizes.**

To test the effect of size of sliding window in the kinematic analysis, five different time window were used to reanalyze symmetric and asymmetric ratio. The reduction of symmetric fraction and increase of asymmetric fraction was consistently observed all different time window.

Supplemental figure 5-3

**Supplemental figure 5-3. No significant difference in Naïve control group.**

(A) Quantitative analysis of the traction of time on bilateral movements $SR \geq 0.5$ during food consumption (left), and the normalized value (right) (paired t-test, $p = 0.293$, $n = 6$). The horizontal bar in the left panel is mean. (B) Quantitative analysis of the traction of time on unilateral movements $SR < 0.5$ during food consumption (left), and the normalized value (right) (paired t-test, $p = 0.293$, $n = 6$). (C) Quantitative analysis of the traction of time on symmetric movements $\bar{\theta} \leq \pi/4$ (left), and the normalized value (right) (paired t-test, $p = 0.574$, $n = 6$). The horizontal bar in the left panel is mean. (D) Quantitative analysis of the fraction of time on asymmetric movements $\bar{\theta} > \pi/4$ (left), and the normalized value (right) (paired t-test, $p = 0.574$, $n = 6$). Note the fraction of time of asymmetry is counterpart of symmetric movements.

Chapter 6: Conclusions and Future Directions

The aim of the thesis research was to better-understand the neural mechanism of bimanual coordination. The potential of rodents as an animal model for studying bimanual coordination was explored, by developing a new method for quantifying and classifying three-dimensional bilateral forelimb trajectories in head-fixed rats. The recording system and analytical pipeline revealed that both asymmetric and symmetric bilateral movements occur in food handling behavior, with symmetric bilateral forelimb movements quantitatively more common. Based on a review of the literature, the corpus callosum was hypothesized to play a key role in bimanual coordination. To test this hypothesis, axonal conductance of the anterior corpus callosum (aCC) was pharmacologically attenuated using local anesthetics. Electrophysiological analysis of extracellular field potentials using current source density analysis showed that the lesions were effective. The effects of this lesion on bilateral forelimb movements during food handling was determined using the developed kinetic analysis. The results suggest that the aCC is not required for successful food handling, however, it appears to be necessary for the symmetrical movements involving fine coordination of velocity profiles. Bilateral multiunit recordings showed that neurons were positively correlated with corresponding areas of the opposite cortices in the great majority of cases, with a substantial portion of negatively correlated firing patterns. In addition, putative excitatory neurons were also correlated with putative inhibitory neurons in contralateral hemispheres, suggesting interhemispheric inhibition occur during feeding behavior. Taken together, the thesis research suggests that the aCC plays a crucial role in the bilateral coordination of symmetrical movements by means of both excitatory and inhibitory interhemispheric interaction. However, there are many issues that require further investigation.

The kinematic analysis based on 3-D movement trajectories, while innovative, has its limits. In particular, the trajectories do not report information about muscle activation patterns

or the purpose of movements implied by the concept of bilateral coordination. Further careful analysis of the association of trajectories would be helpful. Closely related to this issue is the choice of task. In the present study, the food object being manipulated, because of its donut shape, may have contributed to the excess of symmetrical movements. Future studies should investigate a range of different objects requiring different forms of coordination.

The effects of the lesion of the aCC were surprisingly subtle given the extent of the drug infusion, and this may indicate that the task did not fully challenge the interhemispheric connections. Further work should examine the effects of aCC lesions on tasks which highly demand interhemispheric connections. In particular, the task used in the present study allowed some access to other information about the positions of the contralateral limb, such as sensory information from the contralateral forelimb. A more challenging task could be developed, which the present research suggests should require a high degree of symmetry, which could be contrasted with a task with a lower demand for symmetrical movement.

Finally, the electrophysiological findings raise many questions for future research. In particular, it is of interest whether correlated interhemispheric neural pairs is functionally similar or not, which shows cellular representation of symmetric and asymmetric bilateral forelimb movements. The role of monosynaptic excitatory connections and polysynaptic inhibitory effects could be further studied using the genetic tools that are available for research with rodents such as optogenetics and chemogenetics. These would allow a more detailed dissection of the different components important for bilateral coordinated movement.

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