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AGE- AND SEX-DEPENDENT ALTERATIONS IN PRIMARY SOMATOSENSORY NEURONAL CALCIUM NETWORK DYNAMICS DURING LOCOMOTION

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AGE- AND SEX-DEPENDENT ALTERATIONS IN PRIMARY SOMATOSENSORY NEURONAL CALCIUM NETWORK DYNAMICS DURING LOCOMOTION MAY CONTRIBUTE TO GAIT DYSREGULATION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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2023

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ABSTRACT OF DISSERTATION

AGE- AND SEX-DEPENDENT ALTERATIONS IN PRIMARY SOMATOSENSORY NEURONAL CALCIUM NETWORK DYNAMICS DURING LOCOMOTION MAY CONTRIBUTE TO GAIT DYSREGULATION

Over the past 30 years, the calcium (Ca^{2+}) hypothesis of brain aging has provided clear evidence that hippocampal neuronal Ca^{2+} dysregulation is a key biomarker of aging. Indeed, age-dependent Ca^{2+}-mediated changes in intrinsic excitability, synaptic plasticity, and activity have helped identify some of the mechanisms engaged in memory and cognitive decline. However, much of this work has been done at the single-cell level, mostly in slice preparations, and in restricted structures of the brain. Recently, our lab identified age- and Ca^{2+}-related neuronal network dysregulation in the cortex of the anesthetized animal. Still, investigations in the awake animal are needed to test the generalizability of the Ca^{2+} hypothesis of brain aging and dementia. Here, we used *in vigilo* two-photon (2P) imaging in ambulating mice, to image GCaMP8f in the primary somatosensory cortex (S1), during ambulation and at rest. In order to investigate aging- and sex- related changes in the neuronal Ca^{2+} network, a continuous wavelet transform (CWT) analysis was developed (MATLAB) to extract measures of network communication while also addressing pair-wise correlations at single-cell resolution. Following imaging, gait behavior was characterized to test for changes in locomotor stability. During ambulation and compared to rest, in both young (4 months) and aged mice (22 months), an increase in connectivity and synchronicity was noted. An age-dependent increase in network synchronicity was seen in ambulating aged males only. Additionally, females displayed a greater number of active neurons, area-under-curve, and neuronal activity compared to males, particularly during ambulation. These results suggest S1 Ca^{2+} dynamics and network synchronicity are likely contributors of locomotor stability. We believe this work raises awareness of central elements at play in S1 where neuronal Ca^{2+} network dysregulation is seen with aging, perhaps highlighting potential therapeutic targets that may help offset age-dependent increases in falls.

KEYWORDS: neuroscience, somatosensation, locomotor stability, two-photon, aging

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04/20/2023
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AGE- AND SEX-DEPENDENT ALTERATIONS IN PRIMARY SOMATOSENSORY NEURONAL CALCIUM NETWORK DYNAMICS DURING LOCOMOTION MAY CONTRIBUTE TO GAIT DYSREGULATION

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04/20/2023
Date

DEDICATION

To my future self:

1. Be unapologetically you.

2. Don't settle for anything less than extraordinary.

3. You are smart, you are kind, and you are important.

4. Create your own happiness.

5. Never lose your sense of wonder.

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CHAPTER 1. INTRODUCTION

1.1 Falls and Gait Dysregulation in the Older Adult

In the United States, falls are a leading cause of both fatal and nonfatal injuries in older adults (Bergen et al., 2016). On average, 30–40% of people over the age of 65 and 50% of people over the age of 80 will experience a fall each year (Rubenstein & Josephson, 2002; Thapa et al., 1996; Tinetti, 2003). Many of these events are associated with injury, with one study reporting that in women >70 years old, 41% of falls resulted in minor injuries while 6% resulted in major injuries (i.e., head trauma, fractures, or lacerations) (Centers for Disease & Prevention, 2003; "Guideline for the prevention of falls in older persons. American Geriatrics Society, British Geriatrics Society, and American Academy of Orthopaedic Surgeons Panel on Falls Prevention," 2001; Rubenstein & Josephson, 2002). Additionally, falls account for >60% of emergency room visits in patients who are 65 or older (Centers for Disease & Prevention, 2003), of which 5% conclude with hospitalization ("Guideline for the prevention of falls in older persons. American Geriatrics Society, British Geriatrics Society, and American Academy of Orthopaedic Surgeons Panel on Falls Prevention," 2001). The likelihood of incurring a fall-related injury depends on a variety of factors, such as the height and velocity of the event (Lee et al., 2018; Wong et al., 2019) and the overall health of the individual (Ek et al., 2018; Hatcher et al., 2019). Interestingly, sex also appears to play a role as older women are less

likely to experience a fall (Aryee et al., 2017; Ek et al., 2019) but more likely to sustain an injury (either minor or major) compared to older men (Bergen et al., 2016). In addition to elevating the risk of short-term injury, falls in older adults also lead to increased risk of morbidity, chronic medical complications, and admission into long-term care facilities (Close et al., 2012; Gill et al., 2013; Rubenstein, 2006; Vieira et al., 2016). While death from falls is much less common, long-term complications associated with fall events are still a significant contributor to mortality in older populations (Sattin, 1992). In fact, approximately 2% of injurious falls in older adults result in death (Sattin et al., 1990), and this statistic increases with age. Further, fear and anxiety surrounding the outcome of these events seems to be more prevalent in this particular population, with one study reporting that 80% of older women would prefer death over an injurious fall and placement into a long-term care facility (Salkeld et al., 2000).

While the increase in falls with age is undisputed, central mechanisms responsible for this have not been investigated thoroughly in animal models of aging. Recently, however, it has become clear that chronic conditions, including stroke, diabetes and dementia are intrinsic risk factors that are not only critical when considering the etiology of falls with aging, but importantly, may also represent therapeutic targets for reducing fall risk with age. Evidence from a recent meta-analysis investigating over 14,000 patients identified a significant increase in fall risk in patients with diabetes compared with healthy subjects (Yang et al., 2016), further supporting prior evidence from the Longitudinal Ageing Study, that individuals with diabetes exhibit more frequent falls compared to healthy adults (Pijpers et al., 2012). The links between peripheral metabolic dysregulation and falls have been reviewed recently with careful attention to the potential involvement

of sensorimotor dysregulation, musculoskeletal dysfunction, and pharmacological complication. It seems clear that repeated hypoglycemic events in patients with diabetes likely contribute to an increased risk of falls (Crews et al., 2013). More recently, polypharmacy use has been shown to weaken glycemic control, giving rise to increases in dizziness and falls (Remelli et al., 2022).

Clearly, characterizing the pathological changes and mechanisms that underlie age-dependent gait impairments, including processes associated with metabolic dysfunction, is a worthwhile endeavor, as these impairments significantly increase the risk of falling in the older population. However, despite enormous advances in the identification of peripheral mechanisms that contribute to altered ambulatory function and increased fall risk in older individuals, there is still a paucity of information highlighting the potential *central* components of ambulatory distress with age. Additionally, there is currently a lack of effective therapeutic treatments designed to target these central processes. Here, I discuss alterations in central modalities that clearly participate in locomotor activity and may contribute to gait dysregulation with age. While these central functions that control ambulation are a valuable therapeutic target for the prevention of falls with age, many current strategies have fallen short.

1.2 Current Therapeutic and Pharmacological Approaches to Reducing Falls

While it is undeniable that injurious falls significantly impact the quality of life of those affected, these events also exert an immense financial burden on society. In fact, as of 2015, the estimated cost of care associated with fall injuries in individuals ≥ 65 years old was nearly $50 billion in the US (Florence et al., 2018). The use of exercise

interventions such as physical therapy, yoga, and Tai Chi all appear to improve ambulatory performance in the older adult (Gillespie et al., 2012; Sherrington et al., 2019; Youkhana et al., 2016), yet lack of accessibility to these programs as well as low patient compliance limits their potential benefit. Additionally, there are currently very few effective therapeutic approaches available to address fall risk in these individuals beyond recommending nutritional changes and maintenance of optimal vitamin D status (Esquivel, 2018; Medical Advisory, 2008; Patil et al., 2016; Scragg et al., 2016; Shahar et al., 2009; Smith, 2018). While rivastigmine has been shown to reduce fall frequency by 45% in patients diagnosed with Parkinson's disease (PD), these results did not identify potential pathways or cellular targets mediating this effect (Henderson et al., 2016), and it appears that other cholinomimetics do not reliably improve imbalance-related falls (Beauchet et al., 2013; Kapur et al., 2021). Recently, the use of fampridine, a potassium channel blocker, in individuals with multiple sclerosis appeared to be beneficial in im-proving gait speed (Lecat et al., 2017), while memantine, an anti-dementia drug, was able to improve gait variability in patients diagnosed with Alzheimer's disease (AD) (Beauchet et al., 2013). However, it is not clear if any of these drugs could be beneficial to the normal aging population, nor what central modalities they target. Finally, it is important to note that the use of certain prescription medications (e.g., benzodiazepines, α- and β-blockers) appears to correlate with, and often exacerbate, the fall risk in individuals >70 years old (Jiang et al., 2019; Perez-Ros et al., 2019). This produces a significant complication with effectively treating falls, as these medications are often prescribed for other age-associated dysregulations such as insomnia, high blood pressure, and anxiety. Because of this, attempting to identify new therapeutic avenues that more directly target the physiological

processes underlying these events without negatively impacting other factors of daily life is highly relevant.

1.3 Contribution of Peripheral Systems and Special Senses to Gait Dysregulation

While there has been a large amount of work focused on extrinsic physical interactions that can be modified to reduce fall risk in the older adult (i.e., type of shoes, mobility aids, environmental changes, etc.), even more intrinsic factors have been identified; these include peripheral alterations such as sarcopenia and metabolic disorders as well as impairments in special senses, such as vestibular and visual systems, which ultimately reduce activities of daily living.

1.3.1 Peripheral Systems

In cases where physical rehabilitation and balance training are used to improve gait dysregulation in older individuals, it is not clear which physiological changes underlie the benefits of these therapies. This is further complicated by the presence of comorbidities associated with aging. For example, age-dependent musculoskeletal alterations have been thoroughly implicated as a contributing factor to frailty and increased risk of falls (Lang et al., 2010). Lower limb weakness resulting from sarcopenia (the deterioration of muscle tissue with age) has also been shown to impede the ability to stand, reduce gait speed, and impair balance (Dutta, 1997). Further, the link between the increased prevalence of falls and sarcopenia in the older adult is clear, and this underlies many validated approaches for the management of falls in this population, particularly the strengthening of peripheral muscles using physical training and exercises such as Tai Chi (Bula et al., 2011; Hewston & Deshpande, 2018) and yoga (Hewston & Deshpande, 2018; Youkhana et al., 2016).

However, in addition to limiting lower limb function, sarcopenia is also associated with an elevated risk of developing metabolic dysregulation (Dutta, 1997), likely due to decreased peripheral glucose uptake resulting from diminished muscle mass. Interestingly, gait impairments are intensified by peripheral metabolic disorders such as Type-2 diabetes mellitus (T2DM) (Gregg, Engelgau, et al., 2002), particularly in older women (Gregg, Mangione, et al., 2002; Schwartz et al., 2002). Studies in older adults have shown that T2DM is also associated with an increased fear of falling and lower balance confidence (Gregg, Engelgau, et al., 2002; Kaye et al., 1994; Neri et al., 2019). Similarly, one meta-analysis of over 13,000 patients indicated that those with obesity and diabetes had a higher risk of falling and worsened recovery outcomes compared to healthy individuals (Peachey et al., 2018), while another analysis of over 1 million patients reported that obesity increased the likelihood of multiple falls in individuals over the age of 60 (Neri et al., 2019). While much has been learned regarding the role of physical exercise in treating these peripheral alterations, other intrinsic factors, particularly changes in vestibular and visual senses, have also been implicated in mediating poor stability outcomes with aging.

1.3.2 Special Senses

In addition to peripheral dysregulations, special sense impairments, such as vestibular and vision loss, are also associated with falls and poor gait in older individuals (Kaye et al., 1994; Lord et al., 2016). Vestibular signals contribute to balance and walking, where the otolith organs and semicircular canal output converge to guide the control of balance and posture during ambulation. In the older adult, decreases in semicircular canal function result in longer stride length and stance time, in addition to slower cadence (Anson et al., 2019). While vestibular hypofunction in advanced ages can lead to dizziness, postural

instability, and unsteady gait (Chow et al., 2021; Matheson et al., 1999), previous studies show that over 30% of people living at home and over 50% in assisted living facilities experience at least one fall per year, without experiencing dizziness (Blake et al., 1988; Campbell et al., 1981; Masud & Morris, 2001; Prudham & Evans, 1981), suggesting only a partial contribution of vestibular function to gait dysregulation with aging.

The vestibular system communicates regularly with the visual system to maintain stability through reflexes, such as the vestibulo-ocular reflex, which helps to stabilize gaze. Much research has also investigated the impact of vision loss in age-dependent gait alterations, as it plays a large role in coordination and planning of movement in addition to balance. One study recently showed that patients with age-related macular degeneration had significantly slower walking speeds and stride velocities (Varadaraj et al., 2017), while another reported that reduced contrast sensitivity, but not visual acuity, with age is associated with decreased stride lengths (Duggan et al., 2017). Further, slower gait and cadence, shortened stride length, and lengthened double support time are all exacerbated in extreme or changing lighting conditions, and is not shown to be dependent on fear of falling (Bicket et al., 2020).

Currently, a large amount of work has highlighted several central and peripheral intrinsic factors associated with comorbidities of aging that are tied to ambulatory distress, including hypertension, muscle weakness and fatigue, poor visual acuity, loss of vestibular function, weak tendons and/or joints, and reduced sensory modalities. However, while this work has given rise to a rich body of associative clinical studies, few, if any, have directly investigated the role of less-characterized brain regions that are associated with motor and

sensory ambulatory control, such as the basal ganglia or the primary motor and somatosensory cortices (see **Figure 1.1**).

1.4 Neuroanatomical Changes in Gait Processing Centers

The field of brain aging has often focused on cognitive- and memory-associated functions, and most investigations were conducted in the hippocampus and associated cortices (Morrison & Baxter, 2012). However, accumulating evidence shows that superficial layers in the primary somatosensory cortex receive inputs from the thalamus and cortical areas (Lacefield et al., 2019; Petreanu et al., 2009; Wimmer et al., 2010) associated with limb movement and sensory encoding (Cichon & Gan, 2015). Other critical regions include the dorsal basal ganglia and the motor thalamus (Clark et al., 2019; Phillips et al., 1993). Given that age is positively correlated with the number of falls an individual experiences (Hausdorff et al., 2001; Peel, 2011; Rothman et al., 2008), that dysregulation in hippocampal, cortical, and thalamic pathways (all of which are part of the gait processing network) predicts cognitive decline in AD (Albers et al., 2015; Scarmeas et al., 2005), and that cognitive status and falls share common mechanisms, it becomes difficult to ignore central aspects of motor/sensory function in aging.

1.4.1 Cerebellum

The cerebellum is a major brain region regulating balance, movement, vision, and motor learning and a key driver of gait. The cerebellar cortex differs from the neocortex in that it contains unique, expansively-branched Purkinje cells, which integrate large amounts of information from local and distant inputs. Importantly, age-dependent alterations in numbers of Purkinje cells, volume, and performance have been noted (Bernard & Seidler,

2014). Woodruff-Pak and colleagues have shown age-dependent loss of Purkinje cells paired with worsened performance on the cerebellar eyeblink conditioning task, and these changes appear to precede hippocampal atrophy (Woodruff-Pak et al., 2010). Additionally, decreases in cerebellar volume are seen with age, specifically in the cerebellar hemispheres and vermis (Raz et al., 1998; Raz et al., 2001). Interestingly, these same two studies reported larger cerebellar regions in males, compared to females, but these size differences are known to exist in children before puberty (Giedd et al., 1996). Apart from morphological changes, few studies have characterized neurochemical changes in the cerebellum with age, unfortunately. One study has shown increases in total creatine in the cerebellum of older adults (Zahr et al., 2013). While no age-dependent changes in glutamate, a major excitatory neurotransmitter, are seen in the cerebellum, decreases of glutamate are seen in other important brain regions, such as the striatum of the basal ganglia (Zahr et al., 2008; Zahr et al., 2013).

1.4.2 Basal Ganglia

The basal ganglia, including the caudate nucleus and putamen, are heavily involved in the tuning of voluntary motor output from the motor cortex. Specifically, these regions act in unison to determine the most appropriate motor behavior, including learned behavior (i.e., walking, running, etc.). Interestingly, some studies have shown a loss of neurons in the dorsal striatum with aging (Branch et al., 2014; Gibb & Lees, 1991). While this cell loss may be a contributor, often cellular processes become dysregulated as well, and indeed, in these areas, four decades of research have shown age-dependent reductions in dopamine levels as well as reduced D1 and D2 receptor expression (Carlsson & Winblad, 1976; Rinne et al., 1993; Suhara et al., 1991; Wang et al., 1998; Wong et al., 1997).

However, while changes in dopaminergic signaling in the basal ganglia with age have been well-described, few studies have investigated the role of these biomolecular changes on gait function. In older subjects, smaller caudate nucleus volumes have been correlated with slower walking speed (Dumurgier et al., 2012). Additionally, age-related neurological diseases that are associated with altered ambulatory function, such as AD, have also been tied to dysregulations in the basal ganglia, where changes in gait speed are associated with AD pathology, particularly in the posterior putamen (Del Campo et al., 2016). Still, while it is well-known that the basal ganglia contribute to the tuning of motor behavior and that aging can alter basal ganglia function, their impact on gait in the context of normal aging is not well characterized, and it is clear that other neuroanatomical gait centers are at play (Beauchet et al., 2017; Clark & Taylor, 2011).

1.4.3 Primary Motor Cortex (M1)

The M1 is responsible for the execution of movement and is the last cortical area involved in the process of motor output. This area sends long projections to lower CNS structures, including lower motor neurons in the spinal cord that control leg movements. As with the basal ganglia, the M1 is sensitive to aging, and changes in this region have been shown to correlate with impaired gait. For example, decreased M1 dopamine transport and receptor density is seen with aging (Mesco et al., 1991; Volkow et al., 1996), along with reduced M1 volume, which is associated with shorter stride length, longer double support time, and slower gait speed (Dumurgier et al., 2012; Rosano et al., 2008). Further, several studies have reported M1 hypoexcitability with age, including increases in intracortical inhibition and decreases in intracortical facilitation (Kossev et al., 2002; McGinley et al., 2010), which suggests reductions in motor output, perhaps mediated by

loss of functioning upper motor neurons (Clark & Taylor, 2011). Interestingly, studies have shown that during the performance of motor tasks, there is an age-associated increase in the recruitment of accessory processing areas (Naccarato et al., 2006; Rowe et al., 2006), such as the somatosensory cortex.

1.4.4 Primary Somatosensory Cortex (S1)

The S1 coordinates a series of functions including proprioception, pain, heat, and vibration sensation, as well as tactile discrimination. Importantly, information about proprioception, vibration, and tactile discrimination are all carried to the brain from the periphery via the same route, the dorsal column-medial lemniscus pathway (**Figure 1.2**). Although an increase in gait variability along with a reduction in volume in brain regions such as the parietal and sensorimotor cortices has been shown with age, no changes in S1 gray matter volume have been reported. However, it is interesting to note that in S1 slice recordings from aged rats, thalamocortical activation showed increased cellular excitability, as well as increased receptive field size and suppression of responses, compared to adult animals (Hickmott & Dinse, 2013; Spengler et al., 1995). Other animal studies have also shown age-associated increases in S1 neuronal excitability that may be mediated by changes in GABAergic innervation (Hickmott & Dinse, 2013; Popescu et al., 2021; Spengler et al., 1995). Similarly, in the clinic, a significant correlation between increased S1 excitability and impaired tactile acuity in older individuals was reported (Lenz et al., 2012).

It is clear that aging is associated with a decline in both tactile and motor function; however, recent evidence suggests that distinct aging-sensitive mechanisms may underlie the changes reported in these brain regions (Ruitenberg et al., 2019). Thus, future

investigations targeting region-specific processes and pathways that are associated with age-dependent gait dysfunction are warranted. One of the most prominent mechanisms that could be contributing to region-specific alterations affecting gait dysregulation is neuronal calcium (Ca^{2+}) signalling.

1.5 Ca^{2+} Hypothesis of Brain Aging

The Ca^{2+} hypothesis of brain aging is still considered critical, influential, and viable (Alzheimer's Association Calcium Hypothesis, 2017) and neuronal Ca^{2+} dysregulation has been recognized for over 30 years as a key biomarker of aging (Khachaturian, 1989; Landfield, 1987). The importance of Ca^{2+} in the brain extends back to the early work of Kostyuk and colleagues describing the presence of voltage-gated Ca^{2+} channels in the plasma membrane of snail neurons (Kostyuk et al., 1977), which was later confirmed in human skin fibroblasts using radioactive Ca^{2+}-labelling (Peterson et al., 1985). Around this time, many others were characterizing Ca^{2+} processes, including kinetics of Ca^{2+}-ATPases (Michaelis et al., 1984), Ca^{2+} currents (Landfield, 1987) and intracellular Ca^{2+} stores (Murchison & Griffith, 1999), conducting the work in culture and rodent models of aging and Alzheimer's disease. Together, these studies helped frame the "Ca^{2+} hypothesis of brain aging". Details that emerged early during this critical period highlighted the importance of several Ca^{2+}-sensitive proteins that could contribute to alterations in neurotransmission, membrane excitability, and synaptic plasticity (Miller, 1991; Verkhratsky, 2005).

Using biochemical, electrophysiological, molecular, and imaging techniques in the hippocampus, a variety of mechanisms have been identified that are both sensitive to Ca^{2+}

(see **Figure 1.3**) as well as aging. Initial work identified reductions of NMDA receptors with age and emphasized their role in neuronal excitability and synaptic plasticity (Clayton et al., 2002; Foster & Norris, 1997; Magnusson, 1998; Rosenzweig & Barnes, 2003). Around this time, the first reports of age-dependent increases in plasma membrane L-type voltage-gated Ca^{2+} channels (L-VGCC) were identified as regulators of excitability (Campbell et al., 1996; Moyer et al., 1992; Nunez-Santana et al., 2014; Thibault et al., 2001; Thibault & Landfield, 1996). Counterbalancing these inward fluxes of Ca^{2+}, sodium-Ca^{2+} exchangers (NCX) and Ca^{2+}-ATPases (PMCA) were identified as mechanisms that extrude Ca^{2+} into the extracellular space (Berridge et al., 2003) and that were also sensitive to age (Michaelis et al., 1984). Furthermore, within the cell on the endoplasmic reticulum (ER), inositol trisphosphate (IP_3) receptors respond to second messenger signals allowing Ca^{2+} to leave the ER into the cytosol (Berridge et al., 2003). Those, too, have been shown to be enhanced with aging as reviewed in Oh et al. (2010). Similarly, ryanodine receptors (RyR) on the ER, responsible for Ca^{2+}-induced Ca^{2+} release (CICR) also show increases in function in animal models of aging and age-matched controls (Gant et al., 2006; Kumar & Foster, 2005; Murchison & Griffith, 1999; Stutzmann et al., 2006). Once Ca^{2+} is free in the cytosol, it can interact with proteins and structures that can either target downstream pathways or act as buffers to limit Ca^{2+} effects (Berridge et al., 2003); perhaps not surprisingly, these targets also have been shown to be decreased with age (Murchison & Griffith, 1999). Together, these sources and targets are clearly sensitive to Ca^{2+} and aging, are pivotal for our understanding of underlying mechanisms that contribute to alterations in neurophysiology but are also limited. The most prominent limitations that have hindered a more recent expansion of the Ca^{2+} hypothesis of brain aging include: 1) a weakened

network evaluation based on unicellular assessments, 2) the use of *ex vivo* preparations, and 3) a focus on hippocampal-centric approaches.

1.6 Neurons Communicate as a Network

While synaptic communication has been well-characterized in specific brain regions in limited numbers of neurons across age, these techniques cannot report on neuronal Ca^{2+} network status. Subsets of neurons within these neuronal networks work together to perform a specific function or to encode information, such as sensation or memory. These neurons are known to communicate as an "ensemble", where individual neurons can report on limited information but act together to provide a rich characterization of the environment or context (Aery Jones & Giocomo, 2023). Neuronal ensembles can be defined based on their activity patterns, connectivity, and synchronicity (Carrillo-Reid & Yuste, 2020). Furthermore, ensembles are dynamic, reflective of changes over time, and alterations in recruitment in different ways depends on the task, the context, and the state of the subject (Guzowski et al., 1999; Lacagnina et al., 2019; Ryan et al., 2015). Of note, a majority of the literature on brain aging, including studies on neuronal ensembles, has focused on cognitive processes related to hippocampal functions of memory and learning (Guzowski et al., 1999; Lacagnina et al., 2019; Reijmers et al., 2007; Ryan et al., 2015), as these tend to be critically modified behaviors with both normal aging and aging-related neurodegenerative diseases. Whether these changes are mediated by short-term excessive levels of Ca^{2+}, or by a more subtle, yet sustained alteration in Ca^{2+} signalling remains to be determined. However, it is clear that other processes, including gait behavior, are also sensitive to both Ca^{2+} and age. Thus, it seems critical to further investigate central Ca^{2+}

dysregulation with age in these key areas and how these changes may contribute to alterations in ambulatory performance.

1.7 Two-Photon (2P) Imaging

Advances in calcium imaging have provided new ways to investigate the brain in its original state, rather than slice preparation or in culture. New techniques, such as two-photon imaging, allow us to investigate the intact brain while animals are awake and behaving. One-photon and two-photon imaging are both techniques used to visualize biological structures and processes at the cellular level (*e.g.*, neuronal Ca^{2+} signalling), but they differ in the way they excite fluorescent molecules.

In one-photon imaging, a single photon of a specific wavelength is used to excite the fluorescent molecule (**Figure 1.4**, left), causing it to emit a photon of a longer wavelength that can be detected and used to generate an image. This technique is widely used in conventional and confocal fluorescence microscopy, as it is relatively simple and can provide high temporal resolution. However, it has limitations in terms of depth penetration and potential phototoxicity due to the use of high-energy photons.

In two-photon imaging, two photons of longer wavelengths are used to excite the fluorescent molecule simultaneously (**Figure 1.4**, right), causing it to emit a photon of shorter wavelength that can be detected and used to generate an image. This technique has several advantages over one-photon imaging, including increased depth penetration, reduced phototoxicity, and better spatial resolution due to the non-linear excitation process. Two-photon imaging is a tool that is commonly used in neuroscience, where it allows us to visualize the activity of neurons in live animals. By pairing two-photon imaging with

specific genetically encoded, fluorescent indicators, such as those that report on Ca^{2+} dynamics, we are able to characterize similar measures as those in slice or culture across hundreds of neurons, with the addition of being in an awake and behaving animal, providing a more accurate characterization of the neurophysiology.

1.8 Significance and Hypothesis

Much research on neuronal Ca^{2+} dynamics have been focused on investigating cognitive decline with age, and much of these studies focus on the hippocampus and its connections to the prefrontal cortex. However, it is clear that other age-dependent changes in neurophysiology and behavior are seen in our older population, particularly changes in gait, a major risk factor for falls. Fatal and non-fatal falls accounted for an estimated $50 billion in medical costs in just 2015 alone (Florence et al., 2018). Furthermore, with a 30% increase in fall death rates from 2007 to 2016, the CDC anticipates 7 fall-related deaths in the older adult every hour by 2030. Unmet treatment outcomes using mechanical aides, together with evidence of only a partial influence of peripheral factors (muscle weakness, decreased vision, and vestibular degeneration), suggest a central mechanism may be involved in the age-dependent increase in falls. Additionally, given decreased incidence of falls in older women compared to older men (Aryee et al., 2017; Ek et al., 2019), potential sex differences may give clue to specific mechanisms mediating gait dysregulation with age.

Here, and for the first time, we used *in vigilo* two-photon (2P) imaging techniques to measure neuronal Ca^{2+} dynamics at the single-cell and network levels across ~2,000 neurons in S1 of young and aged ambulating mice. Additionally, we investigated all

measures in both males and females to elucidate potential sex differences in neuronal network and behavioral phenotypes. In this study, we test the hypothesis that alterations in S1 neuronal Ca^{2+} networks in ambulating mice impact distinct patterns of gait behavior, and these alterations differ across both age and sex.

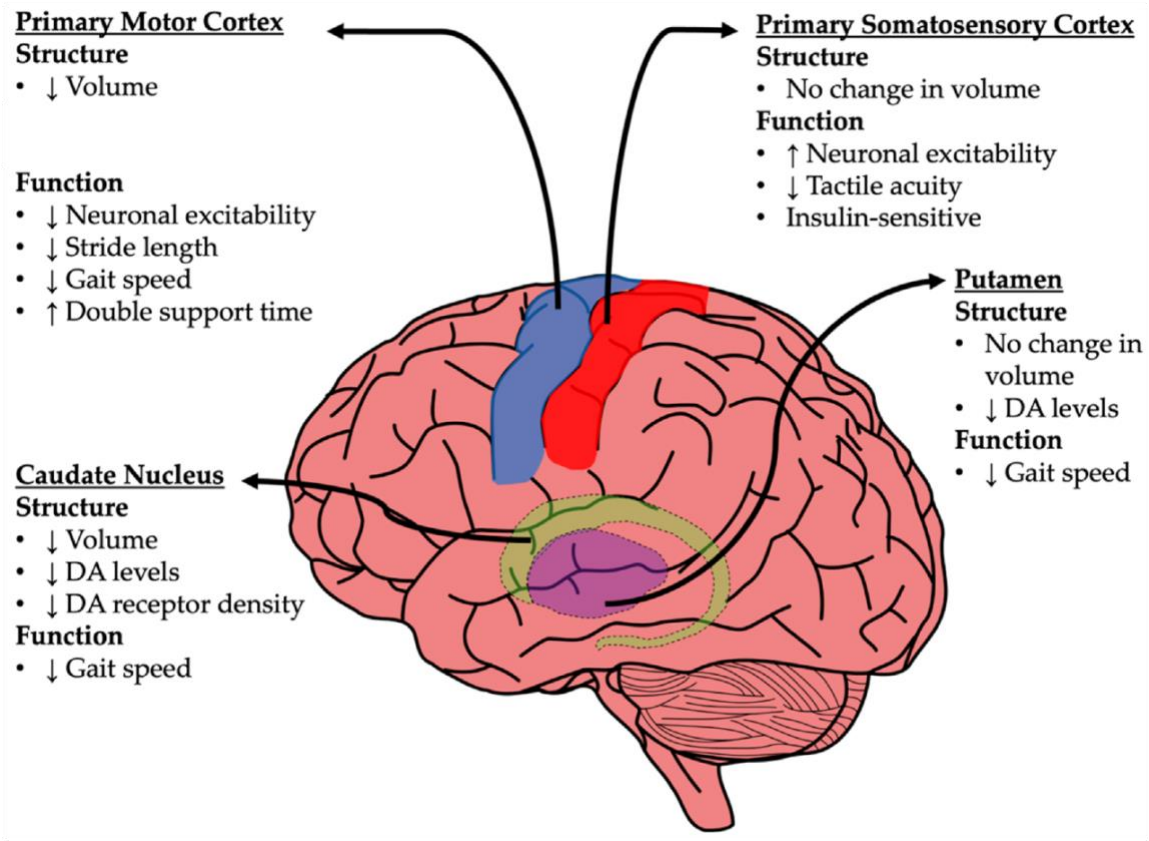


Figure 1.1: Aging-related Structural and Functional Changes in Key Brain Regions that Control Gait

Several key brains regions are involved in awareness, tuning, and initiation of motor movement that help to control gait including the primary sensorimotor cortices and basal ganglia, and these areas have been known to undergo structural and functional changes with age. In the basal ganglia, specifically in the caudate nucleus and putamen, decreases in dopamine levels, the major neurotransmitter in these areas, has been shown to be correlated with decreases in gait speed. Additionally, decreases volume and dopamine receptor density has been reported in the caudate nucleus. In the primary motor cortex (M1), neurons have been shown to be hypoexcitable with age, and atrophy of this region is prominent. These age-related changes in M1 are highly correlated with worse gait

performance. In the primary somatosensory cortex, age-dependent increases in neuronal activity (i.e., hyperexcitability) have been reported, and decreases in tactile acuity in humans are correlated with these alterations.

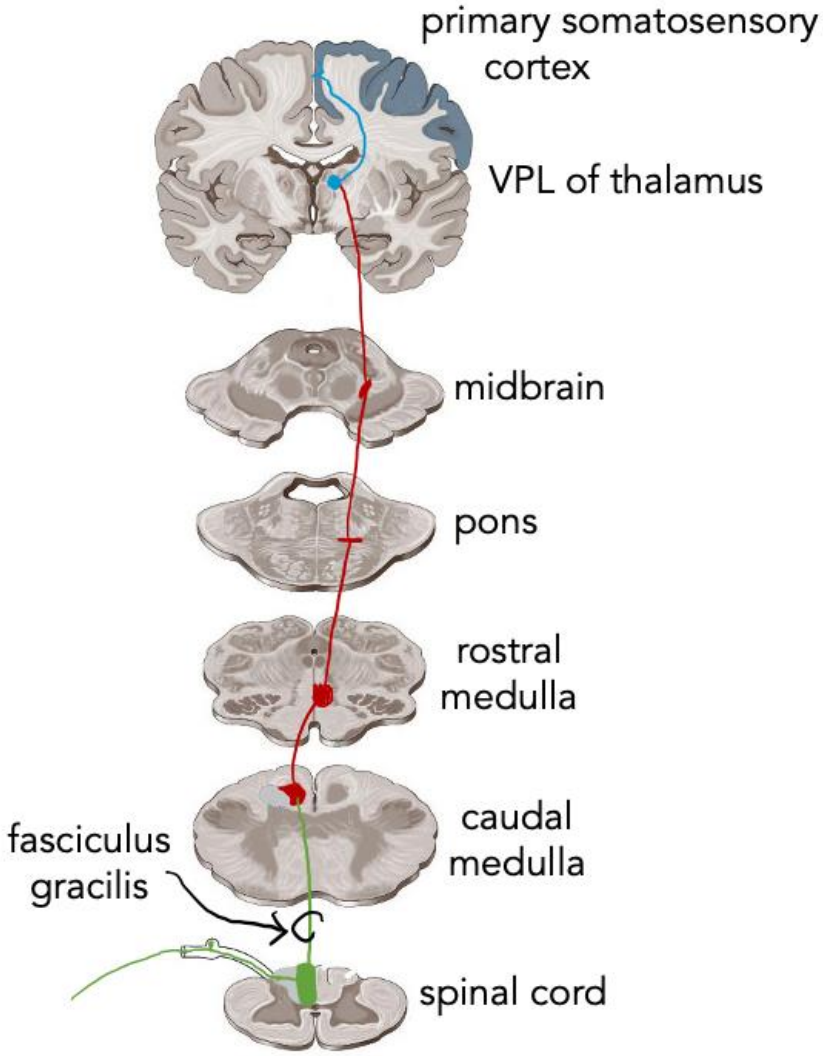


Figure 1.2: Dorsal Column-Medial Lemniscus Pathway

Diagram of the dorsal column-medial lemniscus pathway for sensations from lower limb in human; equivalent to the hindlimb in rodents. First order neurons carry information from the periphery into the spinal cord via the dorsal root ganglia. These neurons ascend as a bundle called the fasciculus gracilis and synapse on second order neurons at the nucleus gracilis located in the caudal medulla. Second order neurons cross as arcuate fibers to the contralateral side of the medulla and form a bundle called the medial lemniscus. The medial

lemniscus continues into the pons, midbrain, and these second order neurons synapse at the ventral posterolateral (VPL) nucleus of the thalamus. Third order neurons in the VPL carry information via the internal capsule to the primary somatosensory cortex, where somatosensory information is processed.

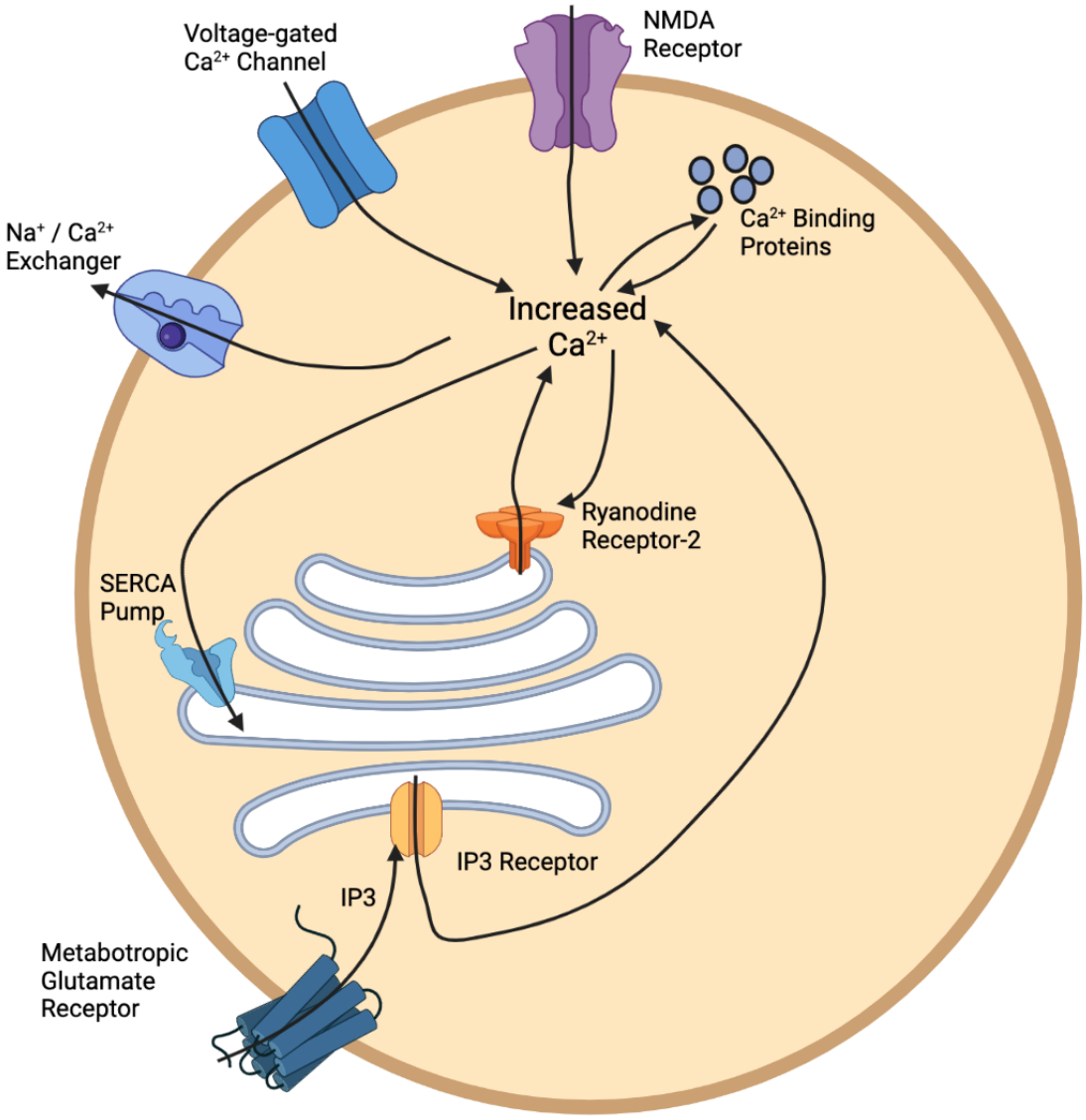


Figure 1.3: Mechanisms of Neuronal Ca^{2+} Transport

Neurons exhibit multiple mechanisms to handle intracellular levels of Ca^{2+}, including those on the plasma membrane, on intracellular membranes, and in the cytosol. On the plasma membrane, there are NMDA receptors and voltage-gated Ca^{2+} channels that bring Ca^{2+} into the cell as well as Na^{+}/Ca^{2+} exchangers that efflux Ca^{2+}. SERCA pumps move intracellular Ca^{2+} into the endoplasmic reticulum (ER), while IP_3 and ryanodine receptors can be activated to release stores of Ca^{2+} from the ER into the cytosol. Within the cytosol,

intracellular proteins can either buffer Ca^{2+} or be activated, which leads to important downstream signalling within the cell.

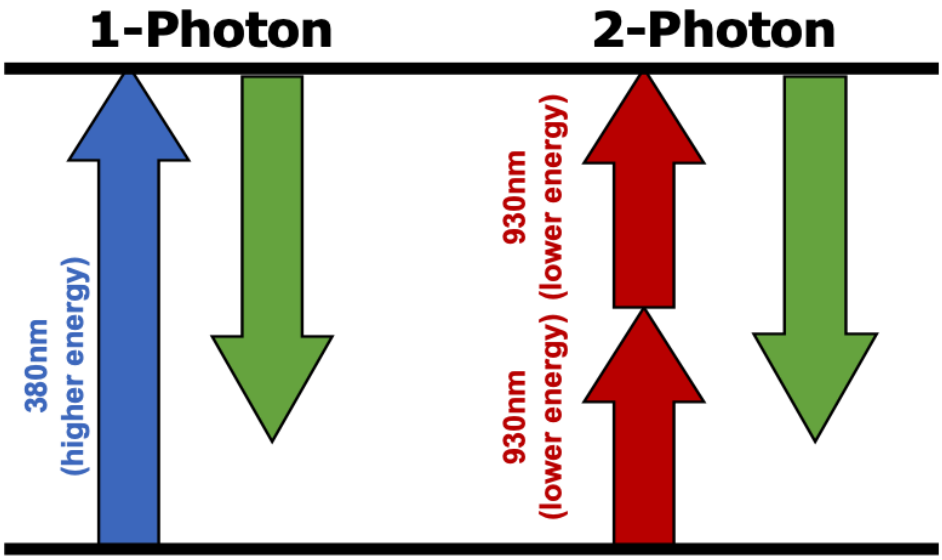


Figure 1.4: One-Photon vs Two-Photon Imaging

In one-photon imaging, a single photon of a specific wavelength is used to excite the fluorescent molecule, causing it to emit a photon of a longer wavelength that can be detected and used to generate an image. This technique is widely used in conventional and confocal fluorescence microscopy, as it is relatively simple and can provide high temporal resolution. However, it has limitations in terms of depth penetration and potential phototoxicity due to the use of high-energy photons. In two-photon imaging, two photons of longer wavelengths are used to excite the fluorescent molecule simultaneously, causing it to emit a photon of shorter wavelength that can be detected and used to generate an image. This technique has several advantages over one-photon imaging, including increased depth penetration, reduced phototoxicity, and better spatial resolution due to the non-linear excitation process.

CHAPTER 2. METHODS

2.1 Animals

The work presented here strictly adheres to our Institutional Animal Care and Use Committee protocol. A timeline of procedures is provided in **Figure 2.1**. On Week 0, young adult (4 months; male n = 11, female n = 12) and aged (22 months; male n = 10, female n = 10) C57BL/6J mice were received from The Jackson Laboratory (Bar Harbor, MN). On Week 1, animals underwent adeno-associated virus (AAV) injection and installation of a chronic cranial window and headplate. During Weeks 2-4, animals were allowed to recover. On Week 5, animals were tested using a grip strength meter to measure forelimb and hindlimb grip strengths. On Week 6, animals were habituated to Neurotar Mobile HomeCage and 2P room and trained to ambulate during head-fixation. On Week 7, following six weeks of GCaMP8f expression, 2P neuronal Ca^{2+} imaging in S1 cortex was obtained. On Week 8, gait parameters were obtained while animals ambulated on the 3-plane visualization walking task apparatus.

2.2 AAV Injection and Chronic Cranial Window Surgery

After one week of acclimation to vivarium conditions, animals received aseptic injections of AAV (1:1 dilution of $1x10^{13}$ vg/mL titer stock in sterile saline) carrying the neuron-specific Ca^{2+} indicator GCaMP8f (pGP-AAV-syn-jGCaMP8f-WPRE; Addgene #162376-AAV9). Briefly, animals were anesthetized (1.5-3.5% isoflurane) and placed on a heated pad (37 °C), then head-fixed with non-penetrating ear bars to a stereotaxic frame (Kopf, Tujunga, CA). Artificial tears (GenTeal®) were placed on each eye and 0.5 mL

warm sterile saline was administered subcutaneously. PhysioSuite (Kent Scientific Corporation, Torrington, CT) was used to monitor physiological outcomes (heart and respiratory rate, body temperature, and O_2 saturation) and to control the heated pad. Hair above the skull was wettened with an alcohol pad, then shaved using a sterile scalpel. After application of alcohol and povidone-iodine to the shaved skin, a circular piece of tissue was removed around the boundaries of the scalp to expose the skull. A motorized hand drill (Foredom K. 1070 High Speed Rotary Micromotor Kit; Blackstone Industries, Bethel, CT) was then used to remove the periosteum from the bone, followed by application of acetone to degrease the skull. Bonding agent (VivaPen®; Ivoclar Vivadent, Schaan, Liechtenstein) was applied to cover the skull and cured by UV light (LY-B200 Dental LED Curing Light). A 3-mm diameter hole was then drilled stereotaxically (Drill and Injection Robot with automatic depth detection; Neurostar, Tubingen, Germany) on either the left or right skull directly above S1 (**Figure 2.2**, left; center of 3-mm hole: ML = ± 1.62 mm; AP = -0.5 mm). The space above the brain was irrigated with cold saline following removal of the 3-mm bone flap. Advancement/retraction speed of the drill was set to 1.0 mm/min. AAV injection (0.25 µL at 0.2 µL/min) was accomplished (**Figure 2.2**, right; ML = ± 1.62 mm; AP = -0.5 mm; DV = -0.40 mm) using a Hamilton® syringe with a 30° bevel as shown in **Figure 2.3**. A 4-mm circular glass coverslip (CS-4R; Warner Instruments, Holliston, MA) and 8.3-mm circular stainless steel headplate (Model 5 Headplate; Neurotar, Helsinki, Finland) were attached to the bone using light-curing dental cement (Fusion Flo; Prevest Denpro Ltd., Jammu, India) as shown in **Figure 2.4**. Following this, animals received subcutaneous injections of meloxicam (10 mg/kg) and buprenorphine (0.2 mg/kg) and were temporarily placed in a heated cage for recovery. Once fully awake and ambulating normally, animals

were returned to their home cage. Post-operative inflammation was controlled by re-administration of subcutaneous meloxicam (10 mg/kg) 24 hours following surgery. Animals recovered for 3 weeks to allow inflammation reduction and adequate expression of GCaMP8f.

2.3 Grip Strength Testing

To test neuromuscular function between age groups, all animals underwent grip strength testing of forelimb and hindlimbs using a digital grip strength meter (Columbus Instruments; Columbus, OH) showing in **Figure 2.5**. For forelimb measures, the mouse was lowered over a horizontal grid, allowing only its forepaws to attach, then gently pulled back by the tail until a maximum grip strength value was recorded. For hindlimb measures, the mouse was lowered over a downwards tilted (30º angle) grid allowing only the hindlimbs to attach, then measuring the maximum force when the animal pushed off to jump from the grid. For all limbs combined, the mouse was lowered over the horizontal grid, allowing both the forepaws and hindpaws to attach, then gently pulled back by the tail until a maximum grip strength value was recorded. All measures were repeated three times per animal, then averaged to yield one value per measurement per animal. We present grip strength measures collected from 36 GCaMP8f-treated animals (young male n = 8, young female n = 11, aged male n = 9, aged female n = 8).

2.4 Neurotar Mobile HomeCage Environment

To characterize neuronal Ca^{2+} network during ambulation across multiple surfaces, animals were trained to ambulate in a floating carbon-fiber cage environment (Neurotar

Mobile HomeCage Large). Six days prior to 2P imaging, animals were habituated to the environment in the 2P imaging room for two days, then handled to allow habituation to brief head-fixation (using fingers to grab headbar for periods of 5 seconds) for an additional two days. Animals then underwent acclimation to 20-minute periods of head-fixation in the Neurotar environment for two days prior to imaging, in which they could navigate the donut maze (**Figure 2.6**). Animals that did not ambulate around the donut after a 20-minute period were considered to have failed to adapt to the task and were removed from the study.

2.5 Two-photon Microscopy

2P imaging was accomplished in layers 2/3 of S1 (~200 μm below the dura) using a Scientifica Hyperscope (Uckfield, United Kingdom) equipped with scanning mirrors (1 resonant, 2 galvos), a large back aperture objective (16X, NA = 0.8; WD = 3.0 mm; Nikon, Tokyo, Japan), and a GaAsP detector mounted inside a multiphoton detection chamber (MDUXL) housing dichroic and infrared blocking filters. All hardware and data acquisition were controlled by ScanImage (v2021.0.0; Vidrio Technologies, Leesburg, VA) running under MATLAB (vR2020b; MathWorks, Natick, MA). GCaMP8f was excited at 930 nm using an InSight X3 dual-wavelength femtosecond-pulsed laser (Spectra-Physics, Milpitas, CA). Image acquisition (512 x 512 pixels at 30 Hz) was accomplished during ambulation and rest. Velocity of animal movement was collected simultaneously using a locomotion tracking software (Neurotar, Helsinki, Finland). One field of view (FOV) was randomly selected within the boundaries of the hindlimb area of S1 (left or right hemisphere, equally distributed across all animals), and was stored for further analysis and an example of a FOV is shown in **Figure 2.7**.

2.6 2P Image Processing and Extraction

Signal processing and data extraction were accomplished using a custom MATLAB pipeline as previously described (Lin et al., 2022). Image stacks of each file were imported as cubes (**Figure 2.8**, left). X and Y [pixels] and timepoints). For network and ΔF/F Ca^{2+} analysis, regions of interest (ROIs) of individual neurons were selected (**Figure 2.8**, right) using adaptive thresholding (sensitivity at 0.35) to binarize potential ROIs followed by size (>60 pixel) and shape (0-0.9 eccentricity) filters to remove any undersized or irregularly-shaped ROIs, as these likely represent imaging artifacts or cellular debris. All fields of view (FOVs) were checked for the presence of clear, morphologically distinct single neurons. The thresholded and filtered image was then used to extract raw GCaMP8f signal intensities across time (traces) for all ROIs in the FOV. Across all 2P Ca^{2+} data, we analyzed a total of 564 neurons from 7 young males, 423 neurons from 5 aged males, 574 neurons from 6 young females, and 498 neurons from 5 aged females.

2.7 Network Analysis

For each ROI, the raw trace underwent a continuous wavelet transform (CWT) using a Morse wavelet to extract power across frequencies (0.05–14 Hz), shown in **Figure 2.9**. The peak magnitude within discrete frequency bands (*i.e.*, 0.1-0.5, 0.5-1.0, 1.0–2.0 Hz, etc.) of the CWT spectrum was then determined for each frequency band across time, then binarized. An event was identified if the power was >2 times the standard deviation from the mean power, providing a dataset of ones (events) and zeroes (non-events) across time. Measures of active neurons were calculated by dividing the number of active neurons with at least 1 event during ambulation in each FOV by the FOV's area in mm^2. Binarized events

were used to calculate a correlation coefficient (CC) for each pair of neurons across time, shown in **Figure 2.10**. We initially selected a thresholded CC value of >0.4, allowing us to select for pairs of neurons 40% with coinciding activity (considered an active connection). Connectivity was defined as the number of active connections per mm^2. Connection length was defined as the average distance (in μm) between each pair. Network synchronicity was derived by calculating a ratio of the number of events divided by the total number of possible events for each neuron and is provided as a percentage of the time. To calculate the total number of possible events, we used the maximum of each frequency range. For instance, to analyze 1-2 Hz range, we multiply 2 Hz by the length of the period (the animal was either ambulating or resting) to yield the number of total possible events per neuron (*e.g.*, 2 Hz x 45 s = 90 total possible events). We report network measures collected from 23 GCaMP8f-treated animals (young male $n = 7$, aged male $n = 5$, young female $n = 6$, aged female $n = 5$).

2.8 A Test: Pacing the Neuronal Network

Using the F344 rat from a previous experiment, we wanted to see if we could peripherally pace the neuronal Ca^{2+} network. In this test, we created a cranial window over the right S1 in the anesthetized rat and stimulated the contralateral paw pads during imaging at 1 Hz, 2 Hz, and 3 Hz for 5 seconds. We then used the network analysis to examine which frequencies these neurons were communicating at, as shown in **Figure 2.11**. Importantly, the network was reliably paced to the peripheral frequency, validating our measures of network frequencies using the CWT method.

2.9 ΔF/F Analysis

For ΔF/F analysis, the threshold for an event in each ROI was set to >2 times the standard deviation from the mean GCaMP8f intensity during resting (*i.e.*, animal stationary). Once identified, Ca^{2+} transients were then characterized for measures of neuronal activity (# of events detected per second), area-under-curve, rise-time, and decay-time constants, shown in **Figure 2.12**. Each trace was normalized to pre-peak baselines (%ΔF/F). One young female displayed values on two measures (area-under-curve [**Figure 3.6B**] and peak amplitude [**Figure 3.6C**]) reported as outliers, we therefore report ΔF/F measures collected from 22 GCaMP8f-treated animals (young male n = 7, aged male n = 5, young female n = 5, aged female n = 5).

2.10 Gait Behavior

For comparison of gait across age, animals were recorded using a digital camera (3840 x 2160 pixels at 25 Hz) ambulating across a 3-plane visualization walking task (**Figure 2.13**) built in-house (Lin et al., 2022). Animals were recorded ambulating across four surfaces (3 mm, 4 mm, and 5 mm plastic mesh; and flat control). Animals that stopped midway through the task were placed at the beginning of the corridor and re-tested until they completed at least four consistent, consecutive steps per surface. We report gait measures collected from 31 GCaMP8f-treated animals (young male n = 8, aged male n = 6, young female n = 10, aged female n = 7).

2.11 Quantification of Gait Parameters

We used previously published methods (Lin et al, 2022) to quantify gait behavior using a reduced corridor width (4.2 cm) in order to accommodate for the smaller animals used here. Briefly, we analyzed the portion of videos with four consistent, consecutive steps in FIJI (ImageJ2 v2.3.0/1.53q) to extract the X and Y position of each paw across time. Measures of locomotor stability were calculated as previously described in Lin et al. (2022) including: ambulation velocity (cm/sec), deviance from center index – the sum of all paw distances (in cm) from the center of the corridor divided by four steps, paw precision index – average of r^2 values derived from each paw's linear regression across four steps, and stride length deviance index – standard deviation of absolute distances between step-to-step paw placements for each paw. Additionally, stride length and a stride time deviation were calculated as follows: stride length – average distance (in cm) between step-to-step paw placement for each paw across four steps; stride time deviation – the standard deviation of time (in seconds) between step-to-step paw placements for each paw across four steps. Diagrams of gait measures are shown in **Figure 2.14**.

2.12 Experimental Design and Statistical Analysis

Due to the complexity of the experiment design which includes young and aged animals undergoing a chronic craniotomy, training on the Neurotar HomeCage environment, 2P imaging of ambulating animals, and collection of grip strength and gait measures, some animals do not have complete datasets for every measure (see **Table 2.1**). All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA). All datasets were tested for significance using either a 2-way or a 3-way

ANOVA (with or without repeated measures [RM]). For all measures of gait (**Figure 3.7**), one surface (4-mm) was missing from one young female due to a computer recording error; thus, a mixed effects (REML) analysis was used. Significance for all measures was defined as $p < 0.05$. All data are presented as means ± standard error of the mean. For all graphs with $n < 10$, we included individual datapoints.

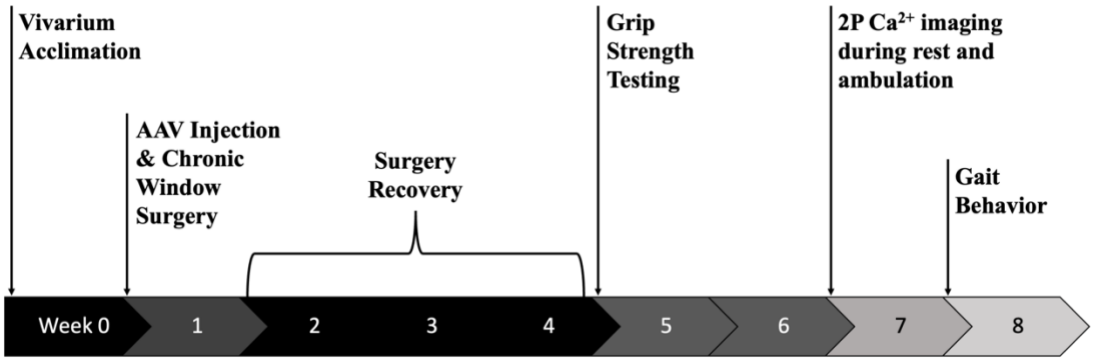


Figure 2.1: Timeline of Procedures and Experiments

On Week 0, animals will acclimate to the vivarium conditions. On Week 1, animals will undergo AAV injection and installation of a chronic cranial window and headplate. During Weeks 2-4, animals recovered from surgery to allow post-operative inflammation to decrease and for adequate expression of the GCaMP8f nanosensor. On Week 5, animals were tested using a grip strength meter to measure forelimb, hindlimb, and fore/hindlimb combination grip strengths. On Week 7, two-photon imaging of GCaMP8f fluorescence in the primary somatosensory cortex during ambulation of animals across the Neurotar environment was be obtained. On Week 8, animals walked down the 3-plane visualization walking task to measure aspects of gait behavior.

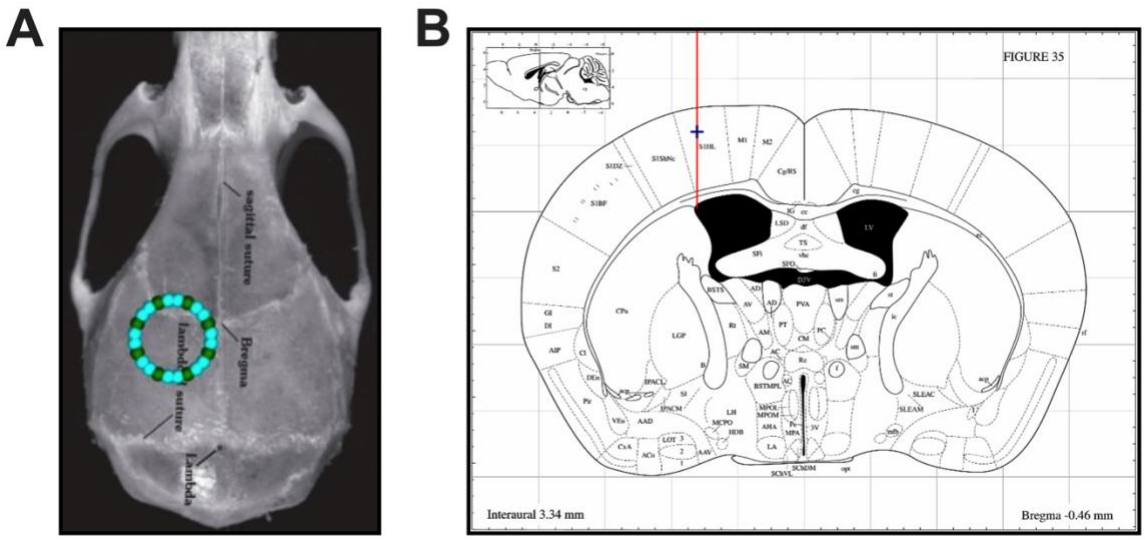


Figure 2.2: Location of AAV Injection and 3-mm Cranial Window

(**A**) 3-mm hole was drilled above the primary somatosensory cortex (center: ML = ± 1.62 mm; AP = -0.5 mm) to expose the brain of a mouse. (**B**) Target of AAV injection of GCaMP8f was layers 2/3 of the hindlimb area of the somatosensory cortex (ML = ± 1.62 mm; AP = -0.5 mm; DV = -0.40 mm).

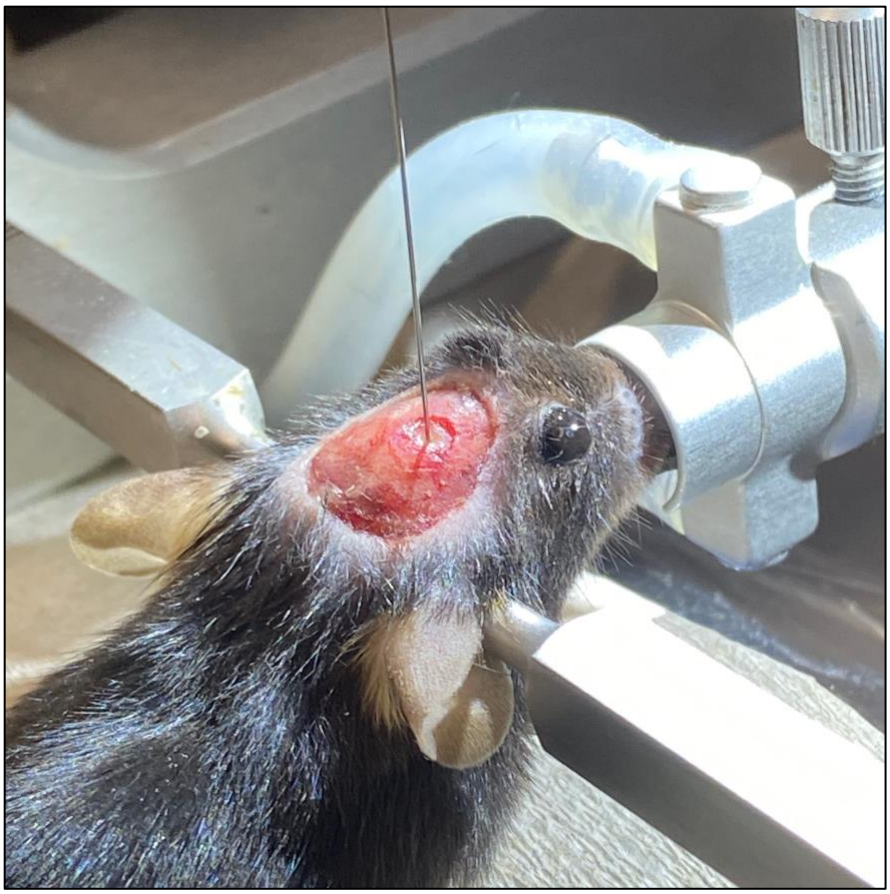


Figure 2.3: Injection of GCaMP8f AAV in Layers 2/3 of Mouse S1

AAV injection (0.25 µL at 0.2 µL/min) was accomplished (ML = ± 1.62 mm; AP = -0.5 mm; DV = -0.40 mm) using a Hamilton® syringe with a 30° bevel.

Figure 2.4: Neurotar Headbar Fixed to Skull of Mouse

Following AAV injection, a 4-mm circular glass coverslip was used to cover the 3-mm hole and it was sealed with UV light-curing dental cement. Then, a stainless steel headbar with an 8.3-mm circular stainless steel headplate were attached to the bone using light-curing dental cement.

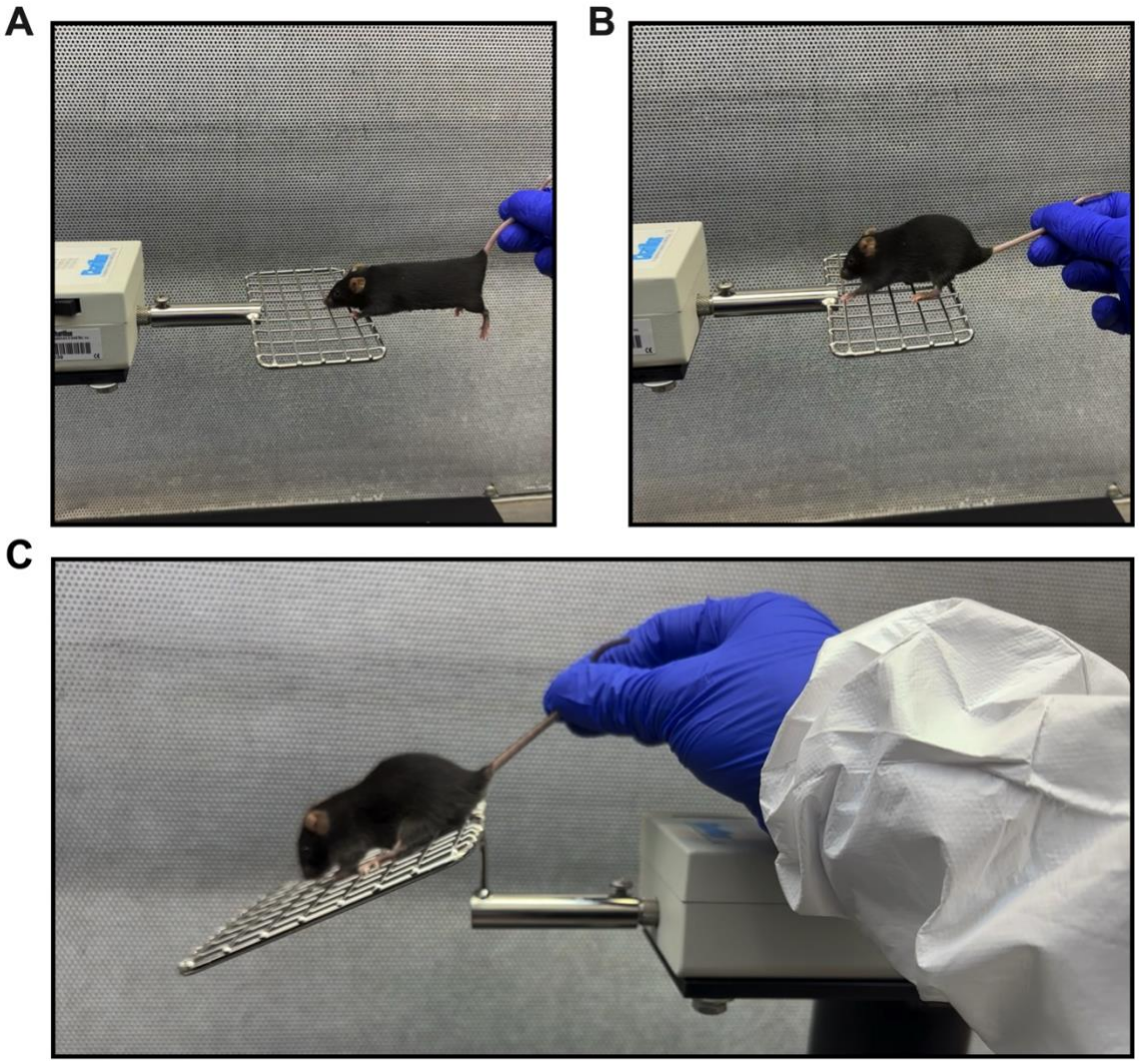


Figure 2.5: Grip Strength Meter

Measures of grip strength were collected using a digital meter across three conditions: (**A**) forelimb only, (**B**) forelimb and hindlimb combined, and (**C**) hindlimb only.

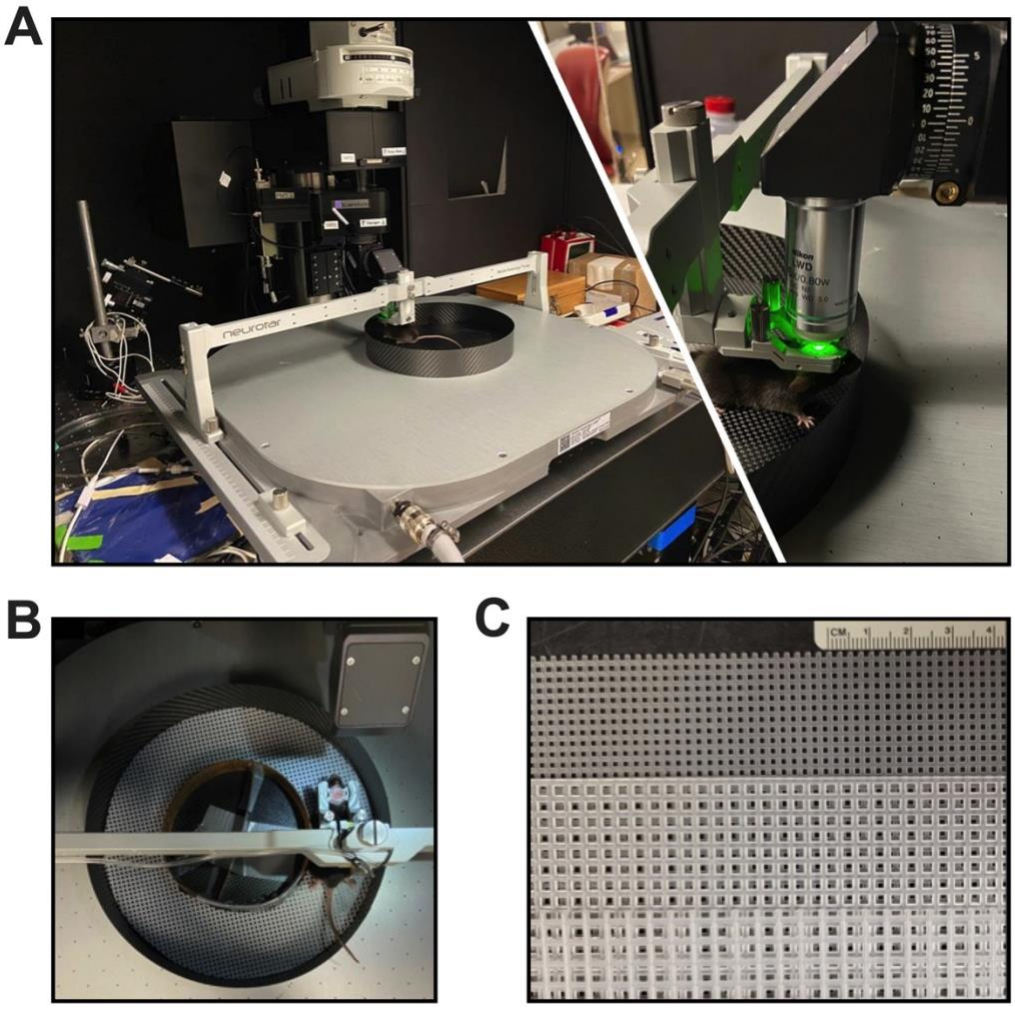


Figure 2.6: Neurotar Mobile HomeCage Large Environment

(**A**) Side view of Neurotar Mobile HomeCage Large environment and zoomed in on head-mount and 2P objective. (**B**) Bird's eye view of animal ambulating across a 4-mm plastic mesh surface. (**C**) 3-mm, 4-mm, and 5-mm plastic mesh surfaces.

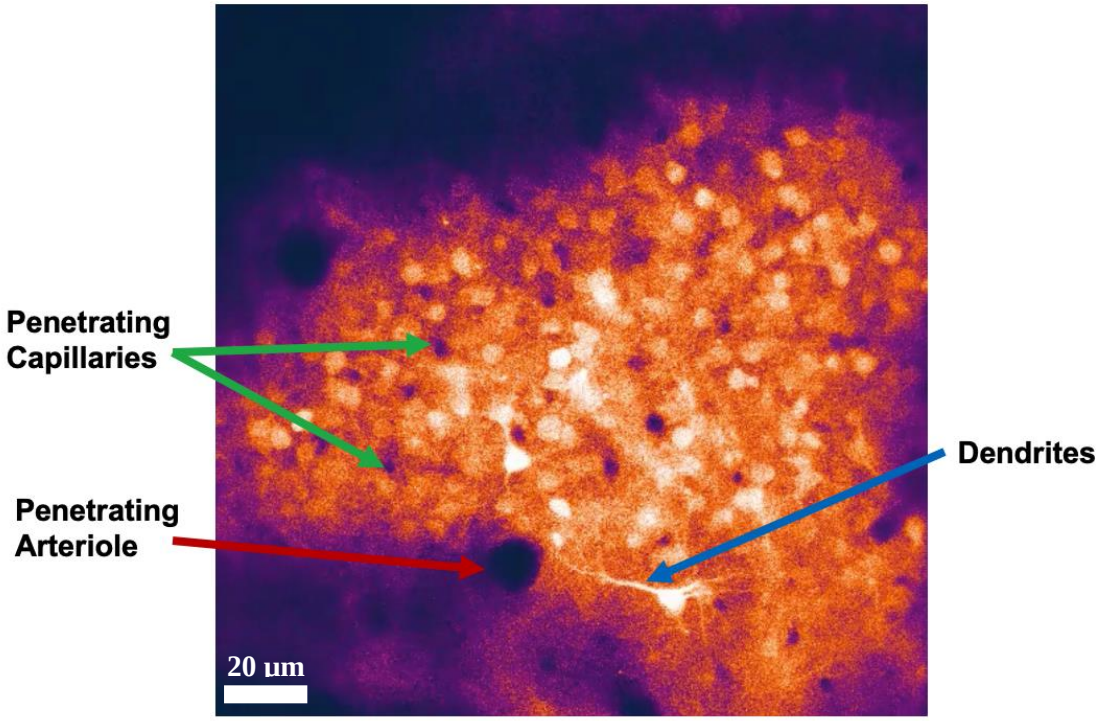


Figure 2.7: FOV Obtained in Layer 2/3 of S1 in an Awake Mouse with High Spatial Resolution

Example pseudo-colored photomicrograph of a field of view obtained from layers 2/3 of primary somatosensory cortex in a C57BL/6J mouse during ambulation with high spatial resolution. Distinct vasculature and neuronal dendrites can be seen. Additionally, a prominent, penetrating arteriole is seen, and during ambulation, the shadow of the penetrating arteriole changes circumference, representative of neurovascular coupling.

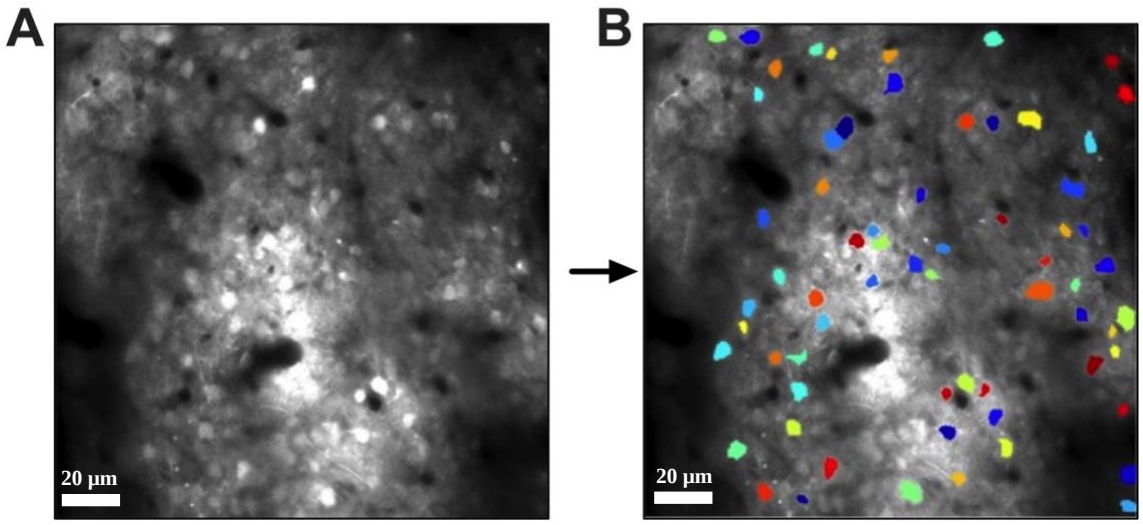


Figure 2.8: Selection of ROIs from FOV

Using a MATLAB based routine, neurons from a field of view (**A**) are selected using adaptive thresholding (sensitivity at 0.35) to binarize potential ROIs (**B**) followed by size (>60 pixel) and shape (0-0.9 eccentricity) filters to remove any undersized or irregularly-shaped ROIs, as these likely represent imaging artifacts or cellular debris. All fields of view were checked for the presence of clear, morphologically distinct single neurons.

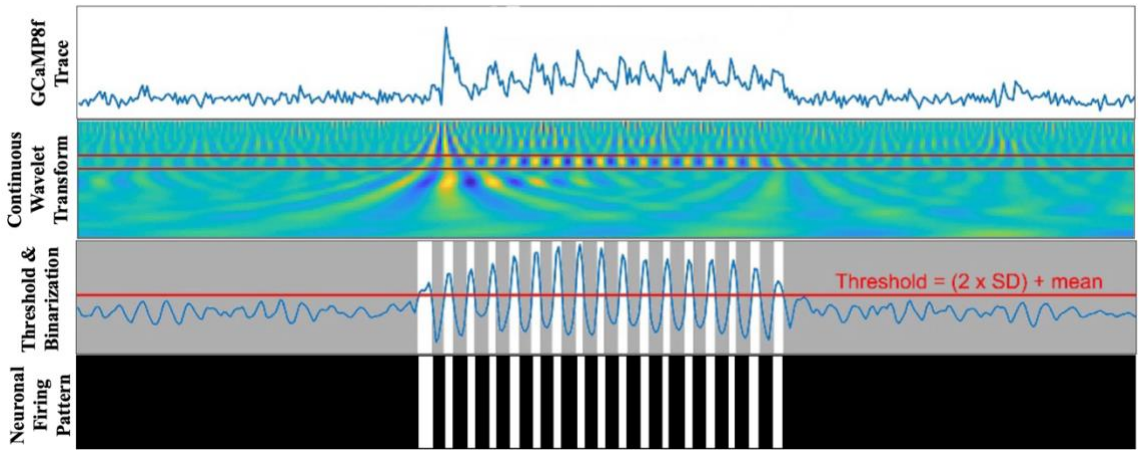
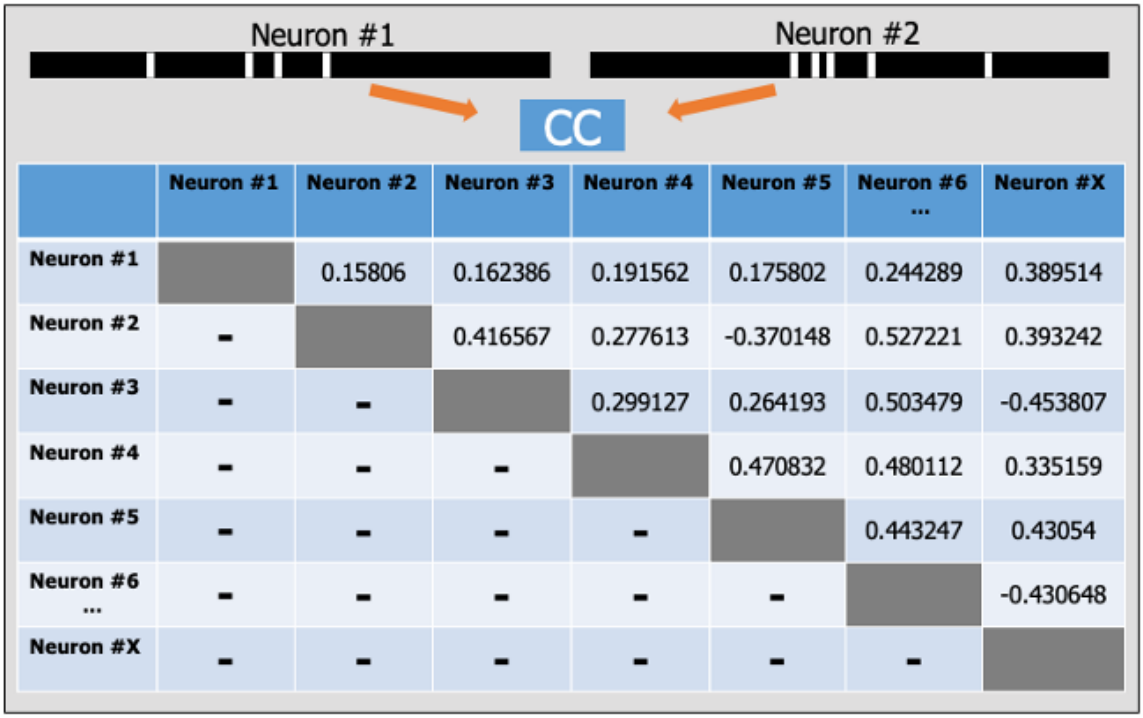


Figure 2.9: Binarization of Trace (1-2 Hz Range) Using CWT Routine

For each ROI, the raw trace (**Panel 1**) underwent a continuous wavelet transform (CWT) using a Morse wavelet to extract power across frequencies (**Panel 2**, 0.05–14 Hz). The peak magnitude within discrete frequency bands (*i.e.*, 0.1-0.5, 0.5-1.0, 1.0–2.0 Hz, etc.) of the CWT spectrum was then determined for each frequency band (**Panel 2,** red rectangle) across time, then binarized (**Panel 3**). An event was identified if the power was >2 times the standard deviation from the mean power (**Panel 3**, red line), providing a dataset of ones (events) and zeroes (non-events) across time.



	Neuron #1	Neuron #2	Neuron #3	Neuron #4	Neuron #5	Neuron #6 ...	Neuron #X
Neuron #1		0.15806	0.162386	0.191562	0.175802	0.244289	0.389514
Neuron #2	-		0.416567	0.277613	-0.370148	0.527221	0.393242
Neuron #3	-	-		0.299127	0.264193	0.503479	-0.453807
Neuron #4	-	-	-		0.470832	0.480112	0.335159
Neuron #5	-	-	-	-		0.443247	0.43054
Neuron #6 ...	-	-	-	-	-		-0.430648
Neuron #X	-	-	-	-	-	-	

Figure 2.10: CCs between Pairs of Neurons in a Field of View

Binarized events were used to calculate a correlation coefficient (CC) for each pair of neurons across time for all neurons in a field of view. CC values ranged from 0 to 1, where 0.4 would represent neurons that fire together 40% of the time. We chose to only investigate neuronal pairs with positive CC values; however, negative CC values may represent inhibitory connections. In this example, two representative binarized traces are shown at the top. Neuron #1 has four events, while Neuron #2 has five events; however, these events are not occurring around the same times, and therefore, have a low CC (0.15806).

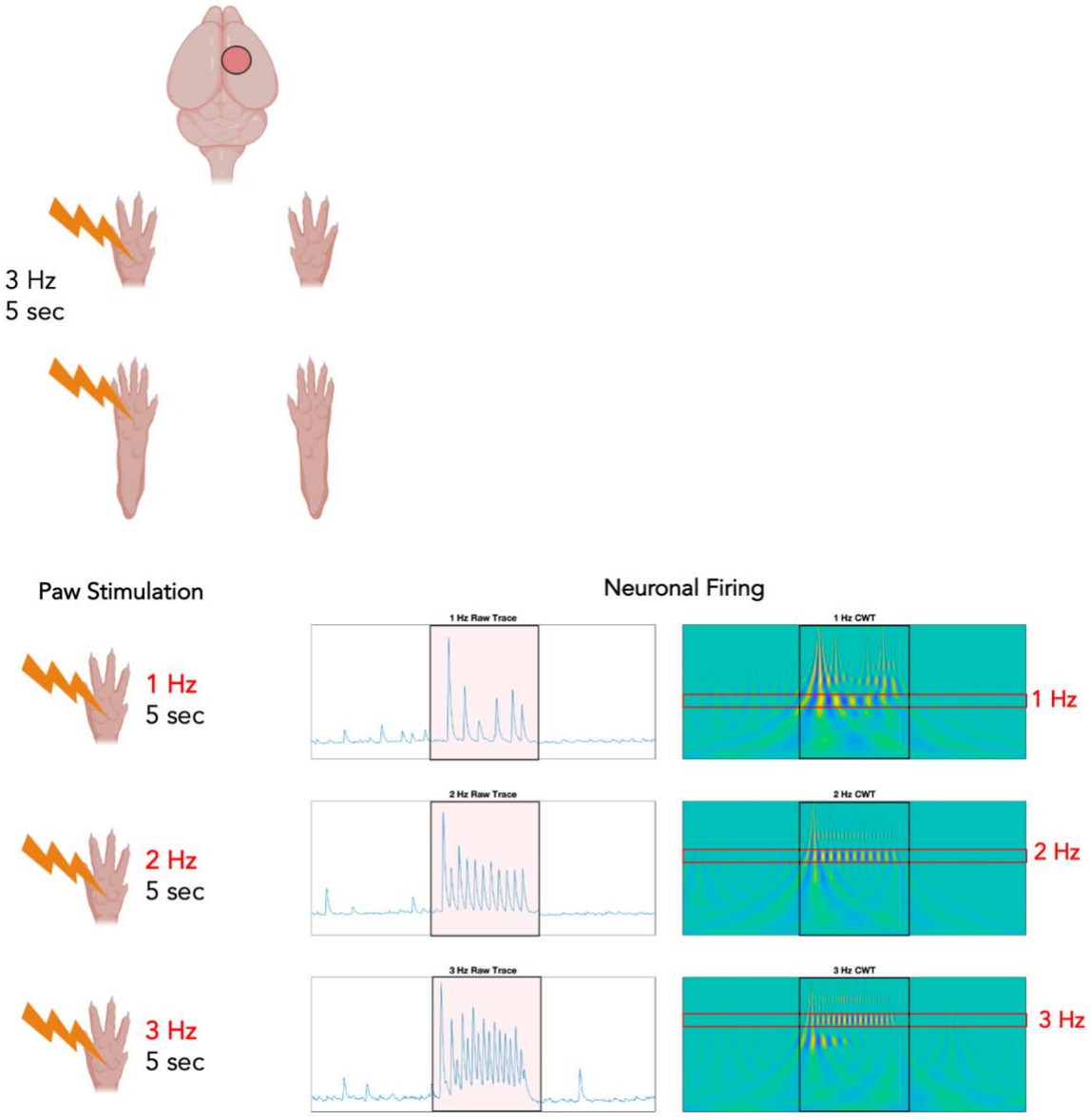


Figure 2.11: Peripherally Pacing the Neuronal Ca^{2+} Network

We stimulated the contralateral paw pads of an anesthetized F344 rat during imaging at 1 Hz, 2 Hz, and 3 Hz for 5 seconds to see if we could peripherally pace the neuronal Ca^{2+} network. We then used the network analysis to examine which frequencies these neurons were communicating at. Here we show that the network can be peripherally paced at 1, 2, and 3 Hz.

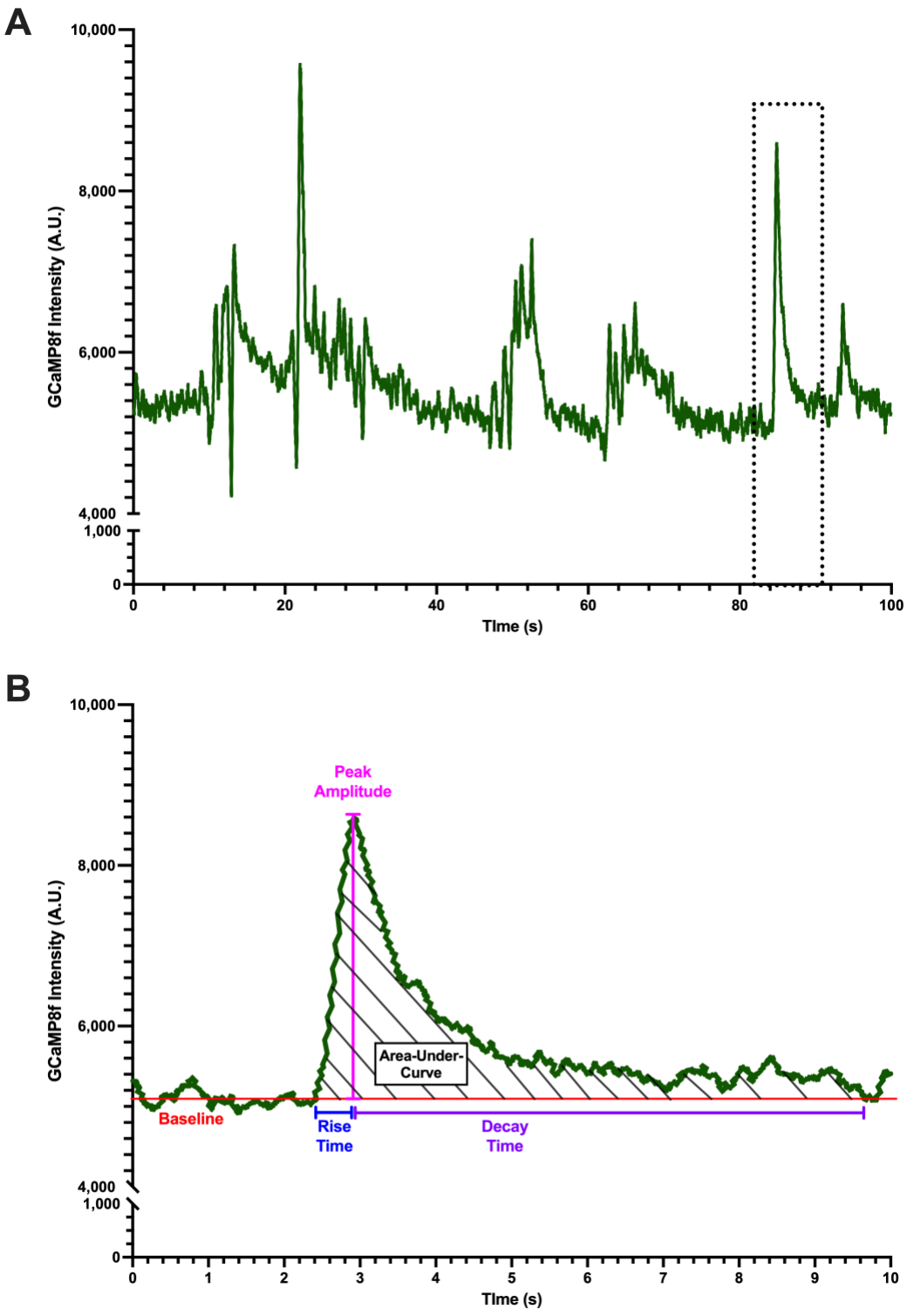


Figure 2.12: ΔF/F Analysis of Ca^{2+} Transients

(A) Representative traces of calcium transients from a neuron in an ambulating animal. (B) Zoomed in look at calcium transient highlighted with dotted box in panel A, showing measures of calcium dynamics include peak amplitude, rise time, decay time, and area-under-curve.

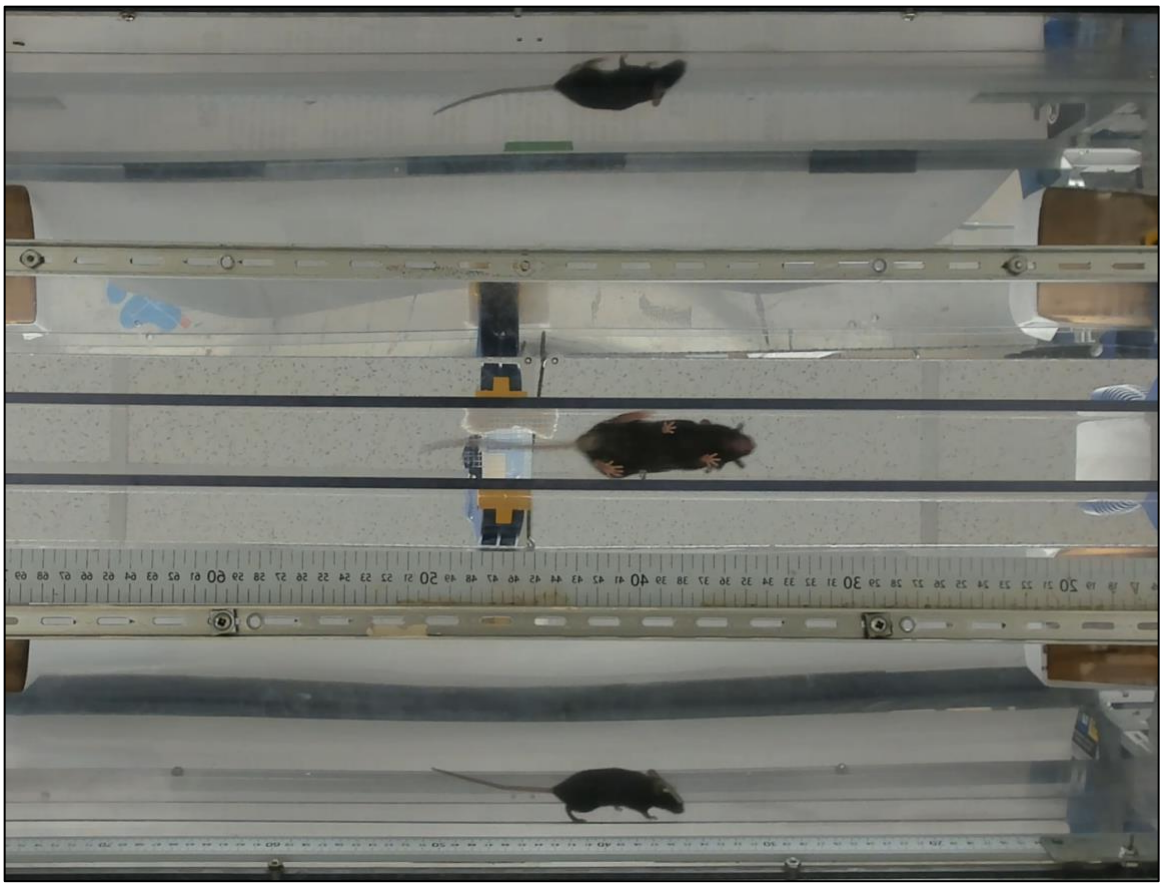


Figure 2.13: 3-Plane Visualization Walking Task

For comparison of gait across age, animals were recorded using a digital camera (3840 x 2160 pixels at 25 Hz) ambulating across a 3-plane visualization walking task built in-house that allows us to map paw placements in X, Y dimensions across time. Animals were recorded ambulating across four surfaces (flat control [shown here], 3-mm, 4-mm, and 5-mm plastic mesh).

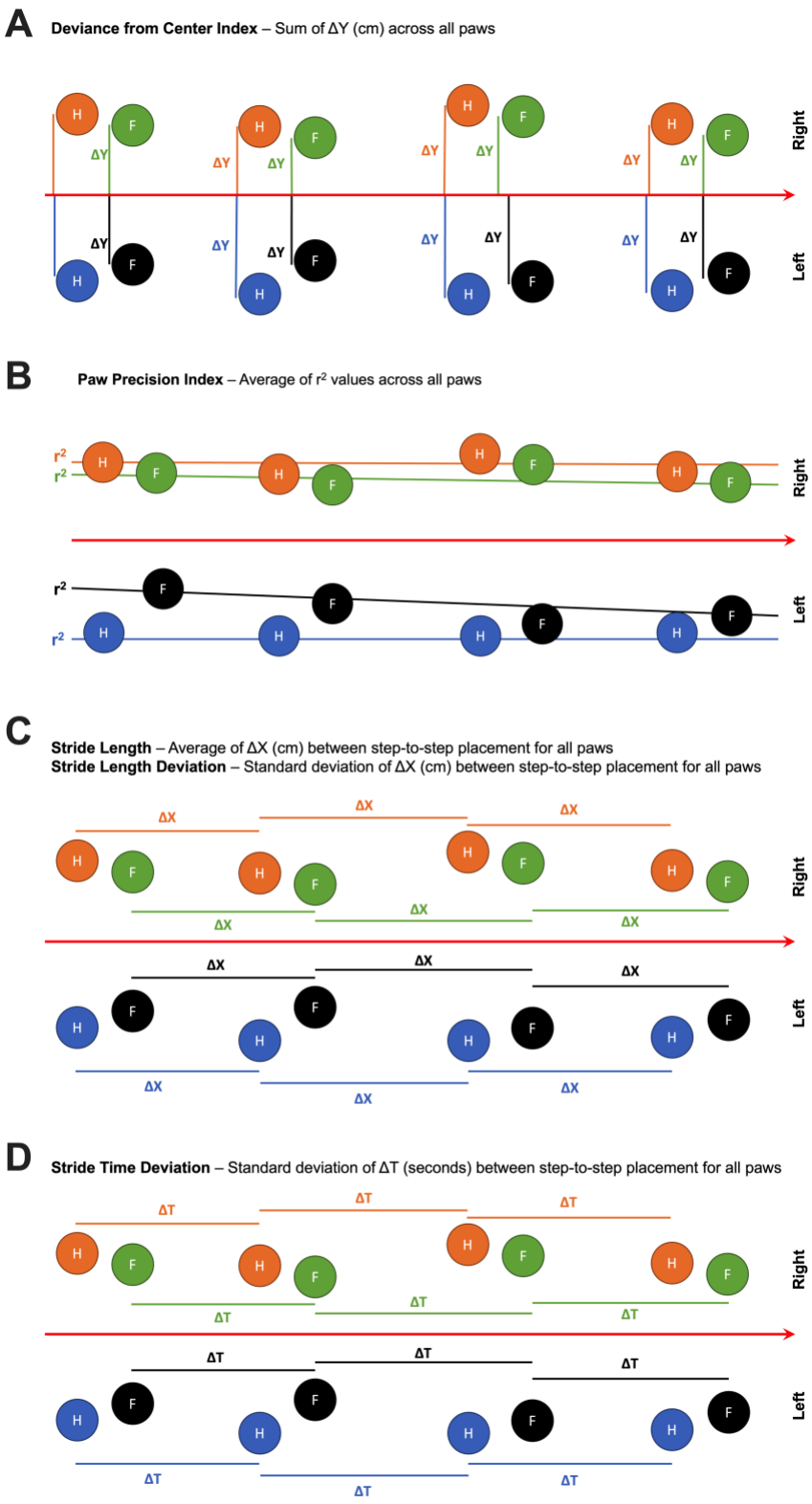


Figure 2.14: Quantification of Gait Parameters

Measures of locomotor stability were calculated as follows: (**A**) *deviance from center index* – the sum of all paw distances (in cm) from the center of the corridor divided by four steps; (**B**) *paw precision index* – average of r^2 values derived from each paw's linear regression across four steps; (**C**) *stride length* – average distance (in cm) between step-to-step paw placement for each paw across four steps, and *stride length deviance index* – standard deviation of absolute distances between step-to-step paw placements for each paw; and (**D**) *stride time deviation* – the standard deviation of time (in seconds) between step-to-step paw placements for each paw across four steps.

Table 2.1 Number of Subjects per Group for Each Measure

	Males		**Females**	
	Young	**Aged**	**Young**	**Aged**
Grip Strength	8	9	11	8
Neuronal Ca^{2+} Network Dynamics	7	5	6	5
ΔF/F Neuronal Ca^{2+} Dynamics	7	5	5	5
Gait Behavior	8	6	10	7

CHAPTER 3. RESULTS

3.1 Grip Strength Testing

Given that prior literature has shown an increase in sarcopenia with age in both preclinical and clinical models (Shavlakadze et al., 2010; Walston, 2012), and that we were quantifying measures of ambulation, it was important to test for sex or age differences in the grip strength (in Newtons) of forelimbs and hindlimbs for each animal in the study. Results indicated no significant differences in grip strength across age or sex, but a significant limb difference was noted (**Figure 3.1**; 3-way RM ANOVA; $F_{[2,64]} = 47.25$; $p < 0.0001$), suggesting that forelimb, hindlimb, and all limbs combined contribute differentially to overall limb strength. We specifically did not normalize these data to body weight and report the numbers in neurons as the females (average young weight: 23.3 ± 0.4 grams; average aged weight: 32.5 ± 1.4 grams) were much smaller (*data not shown*; 2-way ANOVA; $F_{[1,32]} = 29.0$, $p < 0.0001$) than males (average young weight: 30.0 ± 0.7 grams; average aged weight: 42.2 ± 2.6 grams), which would have artificially inflated their measures of grip strength compared to male, if normalized to body weight (Bonetto et al., 2015).

3.2 Neuronal Ca^{2+} Network Dynamics

We used previously published methods (Lin et al, 2022) to acquire, extract, and binarize GCaMP8f events during ambulation on a flat surface (**Figure 3.2A-E**). These data were obtained concomitantly to the velocity trace (**Figure 3.2F**) from the animal's recorded ambulation on the Neurotar Mobile HomeCage. Briefly, using the CWT to extract power

of GCaMP8f intensity across frequencies, we note here significant differences across sex, age, and locomotion status in the 0.1-2 Hz range. This frequency range was selected based on prior literature and data presented in **Figure 3.3** and **Figure 3.4**. A significant main effect of sex was identified for measures of active neurons engaged during movement (**Figure 3.2G**; 2-way RM ANOVA, $F_{[1,19]} = 6.53$, $p = 0.0193$), connectivity (**Figure 3.2I**; 3-way RM ANOVA, $F_{[1,19]} = 4.717$, $p = 0.0427$), and synchronicity (**Figure 3.2J**; 3-way RM ANOVA, $F_{[1,19]} = 6.903$, $p = 0.0166$). Additionally, a main effect of locomotion status was noted with increased measures of connectivity (**Figure 3.2I**; 3-way RM ANOVA, $F_{[1,19]} = 20.55$, $p = 0.0002$) and synchronicity (**Figure 3.2J**; 3-way RM ANOVA, $F_{[1,19]} = 103.8$, $p < 0.0001$), showing that the neuronal Ca^{2+} network dynamics are increased during ambulation. A trend was noted for measures of connection length (**Figure 3.2H**; 3-way RM ANOVA, $F_{[1,19]} = 3.481$, $p = 0.0776$) during ambulation. Interestingly, a significant effect of age was noted for measures of synchronicity (**Figure 3.2J**; 3-way RM ANOVA, $F_{[1,19]} = 4.194$, $p = 0.05$), where aged males showed increased synchronicity during ambulation only. From these measures (**Figure 3.2G-J**) obtained across sex or age, none showed significant differences during resting, suggesting neuronal Ca^{2+} network changes at rest are likely subtle or inconsequential.

One advantage of using the CWT to extract GCaMP8f signals is that it allows for extraction of power across multiple frequency domains (0.05-14 Hz) and investigation of the alignment between ambulatory behavior and network activity across these frequencies (**Figure 3.4**). Here, we show a main effect of locomotion (**Figure 3.3A**; 3-way RM ANOVA, $F_{[1,20]} = 9.149$, $p = 0.0067$; **Figure 3.3B**; 3-way RM ANOVA, $F_{[1,18]} = 13.21$, $p = 0.0019$) and frequency (**Figure 3.3A**; 3-way RM ANOVA, $F_{[1.708,34.17]} = 9.609$, $p =$

0.0008; **Figure 3.3B**; 3-way RM ANOVA, $F_{[1.879,33.82]} = 6.057$, $p = 0.0065$) on measures of connectivity, and it appears that during ambulation, an increase in connectivity in the lower frequency domains was seen (0.1-2 Hz). Additionally, a main effect of locomotion (**Figure 3.3E**; 3-way RM ANOVA, $F_{[1,20]} = 105.9$, $p < 0.0001$; **Figure 3.3F**; 3-way RM ANOVA, $F_{[1,18]} = 42.14$, $p < 0.0001$), frequency (**Figure 3.3E**; 3-way RM ANOVA, $F_{[1.979,39.58]} = 128.6$, $p < 0.0001$; **Figure 3.3F**; 3-way RM ANOVA, $F_{[1.238,22.28]} = 53.3$, $p < 0.0001$), and age, carried mostly by males (**Figure 3.3E**; 3-way RM ANOVA, $F_{[1,20]} = 15.25$, $p = 0.0009$), was noted for measures of synchronicity. As previously reported in other brain regions, we show that irrespective of frequency, aged males display greater connection length (**Figure 3.3C**; 3-way RM ANOVA, $F_{[1,20]} = 7.841$, $p = 0.0111$) in the activated network of S1, compared to young males. This result is in line with the notion of larger receptive field size as well as reduced place cell specificity in aged animals (Beauchet et al., 2019; David-Jurgens et al., 2008; Mehta et al., 1997). Due to the nature of our statistical analysis (3-way RM ANOVAs), we purposely focused on aging differences within each sex, and therefore, could not identify main effects of sex (*i.e.*, 4-way ANOVA). It is interesting to note, however, that males appear to show greater connectivity and synchronicity compared to females, which was shown to be significant on the flat surface alone (**Figure 3.2I-J**). Furthermore, we compared raster maps (*i.e.*, activity) across each frequency with the corresponding animal's velocity trace for that recording (**Figure 3.4**), which highlighted that neuronal Ca^{2+} activity in lower frequencies of 0.1-2 Hz is robustly aligned with ambulatory behavior.

We use neuronal network analyses during ambulation across different substrates. As a proxy to measures of tactile discrimination and proprioception, specifically, we used

a flat surface, or plastic mesh with 3-mm, 4-mm, or 5-mm spacing (see **Figure 3.5A**), to test for differences in measures of neuronal network communication. While **Figure 3.5B** shows that there were no differences in the number of active neurons engaged during ambulation across surface, age, and sex at any time, our results show a main effect of locomotion on all measures (**Figure 3.5C-F**), with increases in activity during ambulation irrespective of age or sex. For measures of synchronicity in both sexes, again, we showed a main effect of locomotion; however, there was also a main effect of age, where males appeared to display increases in synchronicity with age (**Figure 3.5G**; 3-way RM ANOVA; $F_{[1,10]} = 5.565$, $p = 0.04$), while females appeared to show decreases in synchronicity with age (**Figure 3.5H**; 3-way RM ANOVA; $F_{[1,9]} = 5.41$, $p = 0.045$). These effects only appear during periods of ambulation as *post-hoc* analyses revealed that none of the measures presented in **Figure 3.5** were significant during the resting period.

While the CWT routine provided depth and clarity on analyses of neuronal Ca^{2+} dynamics across frequencies and also reported on network-level measures of connectivity and synchronicity across hundreds of neurons, the binarization routine prevented us from addressing more traditional methods of Ca^{2+} dynamics, including measures of amplitude, area-under-curve, rise time, and decay time of the Ca^{2+} transients. For these reasons, and to address rigor and reproducibility, we also performed a ΔF/F analysis (**Figure 3.6**) of the same dataset that was used to extract measures using the CWT routine. Using a ΔF/F method to identify neuronal activity, we showed a main effect of sex and locomotion on the number of Ca^{2+} events identified per second. We chose to represent this data as events/s, since animals spend different amounts of time either resting or ambulating during the imaging session, and focused on the flat surface, since we found no prior main effect of

surface in **Figure 3.5**. As with CWT measures, a main effect of locomotion was noted for measures of neuronal activity (**Figure 3.6A**; 3-way RM ANOVA; $F_{[1,18]} = 74.08$, $p < 0.0001$), area-under-curve (**Figure 3.6B**; 3-way RM ANOVA; $F_{[1,18]} = 18.57$, $p = 0.0004$), rise time (**Figure 3.6C**; 3-way RM ANOVA; $F_{[1,18]} = 21.95$, $p = 0.0002$), and decay time (**Figure 3.6D**; 3-way RM ANOVA; $F_{[1,18]} = 17.88$, $p = 0.0005$). As seen with measures of connectivity and synchronicity using the CWT routine, where we saw a significant sex difference, we saw a main effect of sex for measures of neuronal activity (**Figure 3.6A**; 3-way RM ANOVA; $F_{[1,18]} = 10.99$, $p = 0.0039$) and area-under-curve (**Figure 3.6B**; 3-way RM ANOVA; $F_{[1,18]} = 7.877$, $p = 0.0117$. The alignment between these two methods of analyses further translated to the directionality of the effect, where females showed an increase in number of active neurons and events/s (**Figure 3.2G** and **Figure 3.6A**) as compared to males, but also showed decreases in connectivity and synchronicity (**Figure 3.2I-J**) paired with increases in Ca^{2+} transient area-under-curve (**Figure 3.6B**).

3.3 Gait Behavior Testing

Following 2P neuronal Ca^{2+} imaging, we used a 3-plane visualization walking task (Lin et al., 2022) to obtain measures of gait coordination, velocity, and stride across age, sex, and surface. A main effect of age was seen for measures of velocity (**Figure 3.7A**; 3-way RM ANOVA; $F_{[1,27]} = 16.75$, $p = 0.0003$) and stride time deviance index (**Figure 3.7C**, 3-way RM ANOVA; $F_{[1,27]} = 6.162$, $p = 0.0196$), highlighting that aged animals ambulate slower and exhibit impaired gait rhythm. Furthermore, a main effect of sex was noted for measures of stride time deviance index (**Figure 3.7C**; 3-way RM ANOVA; $F_{[1,27]} = 4.334$, $p = 0.0470$) and stride length (**Figure 3.7E**; 3-way RM ANOVA; $F_{[1,27]} =$

9.445, $p = 0.0048$) where females show less deviance and slight increases in stride length compared to males. We detected a main effect of surface on measures of velocity (**Figure 3.7A**; 3-way RM ANOVA; $F_{[3,80]} = 3.915$, $p = 0.0116$), suggesting this behavioral task is able to identify surfaces that present with greater challenges for the aged animals.

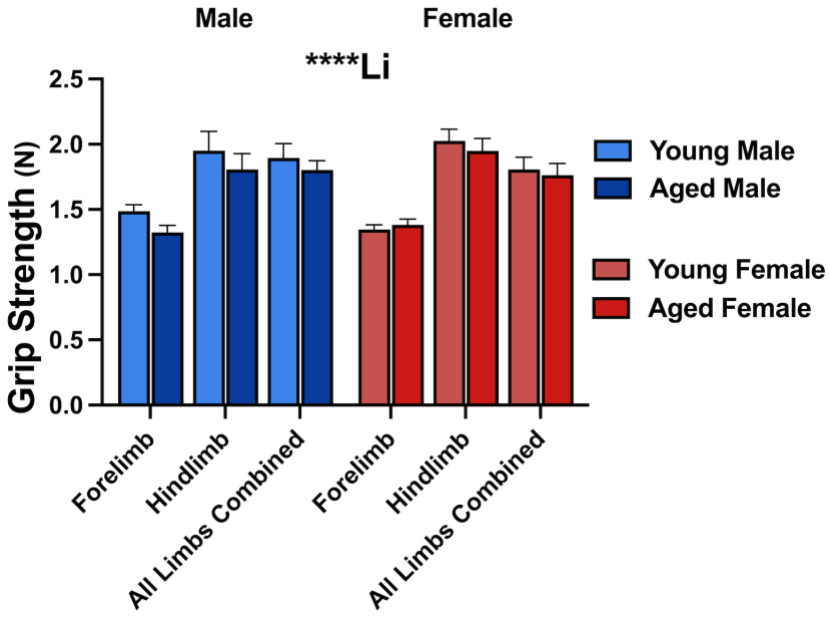


Figure 3.1: Grip Strength in Young and Aged Mice

Measures of grip strength for forelimb, hindlimb, and all limbs combined were obtained in young male (n = 8), aged male (n = 9), young female (n = 11), and aged female (n = 8) C57BL/6J mice. While a limb effect (*i.e.*, forelimb vs hindlimb) was noted, no sex- or age-associated changes were present. Data is expressed in Newtons (N). ****Li indicates a main effect of limb with $p < 0.0001$.

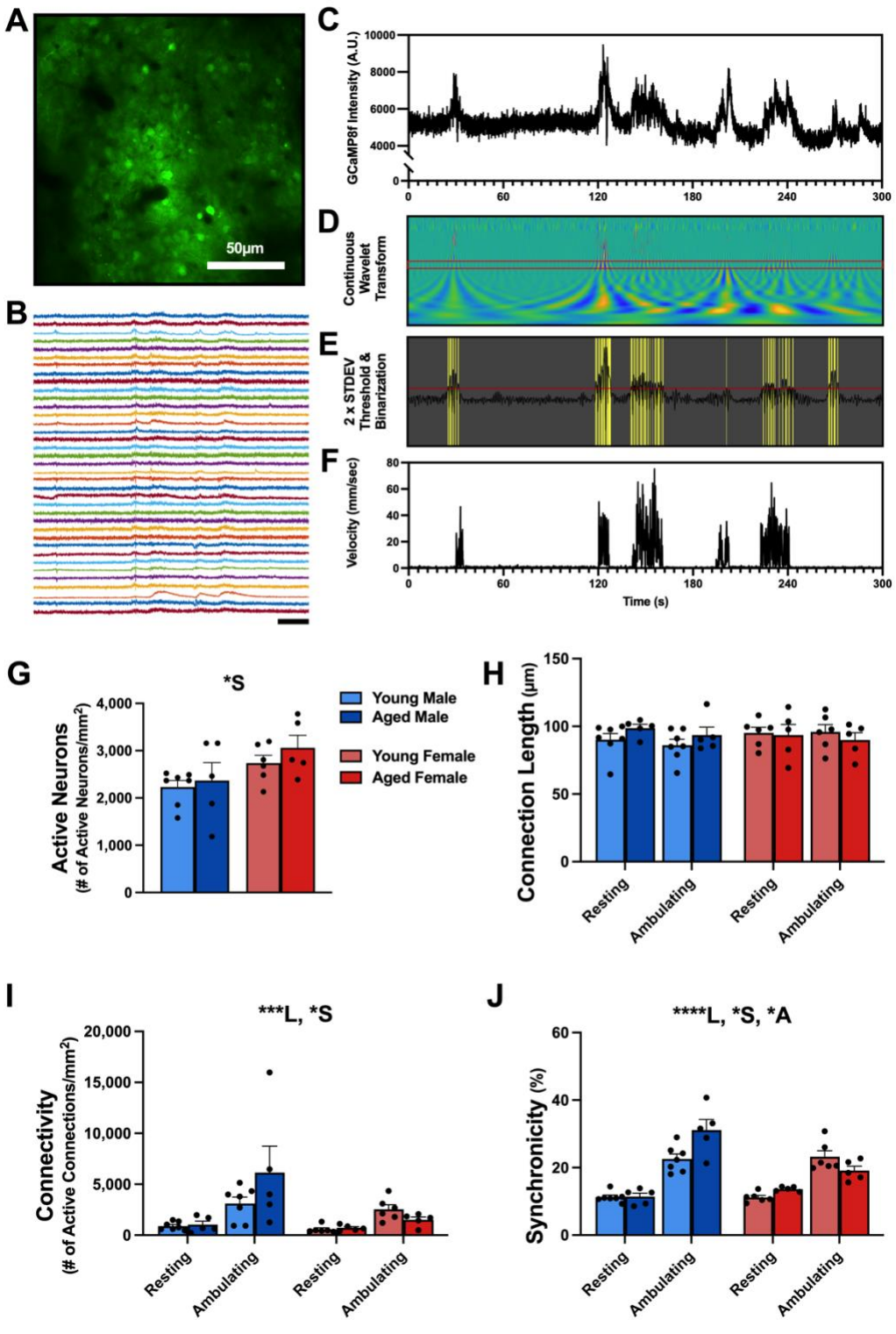


Figure 3.2: S1 Neuronal Ca^{2+} Network Dynamics in Young and Aged Mice on a Flat Surface

(**A**) Individual GCaMP8f-positive neurons are distinguishable with high spatial resolution in S1 in an aged male during ambulation and rest across a flat surface. Dark circular areas reflect penetrating blood vessels. (**B**) Representative GCaMP8f intensity traces from ROIs identified in panel **A** during periods of rest and ambulation with high temporal resolution. Rectangular scale bar represents 30 secs. (**C**) Individual raw GCaMP8f signals during rest and ambulation underwent a continuous wavelet transform (**D**), then thresholded and binarized (**E**), to extract events across multiple frequencies. (**F**) Corresponding animal's ambulation velocity trace across time. Measures of network communication (**G-J**) were obtained in young male (n = 7), aged male (n = 5), young female (n = 6), and aged female (n = 5) C57BL/6J mice and here, we report measures in the 0.1-2 Hz frequency domain. A main effect of sex was detected on measures of active neurons (**G**), connectivity (**I**), and synchronicity (**J**). A significant locomotion effect (ambulating vs resting) was noted for measures of connectivity (**I**) and synchronicity (**J**). Additionally, a main effect of age was reported for measures of synchronicity (**J**), with age males having a significantly greater synchronicity compared to young males. No significant changes were detected on measures of connection length ($p > 0.05$). *, ***, and **** indicate a main effect with a $p < 0.05$, $p < 0.001$, and $p < 0.0001$, respectively. *A indicates a main effect of age. ***L and ****L indicate a main effect of locomotion status. *S indicates a main effect of sex.

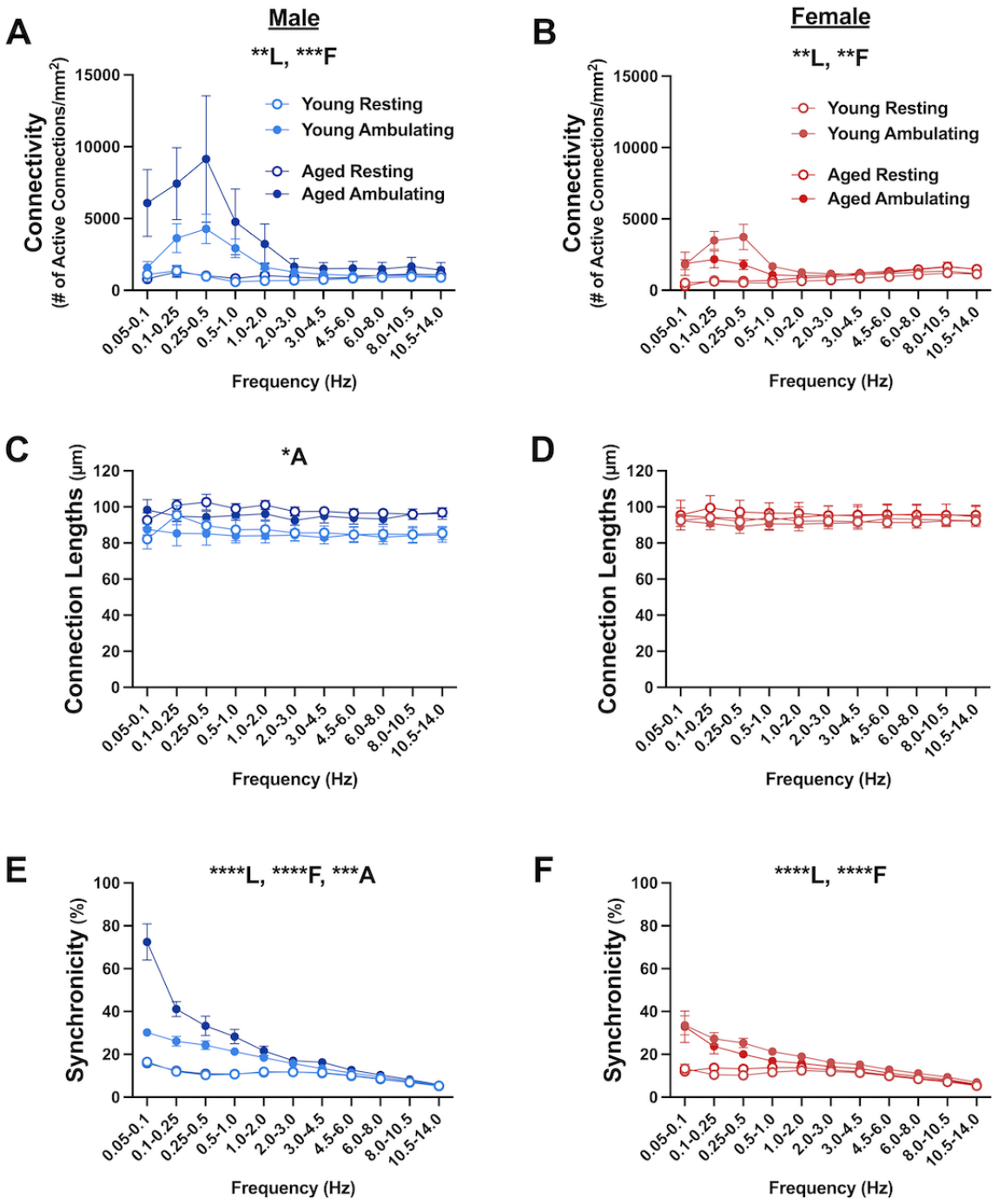


Figure 3.3: Neuronal Ca^{2+} Network Dynamics Across Frequency on a Flat Surface

Measures of network communication were obtained in young male (n = 7), aged male (n = 5), young female (n = 6), and aged female (n = 5) C57BL/6J mice across multiple frequency domains during ambulation and rest. Main effects of locomotion status and frequency were

detected on measures of connectivity (**A** & **B**) and synchronicity (**E** & **F**) in both males and females. A main effect of frequency was noted for measures of synchronicity. Interestingly, we note a significant effect of age on measures of connection length (**C**) and synchronicity (**E**) in males only, where aged males had greater lengths of connections and synchronicity than young males. No significant changes were detected on measures of connection length in female mice ($p > 0.05$). *, **, ***, and **** indicate a main effect with a $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. *A and ***A indicate a main effect of age. **L and ****L indicate a main effect of locomotion status. **F, ***F, and ****F indicate a main effect of frequency.

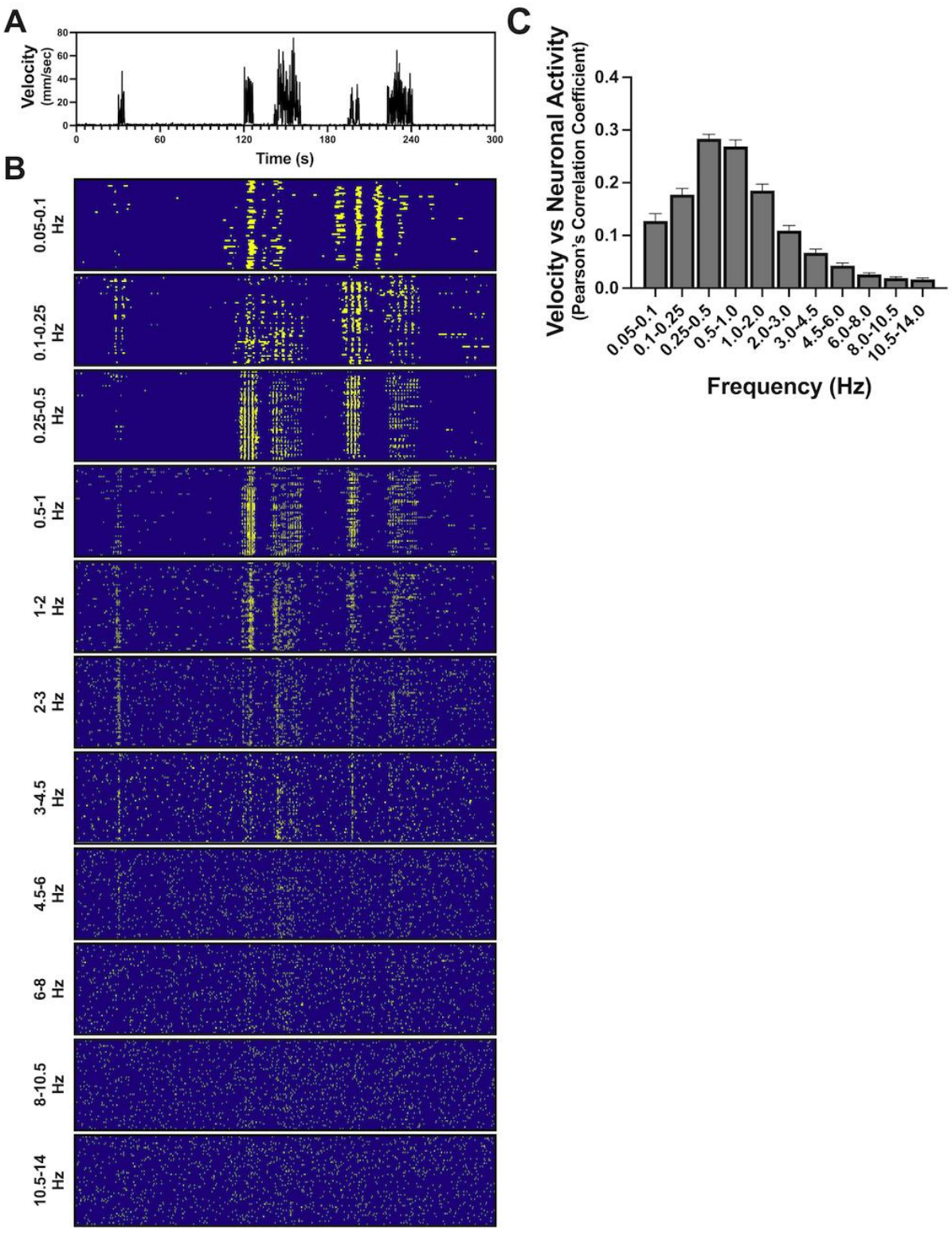
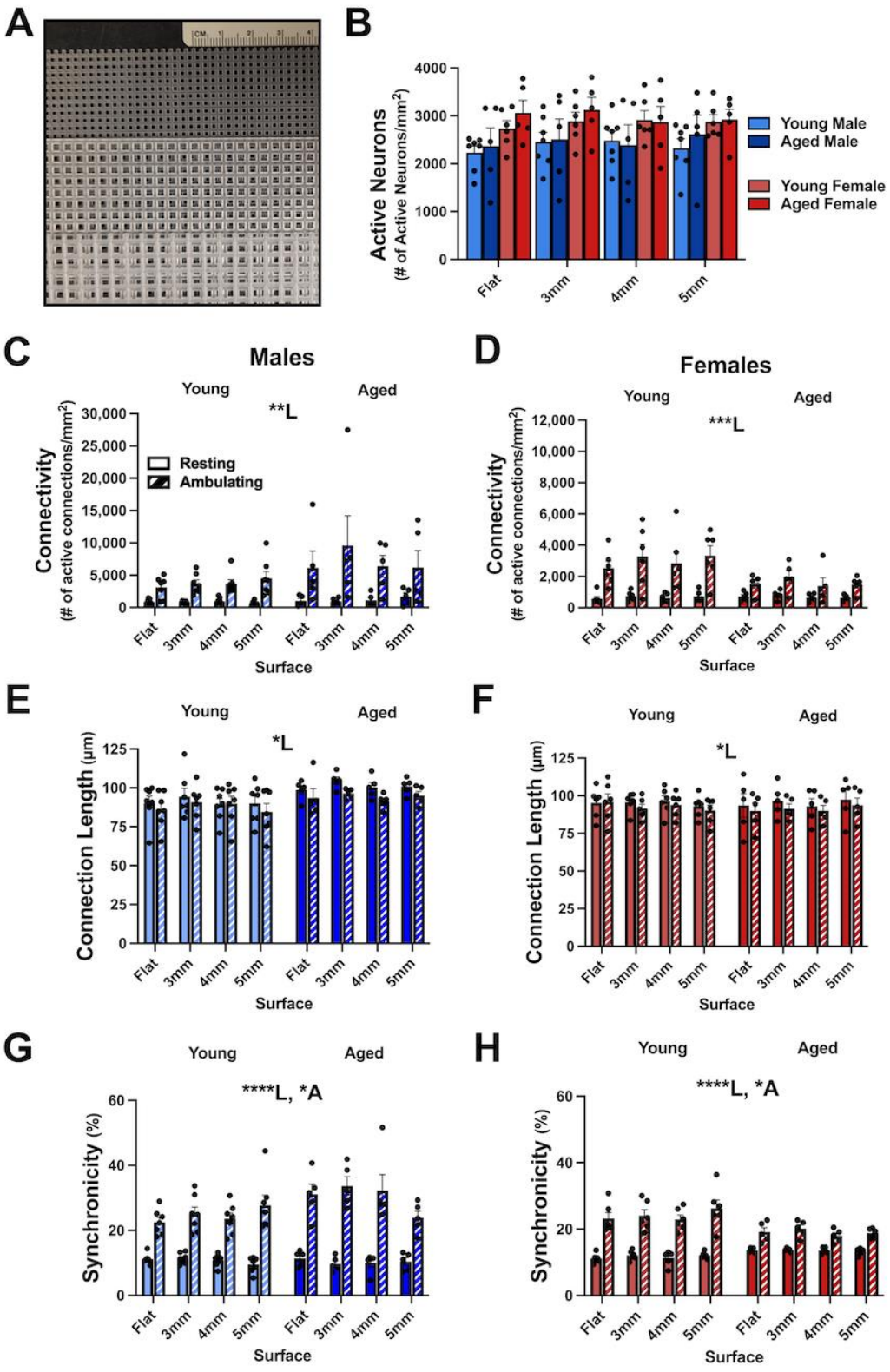


Figure 3.4: Alignment of Velocity with Neuronal Ca^{2+} Events Across Multiple Frequency Domains

(**A**) Representative velocity trace of aged male walking across a flat surface. (**B**) Corresponding raster maps of individual neuronal Ca^{2+} events across time (where each Y-axis value represents an individual neuron) derived using the CWT routine across multiple frequency domains. (**C**) Plot of correlation coeffects between velocity and neuronal activity across multiple frequencies, highlighting that neuronal activity in lower frequencies of 0.1-2 Hz is clearly aligned with ambulatory behavior.


A
B
Active Neurons
(# of Active Neurons/mm²)
4000
3000
2000
1000
0
Flat
3mm
4mm
5mm
Young Male
Aged Male
Young Female
Aged Female
C
Males
Young
Aged
**L
Connectivity
(# of active connections/mm²)
30,000
25,000
20,000
15,000
10,000
5,000
0
Resting
Ambulating
Flat
3mm
4mm
5mm
Surface
D
Females
Young
Aged
***L
Connectivity
(# of active connections/mm²)
12,000
10,000
8,000
6,000
4,000
2,000
0
Surface
E
Young
Aged
*L
Connection Length (µm)
125
100
75
50
25
0
Surface
F
Young
Aged
*L
Connection Length (µm)
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100
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Synchronicity (%)
60
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Surface
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Young
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Synchronicity (%)
60
40
20
0
Surface

Figure 3.5: Neuronal Ca^{2+} Network Dynamics in Young and Aged Mice Across Multiple Surfaces

Measures of network communication were obtained in young male (n = 7), aged male (n = 5), young female (n = 6), and aged female (n = 5) C57BL/6J mice during ambulation and rest across multiple surfaces (**A**; 3-mm, 4-mm, and 5-mm plastic mesh), and here, we report measures in the 0.1-2 Hz frequency domain. A main effect of locomotion status was detected on measures of connectivity (**C-D**), connection length (**E-F**), and synchronicity (**G-H**) in both males and females. Additionally, we note a significant effect of age on measures of synchronicity in both male and female mice (**G-H**), where aged males had greater synchronicity than young males, while aged females had decreased synchronicity compared to young females. No significant changes were detected on measures of active neurons (**B**) across sex, age, or surface ($p > 0.05$). *, **, ***, and **** indicate a main effect with a $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. *A indicates a main effect of age. *L, **L, ***L, and ****L indicate a main effect of locomotion status.

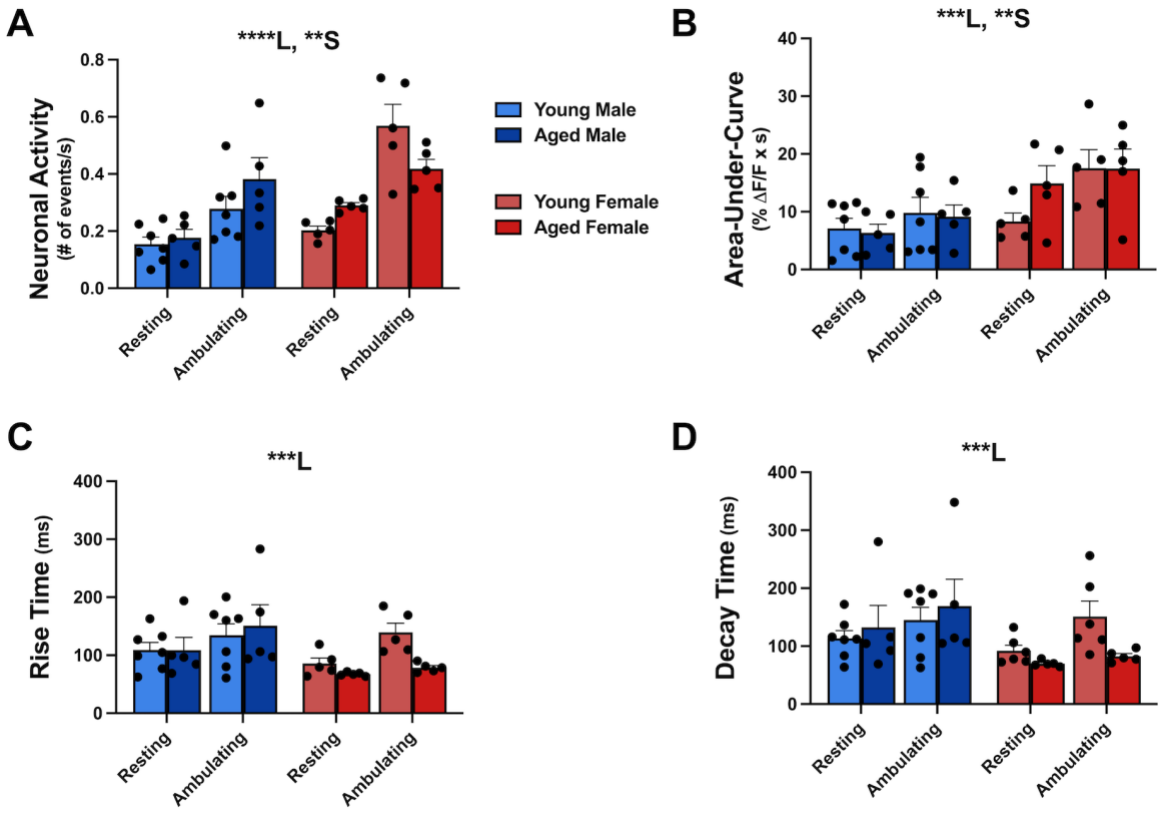

Figure 3.6: Single-cell (ΔF/F) Neuronal Ca^{2+} Dynamics on a Flat Surface

Measures of single-cell (ΔF/F) neuronal Ca^{2+} dynamics were obtained in young male (n = 7), aged male (n = 5), young female (n = 5), and aged female (n = 5) C57BL/6J mice during ambulation and rest across a flat surface. We report a main effect of locomotion status for measures of neuronal activity (**A**), area-under-curve (**B**), rise time (**C**), and decay time (**D**). Additionally, we report a main effect of sex for measures of neuronal activity (**A**) and area-under-curve (**B**). **, ***, and **** indicate a main effect with a $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively. ***L and ****L indicate a main effect of locomotion. **S indicates a main effect of sex.

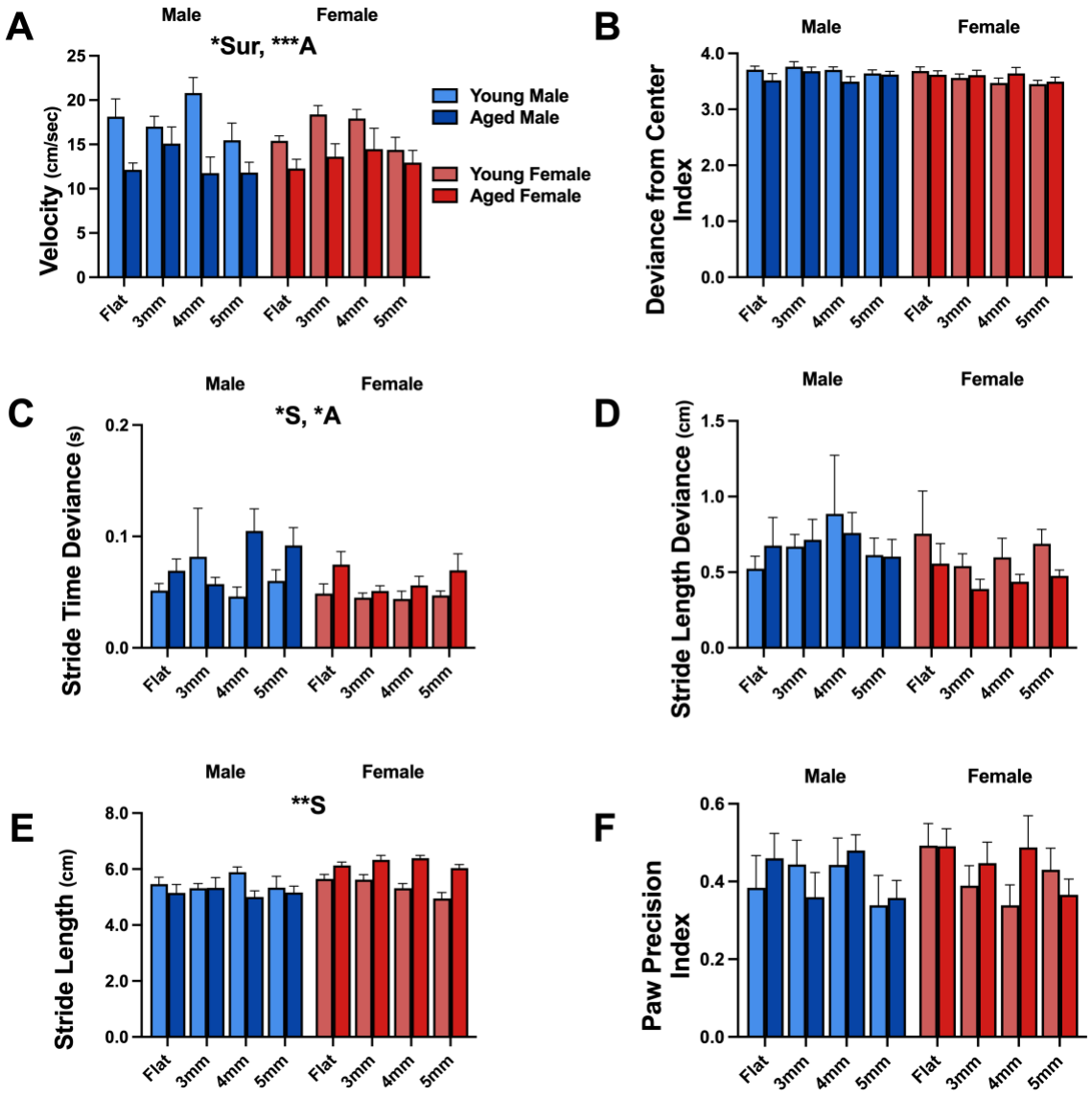

Figure 3.7: Gait Behavior in Young and Aged Mice Across Multiple Surfaces

Measures of gait behavior were obtained in young male (*n* = 8), aged male (*n* = 6), young female (*n* = 10), and aged female (*n* = 7) C57BL/6J mice during ambulation across a flat (control), 3 mm, 4 mm, and 5 mm plastic mesh surface. A main effect of age was noted for measure of velocity (**A**) and stride time deviance (**C**), highlighting that aged animals ambulate slower and perhaps exhibit impaired gait rhythm or cadence. We report a main effect of sex for measures of stride time deviance (**C**) and stride length (**E**). *, **, and ***

indicate a main effect with a $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Interestingly, we report a main effect of surface for measures of velocity (**A**). *A and ***A indicate a main effect of age, *S and **S indicate a main effect of sex. *Sur indicates a main effect of surface.

CHAPTER 4. DISCUSSION

This study examined neuronal Ca^{2+} dynamics across sex in young (4 months) and aged (22 months) ambulating C57BL/6J mice using two methods (CWT and ΔF/F). The work includes over 2,000 active neurons imaged across four groups (young male, aged male, young female, aged female), and reports on changes in layers 2/3 of S1 while investigating the relationship between Ca^{2+} dynamics and gait dysregulation. We specifically tested the hypothesis that age- and sex-dependent alterations in S1 Ca^{2+} neuronal ensembles are present in the awake, ambulating mice, thereby expanding the Ca^{2+} hypothesis of brain aging to *in vivo* settings during behavioral engagement, in new brain regions, and across tens of neurons communicating in local circuits.

Our results align well with previously published work using different methods in awake animals showing cortical signals spontaneously oscillating in the slower delta wave range (Llano et al., 2021; Miller et al., 2014; Poulet et al., 2012; Sun & Dan, 2009; Zhang et al., 2021). To our knowledge, this is likely one of the first analyses of individual neurons showing activity in lower frequency domains (0.1-2 Hz) during ambulation and rest in S1 of young and aged animals. Importantly, within these frequencies, we report on significant, albeit small, age-dependent increases in neuronal synchronicity during ambulation in males (**Figure 3.2J**; **Figure 3.3E**; **Figure 3.5G**). In some cases, synchronicity reached nearly 70% in the aged male, which might be reflective of poor single-cell activity, but depending on the brain regions, it is unclear whether increases or decreases in synchrony are beneficial.

4.1 Reports of Synchrony Across the Field of Neuroscience

Many studies have examined cortical synchrony either within, or across large brain regions using electroencephalogram (EEG) techniques, where neuronal activity and synchrony report on specific behavioral tasks, and are reflective of engagement and activation (Uhlhaas et al., 2009). Increases in synchrony across spatially distinct brain regions are often associated with improved memory performance, specifically in the context of memory encoding where hippocampal connections to the prefrontal cortex are noted (Jones & Wilson, 2005; O'Neill et al., 2013; Stern et al., 2004). Furthermore, with human aging, investigations using EEG and diffusor tensor imaging (DTI) have also described network synchrony as a potential mechanism for reduced cognitive performance (Hinault et al., 2021).

However, within a single brain region, such as S1, prior work has shown the presence of network desynchronization during stimulation (whisker or visual stimuli) compared to quiet wakefulness (Arroyo et al., 2018; Khateb et al., 2021; Poulet et al., 2012), highlighting that while the neuronal network may be synchronous at rest, neurons also exhibit complex individual patterns during activation. This is unsurprising, since it is well known that sensation occurs at the level of individual cells, where neurons are shown to have specificity and tuning to various stimuli (Mehta et al., 1997; O'Keefe & Conway, 1978; Zong et al., 2022). Indeed, prior descriptions based on single- or few-cell measures have shown behavior-dependent changes in neuronal activity (Kang et al., 2010; Salinas et al., 2000; Sun & Dan, 2009; Wirtshafter & Disterhoft, 2022; Zhao et al., 2012; Zhao et al., 2016). Furthermore, much has been learned in the past 30 years from measures of single- and multi-cell recording of the brain during activation, providing a clear picture that

specific brain regions exhibit alterations in intrinsic excitability and spatial mapping specificity with age (Chang et al., 2005; Disterhoft & Oh, 2007; Hickmott & Dinse, 2013; Patrylo et al., 2007; Thome et al., 2016; Wilson et al., 2005). However, the use of single cell measures in these studies precludes analyses of the local network activity (Mohajerani et al., 2010), yielding an important, yet addressable gap between single-cell dynamics and large brain region activity.

4.2 Neuronal Activity in S1

Our measures of network communication report on somatosensory encoding during ambulation and rest within small cortical areas (176 x 176 μm) across hundreds of individual neurons. Importantly, and in alignment with others, we report on dynamic changes in neuronal network communication during ambulation compared to rest, including increases in connectivity and synchronicity. Of note, recent work has highlighted the importance of thalamic input on regulating cortical excitability (Poulet et al., 2012) and suggests that our measures may not necessarily be representative of within-layer connectivity, but rather reflective of synchronization driven by thalamocortical and translaminar inputs. Specifically, neurons in layers 2/3 of S1 act as a local circuit which encodes information received from the thalamus and other movement-associated cortical areas (Cichon & Gan, 2015), while neurons in layer 5 send outputs upward within cortical columns as well as to adjacent areas. Given that tactile discrimination and proprioception are important contributors to ambulation and that encoding of these inputs is layer-specific, characterizing local circuit activity within layers 2/3 of S1 is crucial to our understanding of S1's influence on movement. Prior work in S1 has identified clear changes in neuronal

activity both *in vivo* and *ex vivo* in response to stimulation, either peripherally or locally (Hickmott & Dinse, 2013; Lin et al., 2022; Popescu et al., 2021; Zhao et al., 2016). Many of these studies also highlight age-dependent increases in intrinsic excitability of layer 2/3 and layer 5 neurons (Hickmott & Dinse, 2013; Popescu et al., 2021). Whether our reported increases in synchronicity are mediated by similar changes in intrinsic excitability or depend on the origin of the synaptic input (Zhao et al., 2016), remains to be determined. Irrespective of age, here, we describe movement-dependent increases in neuronal activity in layer 2/3 of S1, reinforcing the role of S1 in encoding of ambulatory-related processes including tactile discrimination and proprioception.

4.3 Comparison of Network and Gait Results in Two Models of Aging

Our lab has previously described alterations in gait performance with aging that reflect on dysregulation in the neuronal Ca^{2+} network using the F344 rat model of aging, where number of active neurons and connectivity were increased with aging (Lin et al., 2022). Here, our results in the C57BL/6J mouse during ambulation highlight similar increases in connectivity with no changes in number of active neurons. Additionally, while we saw large increases in measures of synchronicity with age in the behaving mouse, no alterations in synchronicity were noted in the anaesthetized F344 rat. This is likely because the use of anaesthesia has been shown to increase neuronal synchrony (Goltstein et al., 2015), possibly influencing our ability to detect age-related effects in the anaesthetized rat (*i.e.*, ceiling effect). In both models of aging, however, we have now characterized ambulatory performance using the same 3-plane visualization walking task, showing reduced ambulation speed. Here, we do not see the age-dependent changes in deviance

from center index as reported in the rat (Lin et al., 2022); instead, we note a significantly larger stride time deviance in aged, ambulating mice. This additional parameter of stride irregularity is reflective of impaired gait rhythm as seen in the clinic in the older adult (Kobsar et al., 2014).

4.4 Presence and Importance of Sex Differences

Given that increased risk of falling is driven by gait dysregulation (Krauss et al., 2005), and that some studies show a decreased risk of falls in older females compared to older males (Aryee et al., 2017; Ek et al., 2019), it was necessary to consider sex as a biological variable in this study. Importantly, we report decreased connectivity and synchronicity during ambulation in aged female mice compared to aged males. Importantly, we present evidence for increases in the number of active neurons and events/s in aged females, highlighting the possibility that these active neurons may be less synchronized. Perhaps this is representative of a beneficial network-level trait in encoding in females that maintains distinct neuronal activity during a task, independent of nearby synchronicity. Furthermore, on measures of ambulatory performance, aged females show decreased stride time deviance and increased stride length compared to aged males. Since smaller stride time deviance reflects better gait rhythm regularity (Kobsar et al., 2014) and stride length is known to shorten with gait dysregulation (Duggan et al., 2017), these results strongly support the notion that decreased incidence and risk of falls in older females may be dependent on central changes in network encoding.

4.5 Supporting the Hypothesis

Overall, our gait behavior results align reasonably well with human clinical data and the changes in network communication observed in this study may serve as a potential central mechanism contributing to these changes in ambulatory performance. Specifically, sex differences seen across network communication and gait may highlight a role of S1 in gait dysregulation, where neurons display more activity as well as less connectivity and synchronization in female mice, leading to enriched somatosensory encoding and improved gait. It is encouraging to note that using two separate approaches to characterize neuronal Ca^{2+} function (CWT and $\Delta F/F$), we are able to detect similar results of sex- and locomotion-dependent increases in neuronal activity (**Figure 3.2G** and **Figure 3.6A**). This cross-validation suggests the effects presented are robust and reliable.

Lastly, our study highlights a modern view of the Ca^{2+} hypothesis of brain aging. Contributions toward this hypothesis have focused on characterizing single or multi-cellular assessments in select brain regions and due to this, much of the work across the last 30 years in the field of brain aging may not represented the true *in vivo* neurophysiology and behavior. Certainly, the discovery of age-dependent changes in Ca^{2+} transients, such as increases in the after hyperpolarization, are important for our understanding of neuronal physiology; however, it is clear that ensembles of neurons communicating as an integrated network become altered as well and years of reports showing subtle changes in single-cellular components may be negligible in comparison.

4.6 Limitations and Future Directions

While this study provides important insights, all works do not come without limitations and caveats. Here, I would like to address specific aspects of the study that were limited in nature and how we plan to further develop the results reported.

4.6.1 Mechanisms Underlying Alterations in Ca^{2+} Dynamics

Alterations in Ca^{2+} dynamics reported here highlight the role of age and sex in neuronal network communication, and thus, encoding capabilities in S1. Several mechanisms exist to maintain a low intracellular concentration of Ca^{2+} within neurons (**Figure 1.3**), including NMDA receptors (GluN1) ryanodine receptor subtype-2 (RyR2) and L-type voltage-gated calcium channels (L-VGCC), and these putative targets may participate in greater calcium with age. Future studies examining age- and sex-related changes in quantification, localization, and distribution of these Ca^{2+}-related targets are possible as brain tissue from S1 was collected in approximately 43 of these animals. I will be using this frozen tissue that I collected to perform Western immunoblotting to test for age- and sex-dependent changes in protein levels for these three targets. Additionally, since we know that cortical activity is layer-specific, brain tissue could be collected and stored for use with immunohistochemistry to determine translaminar localization and distribution of these protein targets. Together, these experiments may provide insight on what cellular changes are leading to age- and sex-dependent alterations in single-cell Ca^{2+} transients and network dynamics.

4.6.2 Modalities of S1

While understanding the neurophysiology of cells within S1 was key to this study, individual modalities of S1 (*i.e.*, tactile discrimination or proprioception) were not addressed, limiting our understanding of S1's contribution to gait. Given that S1 encodes multiple modalities including pain, temperature, crude touch, vibration, proprioception, and discriminative touch, it is important to understand which specific modalities lead to alterations in these calcium dynamic changes. Given the environmental temperature remained constant between experiments and that our animals did not express any signs of pain, it would be appropriate to limit our analysis to the dorsal column-medial lemniscus pathway (vibration, proprioception, discriminative touch). Furthermore, we know that vibration was not altered between experiments and that all imaging was performed on an anti-vibration table, so we could focus our analysis on proprioception and discriminative touch. Future projects using local application of lidocaine to the fore and hindpaws of mice could eliminate one modality (discriminative touch) and help determine which of these two modalities are altered with age and sex. I will be performing 2P imaging in three to four C57BL/6J mice before and after application of lidocaine to the fore and hindpaws to look for alterations in neuronal Ca^{2+} networks during ambulation and rest.

4.6.3 Quadrupedal vs Bipedal Gait

Another limitation of this study is the use of the quadrupedal mouse as a model of aging to infer on changes seen in humans, who are bipedal. Quadrupedal and bipedal gait are two different ways that animals move, including both rodents and primates.

Quadrupeds use all four limbs to move, while bipeds use only two limbs. Quadrupeds have greater stability due to their wider base of support, while bipeds require more balance to maintain their posture. Bipedal animals are generally faster over short distances, but quadrupeds are more efficient over longer distances due to their even weight distribution. The muscle activation patterns used in quadrupedal and bipedal gait are different, with quadrupeds using a diagonal pattern of limb movement, while bipeds use a more symmetrical pattern. Quadrupedal animals also have a more pronounced spinal curvature than bipeds, which helps them distribute their weight evenly and maintain their balance. These differences reflect the adaptations that animals have evolved to meet the demands of moving on four versus two limbs; however, highlight a key limitation in this study. In the past few decades, researchers have worked to develop a non-human primate model of bipedal locomotion and one has managed to pair this with unit recording from the brain (Goetz et al., 2012). Future studies using similar bipedal models of aging, including non-human primates, may better reflect age-related gait dysregulation seen in humans, and may explain why only specific measures of gait reported here shown sensitivity to age.

Together, many of these limitations are currently being address in our lab and we hope the results reported here as well as in the future will provide insights on potential therapeutic targets that can help offset gait dysregulation and fall risk in the older adult population.

4.7 Conclusion

Much research on neuronal Ca^{2+} dynamics has been focused on investigating cognitive decline with age, and much of these studies focus on the hippocampus and its

connections to the prefrontal cortex; however, it is clear that other age-dependent changes in neurophysiology and behavior are seen in our older population, particularly changes in gait, a major risk factor for falls. On average, 30–40% of people over the age of 65 and 50% of people over the age of 80 will experience a fall each year (Rubenstein & Josephson, 2002; Thapa et al., 1996; Tinetti, 2003). Unmet treatment outcomes using mechanical aides, together with evidence of only a partial influence of peripheral factors (muscle weakness, decreased vision, and vestibular degeneration), suggest a central mechanism may be involved in the age-dependent increase in falls, and we postulate that this central mechanism is neuronal Ca^{2+} handling.

CHAPTER 5. REFLECTION AND LESSONS LEARNED

Across the past three years, I have worked to address the role of neuronal Ca^{2+} dysregulation in gait dysregulation using rodent models of aging (Case et al., 2022; Case et al., 2023; Lin et al., 2022). Starting with the well-characterized F344 rat model of aging, we showed age-dependent changes in neuronal Ca^{2+} network dynamics in the anesthetized state as well as and gait behavior. Now in the C57BL/6J mouse, we show age- and sex-dependent changes in neuronal Ca^{2+} network, single-cell Ca^{2+} transients, and gait behavior all in the awake, ambulating animal across ~2,000 neurons. However, my contributions to the field of aging neuroscience and *in vigilo* imaging are more than the publications I have submitted. I have a passion for transparency in science and here, I would be remiss if I did not highlight some thoughts, ideas, and discoveries surrounding this project that are important to others who may wish to use similar techniques or want to avoid repeating the same slipups that I encountered.

Many of the methodology presented in this dissertation were new to our lab. To carry out *in vivo* imaging in the awake, behaving animal, I had to design new procedures and trial several ideas and products. Most importantly was the switch from acute (terminal) craniotomies in the rat to the use of chronic (survival) craniotomies. In the rat, we would perform an initial surgery were we injected the GCaMP6f AAV approximately 4-6 weeks prior to 2P imaging, then suture the skin back. Then the day of imaging, we would open the skull back up, create the cranial window, and image the anesthetized animal. In the mouse, we wanted the animal to undergo minimal amounts of anesthesia including during

surgery and imaging, so we performed one surgery where we opened the skull, performed AAV injection of GCaMP8f, then created the cranial window and allowed the animal to heal before imaging. In this case, the headbar had the potential to become detached across 4-6 weeks, so the bonding had to be secure. Initially, we lost several animals to detaching of headbars in their vivarium housing as well as during head-fixation and release during imaging. We had to try 3-4 bonding agents and combinations (powdered cement, Vetbond, Loctite 404) and ultimately revealed that UV light-curing dental cement was extremely strong at holding the headbar to the skull across a two-month period. Additionally, we trialed two sizes of headbars from Neurotar (Model 1 and Model 5) in both young and aged mice across sex to see which sizes worked best for both attached to skull and for the 2P objective. We concluded that Model 5 was larger and had better access for the 2P objective, without being too large for the head of our smallest mice (4-month-old female C57BL/6J mice). Also related to surgery, I was very interested in trying the new GCaMP8f AAV, since we had still been using an earlier GCaMP6f AAV in the lab. GCaMP8F was much brighter improving signal-to-noise ratio and was also much faster. This allowed us to characterize quicker events that would report on activity in the higher frequencies (>7 Hz). I had read some reports of spikes in activity in the 12 Hz range and wanted to examine this frequency range, although we see no changes with age or sex at higher frequencies in this study. I also was able to improve AAV expression and limit cellular debris in this study as we switched from using a high volume (2-4 µL) injection to using a low volume (250 nL) injection in S1. Overall, my impressions with GCaMP8f are great and it allowed us to improve our characterization of neuronal calcium signalling in the intact brain.

Many years ago, our lab had purchased two grip strength meters for mouse and rat; however, they had not been used. I was able to talk with colleagues and find relevant and robust protocols for measuring grip strength using our meter and adding a new aspect to our investigations into gait dysregulation—limb strength. Using this tool, we discovered no age or sex differences, and knowing that whole limb muscle weakness did not differ between animals increased confidence in our other measures. Moving the carbon-fiber cage in the Neurotar setup requires some degree of limb strength, and muscle weakness has also been shown to affect locomotion stability in the rodent.

The Neurotar is the largest addition to our lab's toolkit and leading the setup and troubleshooting for this tool was a unique experience. Animals had to be habituated to not only the 2P environment, but also to being head-fixed and to ambulating during head-fixation for long-periods of time. As I learned previously with gait behavior in the rat, rodents become curious across repeated habituation/training sessions and this impedes the ability to capture mice ambulating consistently for a long enough period of time to be able to analyze (*i.e.*, 10 seconds of 2P imaging during ambulation). To counter this, we limited exposure to head-fixation to two sessions prior to imaging, and restricted 2P imaging to 20-minute sessions for each animal. Additionally, head-fixing the animal into the bridge of the Neurotar is not an easy task. Initially, we used a very brief exposure to isoflurane to relax the mouse before mounting. All anesthesia effects were worn off and the mice were ambulating by the time we located the correct brain area to image. Removing the animal from the bridge was the most crucial part for protecting the headbar-skull bonding. Many animals in my pilot project were sacrificed due to the headbar becoming detached at this

phase. Several times, we used very brief exposure to isoflurane to relax the mouse during removal as well.

Across three years, I learned a wealth of information unrelated to aging neuroscience, but equally as important to this study. From courses in Advanced Neuroscience and Medical Neuroscience—where I learned about the dorsal column-medial lemniscus pathway and somatosensory modalities—to posts on Twitter about using the brand-new GCaMP8f to talking with physical therapists and clinicians about gait measures in their human subjects, I gained tools and ideas that I could bring back to my lab and implement in my own research. Wikipedia states that "those studying for a Doctor of Philosophy degree are required to produce original research that expands boundaries of the knowledge." To me, a PhD entailed both dissemination of original research to the field as well as expanding my own boundaries of knowledge. The process of completing this dissertation has provided me the tools I need to continue learning.

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VITA

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PROFESSIONAL POSITIONS

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PROFESSIONAL PUBLICATIONS

Lin, R-L., Frazier, H.N., Anderson, K.L., **Case, S.L.**, Ghoweri, A.O., & Thibault, O. (2022). Sensitivity of the S1 neuronal calcium network to insulin and Bay-K 8644 *in vivo*: relationship to gait, motivation, and aging processes. *Aging Cell*, 21, e13661. https://doi.org/10.1111/acel.13661

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