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The Neural Activity of the Macaque Motor Cortex During Spontaneous Walking: A Neuroethological Investigation

L'attività neurale della corteccia motoria del macaco
durante la camminata spontanea:
un'indagine neuroetologica

Relatore:

Chiar.mo Prof. LUCA BONINI

Correlatore:

Dott. DAVIDE ALBERTINI

Laureando:

FEDERICA TILI

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Abstract (English)

Walking is a whole-body behavior characterized by a rhythmic movement of the limbs, whose velocity and direction are constantly adjusted to address environmental challenges. While the neural circuits that produce walking pattern are known to lie in subcortical areas, particularly in the spinal cord, little is known about the neural mechanism that shape and adjust the walking cycle in natural conditions. Studies that have investigated the role of Motor Cortex (MC) in walking coordination either adopted the unnatural condition of treadmill or used cats as animal models, leaving unclear the MC's contribution to natural walking in primates. To fill these gaps, we recorded single-neuron activity from the MC of macaques left free to move spontaneously in a large enclosure. We found a high number of neurons ($104/255 = 41\%$) activated during walking, irrespective of walking surface—be it flat ground or more challenging conditions like elevated structure. These neurons exhibited diverse firing patterns relative to the step cycle, with many peaking in activity during the landing phase of the hand contralateral to the recorded hemisphere, and several neurons were modulated by the progression of steps. When electrically stimulated, the sites exhibiting walking-related responses elicited arm movements, but also proximal movements, suggesting that the role of MC in coordinating walking might involve not only distal aspects related to limb placement but also postural adjustments. Furthermore, we found that the neural dynamics typical of walking are partly shared with those recruited during goal-directed reaching, particularly in the phase preceding the contact of the hand with the ground or the object. Taken together, these findings indicate that the MC exerts a fundamental, and previously underestimated, role also in coordinating spontaneous walking in natural conditions.

Abstract (Italiano)

La camminata è un comportamento che coinvolge l'intero corpo, caratterizzato dal movimento ritmico degli arti, la cui velocità e direzione vengono adattate costantemente per affrontare le sfide ambientali. Sebbene sia noto che i circuiti neurali responsabili della generazione del modello locomotorio risiedano nelle aree sottocorticali, in particolare nel midollo spinale, rimane poco chiaro il meccanismo neurale che regola e modula il ciclo del cammino in condizioni naturali. Gli studi che hanno esaminato il ruolo della Corteccia Motoria (MC) nella coordinazione della camminata hanno spesso adottato condizioni non naturalistiche, come l'utilizzo del tapis roulant, o l'impiego di modelli animali quali i gatti, lasciando non chiariti i contributi della MC alla camminata naturale nei primati. Al fine di colmare queste lacune, abbiamo registrato l'attività di singoli neuroni nella MC di macachi lasciati liberi di muoversi spontaneamente in un ampio spazio. Abbiamo osservato un'elevata attivazione neuronale ($104/255 = 41\%$) durante la camminata indipendentemente dalla superficie di appoggio—che fosse terreno piano o condizioni più impegnative come una struttura sopraelevata. Questi neuroni hanno mostrato diversi modelli di attività correlati alle fasi del passo, la maggior parte raggiungevano il picco durante la fase di appoggio della mano controlaterale all'emisfero registrato, e diversi hanno mostrato una modulazione durante la progressione dei passi. Stimolando elettricamente i siti associati alle risposte legate al cammino, abbiamo osservato non solo movimenti del braccio, ma anche movimenti prossimali, suggerendo che il ruolo della MC nella coordinazione della camminata potrebbe coinvolgere non solo aspetti distali legati al posizionamento degli arti, ma anche adattamenti posturali. Inoltre, è emerso che la dinamica neurale tipica del cammino è in parte condivisa con quella osservata durante l'atto di raggiungimento di un oggetto, soprattutto nella fase precedente il contatto della mano con il suolo o l'oggetto. In sintesi, questi risultati indicano che la MC svolge un ruolo fondamentale, e precedentemente sottovalutato, anche nella coordinazione della camminata spontanea in condizioni naturali.

1. Introduction

« *Life's aim is an act not a thought.* » (Sherrington, 1951)

Movement stands as an essential aspect of life, serving as a response to the world and *a means to navigate it.*

1.1. Locomotion as a Fundamental Motor Behavior

Our planet is rich in biodiversity and hosts numerous animals that possess a unique set of motor skills adapted to meet the specific demands of the ecological niche they occupy (Shettleworth, 2001; Tinbergen, 1951). One of the essential functions that has required increasing complexity and diversification for millions of inhabiting organisms is the ability to navigate and traverse the earth through *locomotion.*

The term "*locomotion*" derives from the Latin, where "*loco*" means "*from a place*" and "*motio*" means "*motion, movement*". This term encapsulates the concept of "*movement from place to place*".

The quest for survival has driven species to develop refined locomotor strategies over generations, resulting in the remarkable diversity of locomotion mechanisms observed today (Alexander, 2003; Biewener & Daniel, 2010; Biewener & Patek, 2018; Dickinson et al., 2000; Finkelstein et al., 2016; Pettigrew, 1873). Currently, more than two million species on the planet are actively moving through four main environmental domains (Figure 1): aquatic, terrestrial, fossorial, and aerial.



Figure 1 – Examples of adaptive radiation based on locomotion.

In aquatic environments - including oceans, seas, rivers, lakes, and ponds - inhabitants use specialized structures for swimming, moving on the seafloor, or navigating on the water's surface. On solid surfaces in the terrestrial domain, animals employ various locomotion methods such as walking, running, jumping, and climbing, exploiting the environment's geometry. Fossorial organisms in underground environments engage in challenging locomotion, requiring robust claws, specialized senses for navigating in the dark, or streamlined bodies for movement in confined spaces. In the aerial domain, organisms utilize a diverse range of flying mechanisms, navigating three-dimensionally through the Earth's atmosphere, and using air currents for travelling and hunting. Some animals can also occupy multiple environmental domains, as seen in semi-aquatic creatures and diving birds.

All these locomotor adaptations have been made possible by distinct anatomical structures, such as cilia, legs, wings, arms, fins, and tails. These anatomical features represent ingenious solutions, facilitating interaction with the surrounding environment and ensuring continuous survival. Such solutions often involve the development of *analogous structures* (Figure 2) – superficially similar anatomical features that subserve similar functions but have distinct evolutionary origins and developmental patterns in different organisms.

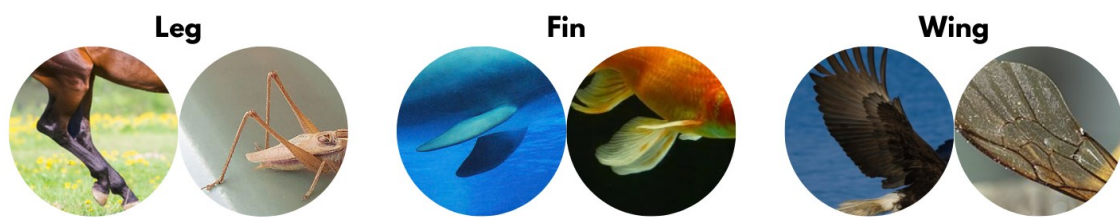


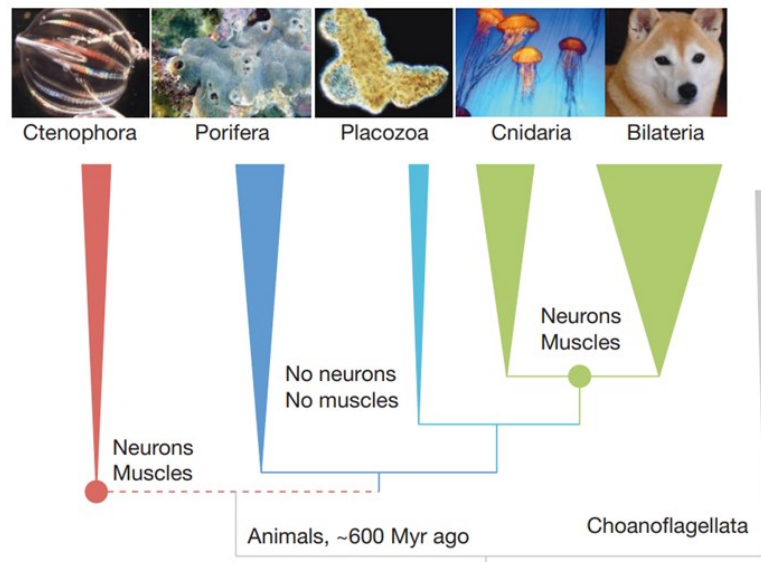
Figure 2 – Comparison of analogous structures for moving in various species.

The sophisticated external anatomy of these structures allows us to recognize the locomotor capacity of individuals possessing them. However, hidden beneath this complex surface is an even more complex nervous system, that serves as the true conductor of movement. The nervous system possesses the remarkable capability to coordinate muscular contractions and generate actions.

This link between the nervous system and movement is fundamental. In the existing literature, a widely accepted hypothesis posits that the primary function of neurons and, consequently, of the nervous system, is to generate and coordinate movement (Llinas, 2001; Wolpert, 2011). This implies that, in the absence of movement, a nervous system might not be necessary (refer to next **BOX 1**).

BOX.1 – Evolutionary Insights: The Interplay Between Movement and Neurons

Recent studies across a wide range of animals strongly support the hypothesis that the nervous system is tightly connected with the capacity of generating movements (Arendt et al., 2016; Butler & Hodos, 2005; Cisek, 2019; Holland, 2003; Jékely, 2011; Liebeskind et al., 2016; Martínez & Sprecher, 2020; Martín-Durán & Hejnal, 2021; Moroz, 2015; Moroz et al., 2014; Willemet, 2013). An illustrative example shedding light on this matter is that of Ctenophores, also known as "comb jellies", a class of marine organisms with a gelatinous morphology and distinctive ciliated combs for movement, that date back approximately 541 million years (Ryan et al., 2016). Ctenophores appear to have followed a unique evolutionary trajectory: they possess neural and muscular systems, structures that subsequently disappear simultaneously in the subsequent phyla, Porifera and Placozoa, only to reappear later in the more advanced phyla of Cnidarians and Bilaterians (refer to the next figure from: Moroz et al., 2014), from which all vertebrates and animals capable of complex movements descend (Martín-Durán & Hejnal, 2021; Moroz et al., 2014).



Adapted from Moroz et al., 2014.

This intriguing neurons-muscles association strongly supports the established hypothesis that the primary role of the neurons is the coordination of movement. The link between the presence – or the absence – of neural and muscular systems suggests that these structures work in tandem. Such hypothesis holds in other species, such as sedentary organisms or those with limited motility, which show comparatively simpler nervous systems (Cisek, 2019; Martín-Durán & Hejnal, 2021). The observed trend reflects nature's economical and adaptive principles: complexity emerges when it serves a clear function, relevant for survival, and it decreases when it is not necessary. Thus, evolution avoids investments in neural intricacies when unnecessary.

1.2. Terrestrial Locomotion: Exploring Walking

One of the common ways animals move on land is by *walking*. Despite variations in mechanisms among species, the widespread adoption of walking establishes it as a fundamental aspect of locomotion (Alexander, 2003; Biewener & Daniel, 2010; Heglund & Taylor, 1988).

Walking can be defined as a whole-body behavior characterized by a rhythmic alternation of forward steps. Among walking animals, we can distinguish between quadrupeds, which use both forelimbs and hindlimbs for walk, and bipeds, which move only using their hindlimbs. Described as a gait, a significant characteristic of walking is the "*duty factor*" corresponding to the fraction of the stride's period during which each foot maintains contact with the ground. In walking, the duty factor exceeds half of the walking time, ranging from 65% for slow walks to 55% for fast walks (Alexander, 2003). Unlike faster gaits like running or galloping, walking demands at least one foot to stay in constant contact with the ground, ensuring exceptional stability. This quality renders walking an ideal mode of movement for activities requiring both mobility and stability.

Walking can be divided into cycles known as "*gait cycles*" (or "*step cycles*"), each comprising two main phases: *stance* and *swing* (Figure 3). The stance phase (approximately 60% of the gait cycle) is defined when the limb is in contact with the ground, and the swing phase (approximately 40% of the gait cycle) is the period when the limb is not in contact with the ground, moving forward, and preparing for the next contact (Leal-Junior & Frizera-Neto, 2022). These two main phases of the gait cycle can be subdivided into additional sub-phases. The stance phase begins with the *initial contact* of the limb with the ground, followed by the *loading response* as the body's weight gradually shifts onto the limb in contact. Next is the

mid-stance phase, where there is complete contact with the ground, supporting the maximum body weight and preparing for the next step. This is followed by *terminal stance*, as the heel lifts from the ground and start the swing phase. This phase begins with *pre-swing*, characterized by lifting the limb in preparation for forward movement, followed by *initial swing*, as the limb moves forward, and *mid-swing*, representing the midpoint of limb advancement. Finally, *terminal swing* involves the limb's free movement for the next ground contact. This pattern applies to both bipeds and quadrupeds (Abhayasinghe & Murray, 2014; D'Août et al., 2002; Granatosky et al., 2018; Leach, 1993; Leal-Junior & Frizera-Neto, 2022); in the latter, the phases of stance and swing involve the two opposite limbs (forelimb and hindlimb on the same side) that alternately coordinate.

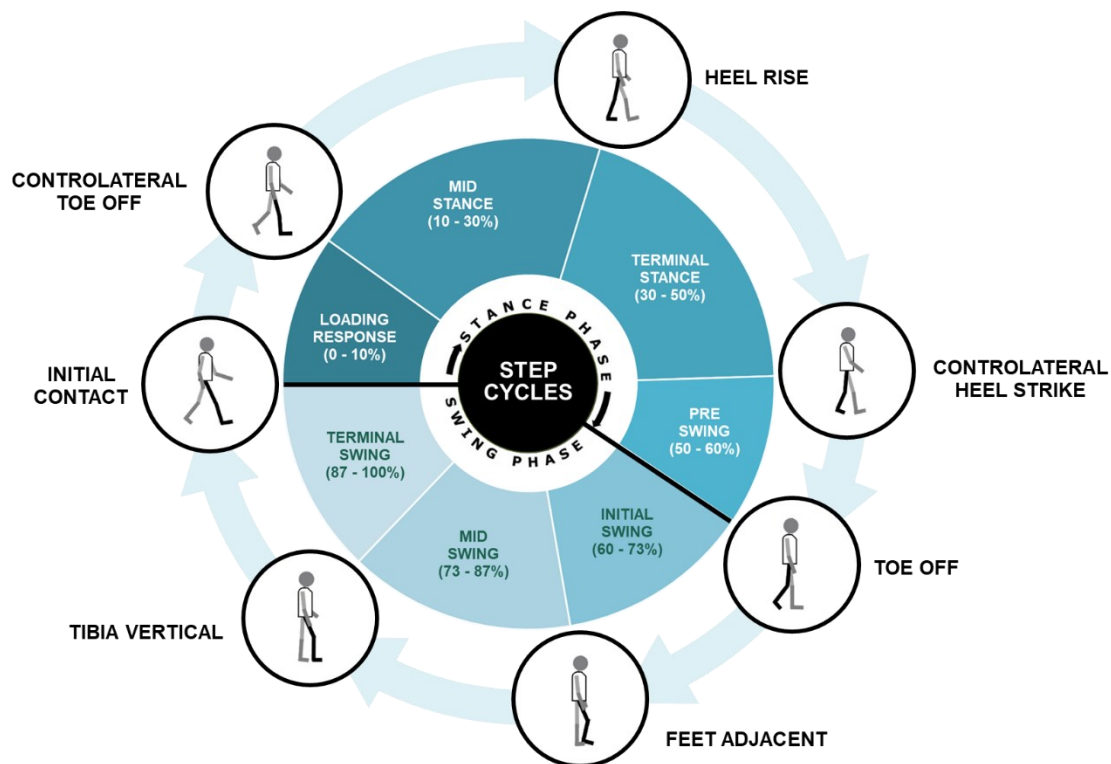


Figure 3 – Step cycle and its subdivisions (adapted from: Leal-Junior & Frizera-Neto, 2022; Moon et al., 2020).

1.3. The Neural Bases of Walking

The neural coordination of walking is a complex process where a cascade of neural events modulates the locomotor pattern (Figure 4).

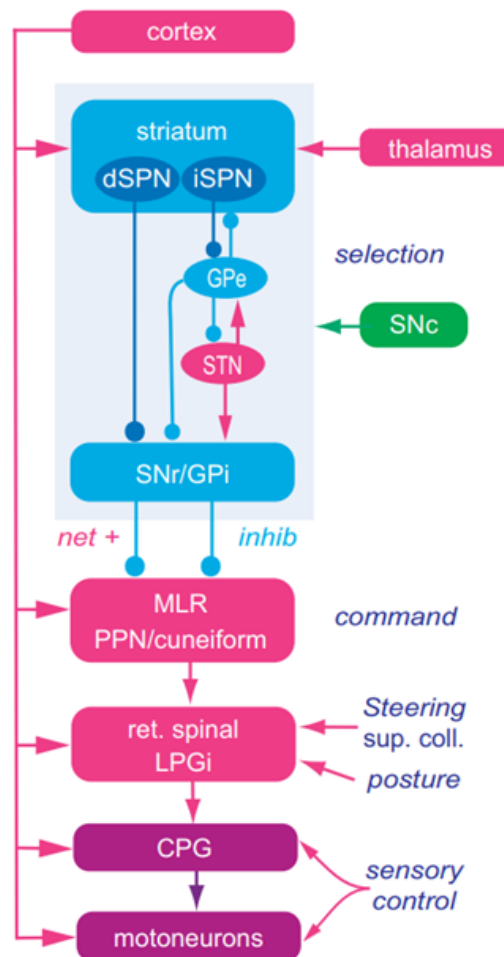


Figure 4 – Diagram of the different components regulating basic aspects of locomotor. Inhibitory structures are depicted in blue, excitatory in red, and dopamine-related elements in green propulsion (Grillner & El Manira, 2020).

The basal ganglia play a significant role in initiating locomotion receiving inputs from the cortex, thalamus, and dopamine neurons in substantia nigra pars compacta (SNc). Through their direct and indirect pathways, the basal ganglia control the initiation and inhibition of locomotion, extending their regulatory influence on neural circuits governing motor programs in both the spinal cord and

brainstem (Grillner & El Manira, 2020; Grillner & Robertson, 2016). The direct pathway (dSPN) triggers a movement, while the indirect pathway (iSPN) appears to inhibit competitor movements through the globus pallidus externa (GPe) and the subthalamic nucleus (STN). Basal ganglia output, involving substantia nigra pars reticulata (SNr) and globus pallidus interna (GPi), influences the mesencephalic locomotor region (MLR), consisting of the pedunculopontine (PPN) and cuneiform nuclei, impacting reticulospinal neurons in the lateral paragigantocellular nucleus (LPGi) that control spinal central pattern generators (CPGs). Finally, the CPGs send input to motoneurons, that drive muscle activation across various joints to generate the walking pattern.

1.3.1. Walking as a Subcortical Process

A substantial portion of walking behavior is regulated by subcortical structures. This organizational pattern - connecting the basal ganglia to the spinal cord - remains consistent across all vertebrates (Figure 5), irrespective of their locomotion type (Grillner, 2011; Grillner & El Manira, 2020; Grillner & Robertson, 2016).

In vertebrates, specialized neural circuits located in the spinal cord named Central Pattern Generators (CPGs) play a crucial role in the propulsive aspect of locomotor movements (Katz, 2016), exhibiting a distinctive capacity to generate rhythmic sequences of neuronal activation, leading to the coordinated contraction of muscles (Arber, 2012; Brown, 1911; Brown, 1914; Grillner & El Manira, 2020; Marder & Bucher, 2001; Marder & Calabrese, 1996).

The CPGs are capable of autonomously producing timed sequences of neuronal activation, even without sensory input or motor commands from the brain.

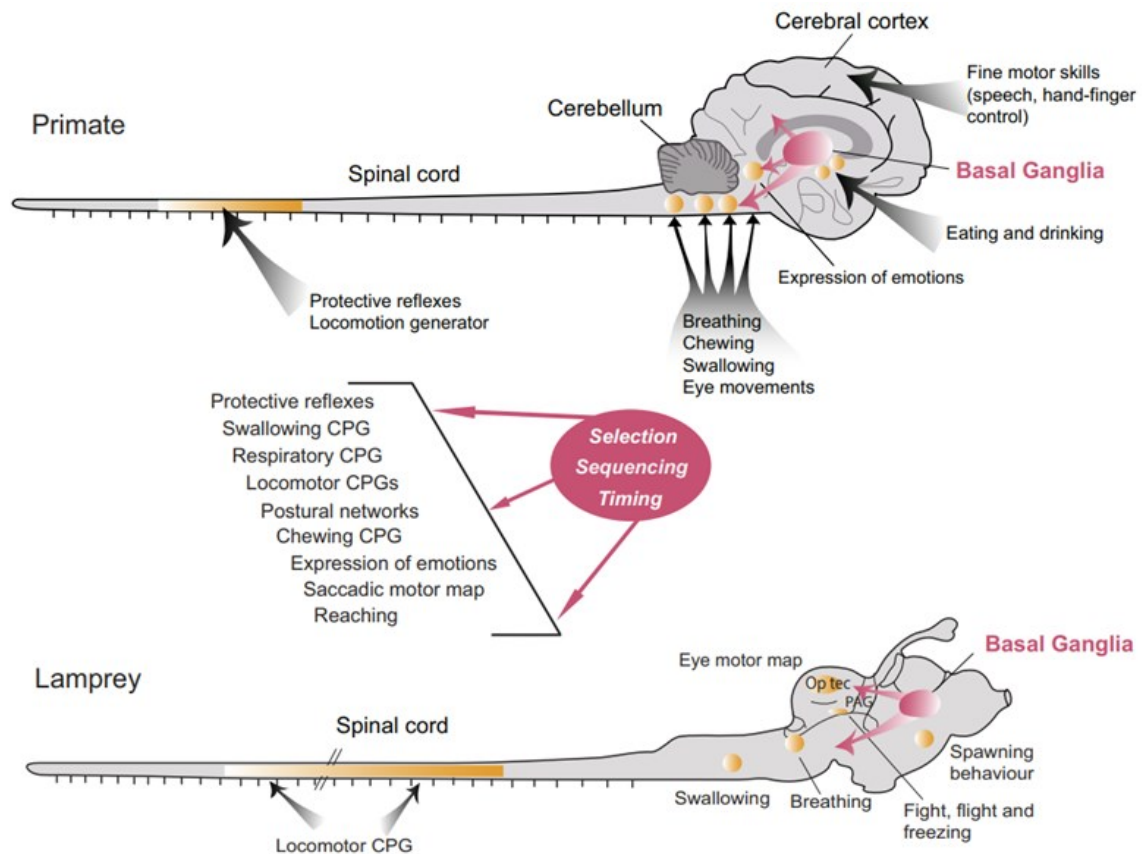


Figure 5 – Common motor infrastructure from lamprey to primates (Grillner & El Manira, 2020).

Experimental evidence confirms that even in cases of deafferentation (Brown, 1911; Grillner & Zangger, 1984; Holst, 1935a, 1935b; Rossignol & Lamarre, 1986; Wilkens, 1994) or curarization (Grillner & Wallén, 1982; Wallén, 1980), CPGs can continue to operate alone, cyclically generating neural impulses corresponding to specific phases of rhythmic behavior (Bagnall, 2022; Grillner & Zangger, 1975; Lindén et al., 2022).

While CPGs can generate patterns alone, their crucial feature lies in their plasticity (Arber, 2017; Grillner & El Manira, 2020). In mammals, CPGs integrate sensory feedback and brain instructive signals, adapting their activity in response to external stimuli or changes in body conditions (Prochazka & Ellaway, 2012). This adaptability allows locomotor patterns to optimize themselves, providing adjustments to variations in environmental conditions or behavioral needs

(Ekeberg & Pearson, 2005; Taga, 1995; Yakovenko et al., 2004). For example, if an obstacle is detected during walking, the sensory feedback modulates CPGs activity to adapt the movement to the new circumstances (Rossignol et al., 2006; Taga, 1998). This real-time correction is facilitated by an efference-copy mechanism, with spinal CPGs sending information to the brainstem via spinocerebellar and spinoreticular pathways (Lambert et al., 2012; Straka et al., 2018). These pathways provide phasic feedback mediated by the cerebellum to major fast-conducting vestibulo-, rubro-, and reticulospinal pathways (originating from the caudal pontine and medullary reticular nucleus) that adapt the movement. Recently, the network of CPGs has been expanded to encompass the incorporation of motoneurons, serving as the final pathway from the spinal cord to skeletal muscles (Ampatzis et al., 2014; Barkan & Zornik, 2019; Song et al., 2018).

1.3.2. The Role of Cortical Regions

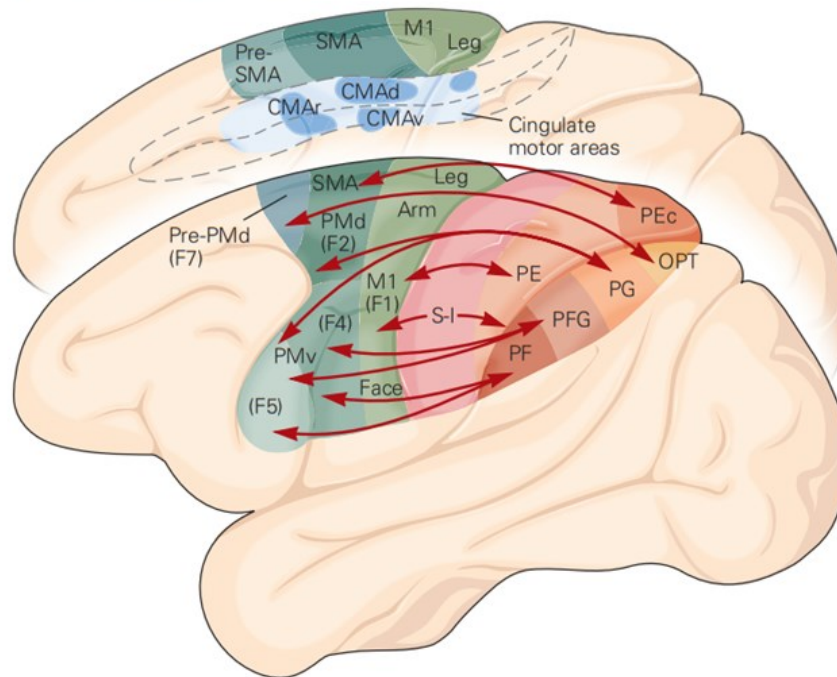
As described above (Figure 5), in primates and other mammals the neural circuit governing locomotion extends to the cerebellum and cerebral cortex. If the cerebellum has been indicated as the stabilizer of movement (Grillner & El Manira, 2020), the role of the cortex (MC) in the coordination of locomotion remains poorly understood.

Situated in the agranular sector of the frontal lobe, the MC encompasses two main regions (Brodmann, 1909): the primary motor cortex (M1/F1), corresponding to Brodmann area 4 (BA4), and the premotor cortex (PMC), corresponding to Brodmann area 6 (BA6), spanning from the central sulcus to the arcuate sulcus. Rizzolatti and colleagues (1998) proposed the further subdivision of these areas based on their cytoarchitectural characteristics. They identified the supplementary (F3) and pre-supplementary (F6) motor areas in the most medial aspect of the PMC,

along with the dorsal premotor (PMd) areas F2 (caudal) and F7 (rostral) and the ventral premotor (PMv) areas F4 (caudal) and F5 (rostral). These areas play a crucial role in coordinating voluntary motor actions (Bufacchi et al., 2023), and its efficiency relies on its tight connectivity with parietal and sensorimotor regions, as illustrated in Figure 6.

These parieto-frontal circuits playing a pivotal role in motor control and the integration of sensory and motor information that underlies various specialized higher-order functions (Rizzolatti et al., 2014; Rizzolatti & Luppino, 2001). The interplay between these regions facilitates precise and adaptive motor responses to sensory information, thereby supporting the effective coordination and execution of movements. At the core of this circuit's functionality is the integration of sensorimotor feedback through afferent circuits that convey information about the state and accuracy of movements, allowing their real-time adaptation. This process is facilitated by the precise cortical encoding of the effector responsible for executing the motor output, as evidenced by the deciphering of topographical cortical maps in both M1 and PMC (Rizzolatti, Scandolara, Gentilucci, et al., 1981; Rizzolatti, Scandolara, Matelli, et al., 1981; Woolsey, 1952). Further studies have suggested that the cytoarchitectonic differences between M1 and PMC are more likely attributable to functional distinctions rather than somatotopic representations (Gentilucci et al., 1988; Kurata & Tanji, 1986).

Areas on the cortical convexity



Areas inside the parietal sulcus

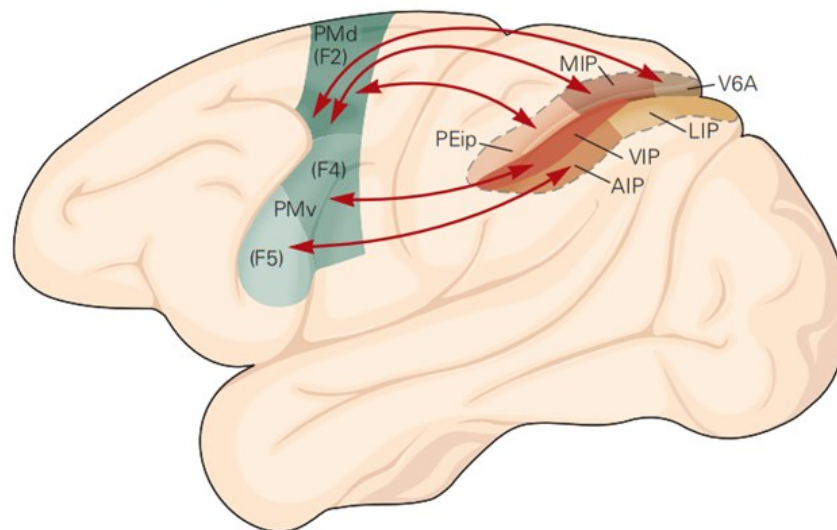


Figure 6 – Frontal motor areas connected with parietal regions. Cortical organization in a monkey model, with the lower panel providing a clear view of the opened intraparietal sulcus. Parietal areas, highlighted in orange and denoted by the letter P, encompass distinct cytoarchitectonic areas: PE; PF and PFG corresponding to BA7b; PG and OPT (occipito-parieto-temporal) corresponding to BA7a. Within the intraparietal sulcus (IP), specific areas include AIP (Anterior), LIP (Lateral), MIP (Medial), VIP (Ventral), PEip (PE), and the visual area V6A. The primary somatosensory cortex is represented in pink as S-I. Motor areas, depicted in green and labeled with the letter F (frontal), consist of the primary motor area (F1) and the premotor area with its subdivisions: dorsal premotor cortex (PMd), ventral premotor cortex (PMv), pre-dorsal premotor cortex (Pre-PMd), and supplementary motor area (SMA). Blue highlights motor areas within the cingulate cortex: rostral cingulate motor area (CMAr), ventral cingulate motor area (CMAv), and dorsal cingulate motor area (CMA). Arrows delineate the primary reciprocal connections between functionally correlated parietal and frontal motor areas (Kandel et al., 2021).

Graziano and colleagues (2007; 2005; 2002) have supported this notion through Intracortical Microstimulation (ICMS) of the monkey's MC, using pulse trains with a duration exceeding 500 ms, which approximately matches the duration of monkey behaviors. Through this method, they revealed a map of complex, multi-joint actions across various regions of the MC. Long-train ICMS induced the monkey to perform ethologically relevant actions commonly found in its behavioral repertoire. These findings suggest that MC are involved in more than simple motor control, encompassing functions typically associated with higher-order cortical regions. Specifically, these motor areas participate in transforming sensory inputs for goal-directed actions, recognizing actions, and making decisions to initiate actions (Rizzolatti & Luppino, 2001).

Based on these premises, subsequent studies have focused on understanding how MC contribute to limbs movements during locomotion.

1.3.3. The Motor Cortex in Skilled Walking

As previously discussed, many studies emphasize the role of subcortical circuits in controlling locomotor behavior, even independently of sensory feedback from the periphery (refer to 1.3.1.), in line with that the retained capacity to walk in animals with cortical lesions (Courtine et al., 2005; Liddell & Phillips, 1944; Muir & Whishaw, 1999). While these findings may suggest limited or no involvement of cortical areas in the locomotor functions, this hypothesis has been challenged by other studies suggesting that the role of the MC during locomotion might be linked to higher-order aspects (Armstrong, 1988; Armstrong & Drew, 1984; Drew et al., 2023; Drew et al., 2002; Georgopoulos & Grillner, 1989).

As highlighted in section 1.1., locomotion is a fundamental motor behavior evolved to adapt to environmental demands, enabling living organisms to navigate

diverse terrestrial landscapes. Animals need to adapt to environmental changes, such as uneven terrains or the presence of obstacles. In this specific aspect of locomotion, the MC appears to play a primary role (Armstrong, 1988).

In 1911, Trendelenburg's experiments with cats and dogs showed that temporarily cooling the MC led to a momentary inability to navigate grids requiring precise limb positioning. Intact animals, in contrast, effortlessly managed such surfaces, underscoring the MC's crucial role in this locomotor task (Trendelenburg, 1911).

In 1944, Liddell and Phillips observed similar outcomes in cats with surgically divided medullary pyramids. After the operation, these cats struggled on narrow beams or ladders, becoming motionless and unable to step without slipping or falling. Their coordination deficits revealed difficulties beyond rhythmic walking, persisting for months post-surgery (Liddell & Phillips, 1944). Further studies on cats consistently revealed similar deficits in animals with permanent lesions of the MC, emphasizing its contribution to walking (Adkins et al., 1971; Beloozerova & Sirota, 1993; Drew, 1988; Drew et al., 1996).

Additional investigations probed the auxiliary functions of the MC within visuomotor circuits, particularly by examining locomotion in cases of lesions to the parietal cortex (Drew & Marigold, 2015; Lajoie & Drew, 2007). These studies emphasized contribution of the parietal cortex to locomotion planning, suggested to dynamically estimating the animal's position relative to objects along its path. The MC leverages this information to modulate the activity of synergistic muscle groups engaged in distinct phases of the gait cycle. Other studies on locomotion following lesions to subcortical structures highlighted the MC's crucial role in compensatory mechanisms (Beloozerova, 2022; Capogrosso et al., 2016). Collectively, these findings support the idea that the MC has a major role in

refining gaits in scenarios that require precise limb placement. This evidence suggests that the integrity of the MC and of the corticospinal tract is pivotal for *skilled* locomotion (Armstrong, 1988). The fronto-parietal network emerges as a key player in facilitating visuomotor coordination, enabling real-time adjustments to locomotion by influencing limb trajectories and guiding their placement (Drew et al., 2008; Drew et al., 2023).

Studies that compared the activity of MC neurons during walking on flat ground versus walking in more challenging conditions requiring precise foot control revealed an overall increase in cortical response, and neurons firing rhythmically and in synchrony with the step cycle increase their firing rate under demanding conditions (Drew, 1988; Drew et al., 1996).

In sum, these studies indicate that the transition from '*stereotyped*' to more '*skilled*' locomotion entails a crucial, but still understood, contribution of the cerebral cortex, and particularly of the MC.

1.3.4. Knowledge Gap: Limited Neurophysiological Studies in Naturalistic Setting

Even after 35 years, the sentiment expressed in the opening lines of Armstrong's 1988 article, "The Supraspinal Control of Mammalian Locomotion," remains appropriate: «...the mammal of my title is therefore essentially a synonym for the cat».

Indeed, most of the literature investigating the responses of single neurons within MC during locomotion is primarily constituted by studies on cats (Amos et al., 1990; Armstrong, 1986; Armstrong & Marple-Horvat, 1996; Beloozerova & Sirota, 1993; Drew, 1988; Drew, 1993; Drew et al., 1996; Nakajima et al., 2019; Palmer et al., 1985; Prilutsky et al., 2005). Only recently some evidence has emerged in rodent studies (Hermer-Vazquez et al., 2004; Omlor et al., 2019; Warren

et al., 2021), but a striking lacking of knowledge remains in the most translationally-relevant animal model for understanding the cortical control of voluntary locomotion in humans, that is, Non-Human Primates (NHPs).

This absence can be attributed, in part, to methodological limitations. In fact, the advancements in wireless recording technologies (see Figure 7) have started to yield some result concerning single neuron firing properties during locomotion in monkeys (Fitzsimmons et al., 2009; Foster et al., 2014; Schwarz et al., 2014; Xing et al., 2019; Yin et al., 2014).

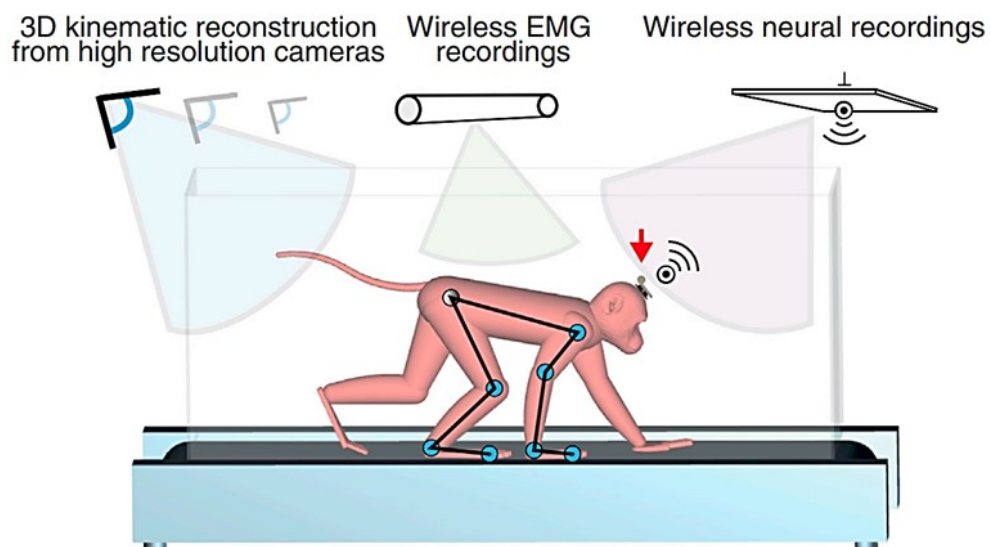


Figure 7 – Non-Human Primate Treadmill Model employed to study neural responses during locomotion tasks (Foster et al., 2014).

However, all these few studies in NHPs, including most of those in cats and rodents, share a common methodological approach: animals were recorded while walking regularly on a treadmill in a rhythmic and stereotyped way, and the neuronal discharge of neurons of the MC was correlated to various movement parameters, including joint kinematics and muscle electromyography (EMG).

For example, Foster and colleagues recorded the activity of single neurons in the dorsal part of the premotor cortex (PMd) in two monkeys as they walked on a

treadmill at various speeds. The results revealed a consistent response in certain neurons during specific phases of the step cycle, regardless of the speed (see Figure 8). This suggests a potential modulation of PMd activity during the stance, ground contact, or swing phase of the walk.

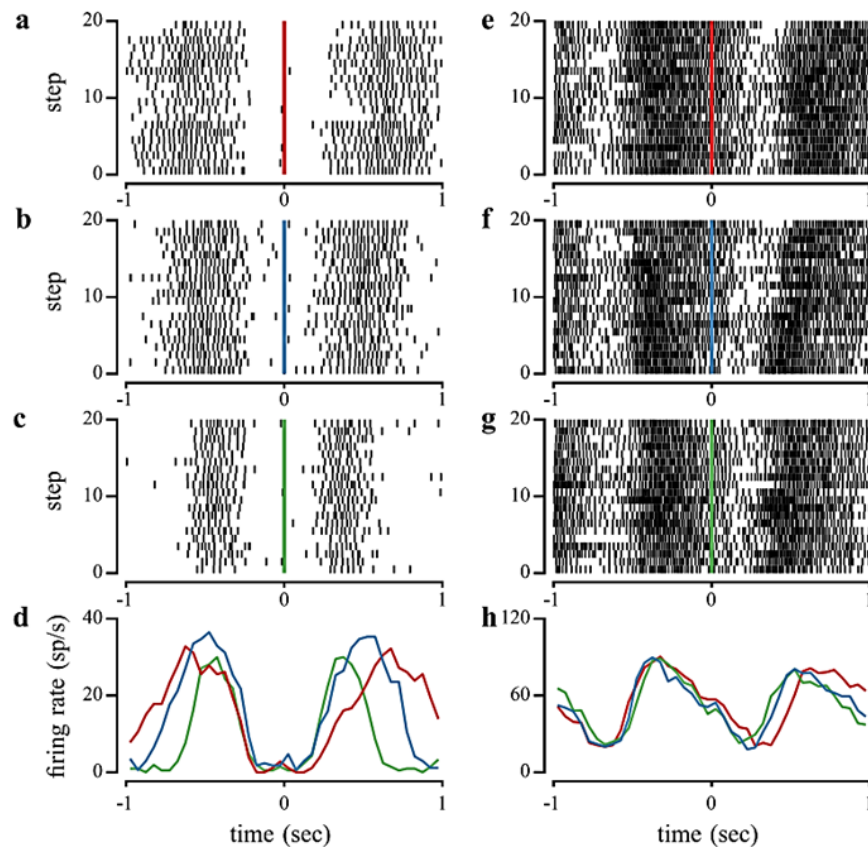


Figure 8 – Neural Correlates of Walking (Foster et al., 2014). Raster plot illustrating the neural activity of a neuron as monkeys walk at slow speed (a = monkey1, e = monkey2), medium speed (b = monkey1, f = monkey2), and fast speed (c = monkey1, g = monkey2). Each row corresponds to a step aligned at $t = 0$ (stance phase), with the right arm contacting the treadmill after taking a step forward. Panels d and h depict the average activations.

While these studies offer valuable contributions to the research, an additional step forward is required. Current literature highlights the necessity of additional studies to overcome the limitations of previous ones (Liang et al., 2023) and a significant expansion of research focused on NHPs, where studies have been notably limited and unchanged for the past decade.

Finally, the proposed approach promotes for the adoption of more naturalistic experimental methodologies, necessitating a shift away from treadmill usage to settings that allow the animal to walk in free non-stereotyped conditions.

1.3.5. The Need for Advancing Research on Walking in NHPs

Expanding the investigation on walking in NHPs assumes significance for both scientific and clinical reasons.

Firstly, research on NHPs enables a more comprehensive exploration of the role of the MC during locomotion compared to studies on cats. This is attributed to the extensive investigation of neural properties in the MC of NHPs (Cisek & Kalaska, 2010; Fogassi et al., 2001; Hoshi & Tanji, 2007; Maranesi et al., 2012; Rizzolatti & Luppino, 2001). Drawing from this literature - outlined in section 1.3.2. - we know that the MC in primates possesses well-defined neural circuits for goal-directed actions. This stands as distinctive feature of primates compared to cats, where the existence of such circuits is hypothesized but debated (Drew et al., 2023; Gertz et al., 2017). For this reason, it is crucial to investigate whether correlates of walking exist in NHPs within the same areas of the network responsible for coding actions - regions demonstrating an ability to abstract specific movement sequences or the effector. A finding of this nature could suggest the presence of a comparable network in cats (as illustrated in Figure 9), though potentially still functionally linked to locomotion (Drew et al., 2023; Fortier-Lebel et al., 2021).

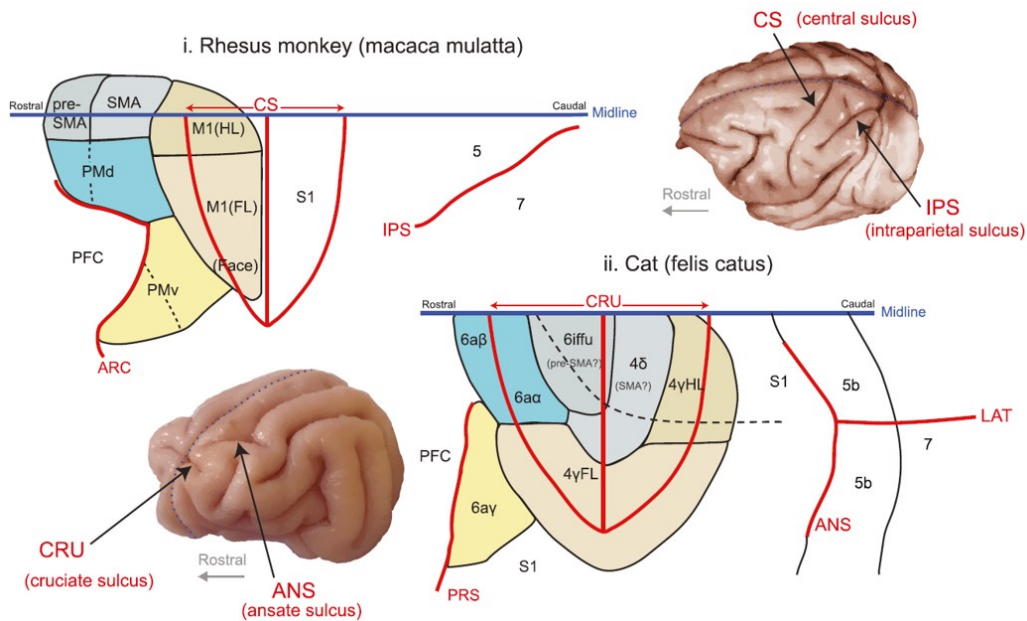


Figure 9 – Lateral views of both monkey and cat brains, accompanied by flattened representations of the fronto-parietal regions. These representations are centered on the fundus of the central sulcus (CS) in monkeys and the fundus of the cruciate sulcus (CRU) in cats. In panel ii, the dotted line outlines the approximate path of the primate midline, determined based on the spatial correspondence of the cat's frontal areas. The color code in the figure indicates hypothetically analogous cortical areas between the two species. This color-coded mapping is derived from the findings of microstimulation studies, cytoarchitecture, cortico-cortical connectivity, and cell discharge characteristics (Drew et al., 2023).

Additionally, the translational value of these studies on NHPs becomes relevant in relation to human rehabilitation. Studying MC circuits in NHPs provides a unique opportunity to understand the interactions between upper and lower motor areas during both locomotion and manual actions. Current studies on humans (Fukuyama et al., 1997; Hanakawa et al., 1999; Malouin et al., 2003; Petersen et al., 2012; Sacheli et al., 2018; Shine et al., 2013; Wagner et al., 2014) have provided valuable insights into the contribution of upper motor areas to locomotion planning but with significant limitations. Existing research fails to fully capture the neural processes involved in locomotion due to the limitation of non-invasive techniques. Otherwise, studies on NHPs offer a unique platform to analyze neuronal activity in detail during complex movements and this information could be fundamental to enhance the understanding of human locomotion disorders and designing more targeted rehabilitation therapies.

1.3.6. The Importance of Treadmill-Free Studies

The use of treadmill for studying walking enables to render the behavior repeatable and stereotyped, facilitating the emergence of its rhythmic component and the comparison of the results with most of the existing literature (Foster et al., 2014). Nonetheless, it introduces a potential drawback. In contrast with the supposed involvement of higher-order cortical areas in naturalistic walking, where precise limb placement is essential (Armstrong, 1988; Georgopoulos & Grillner, 1989), using the treadmill's risks to prevent the identification of the specific contribution of the cortex to naturalistic walking (Grillner & El Manira, 2020). Hence, the current experimental landscape necessitates evidence allowing the exploration of genuine, unrestricted walking (Drew et al., 2023; Liang et al., 2023; Xing et al., 2019), providing insights into cortical activity when animals move in contexts similar to natural environments.

The only recent study that did not make use of a treadmill has been carried out by Tia and colleagues (2021). They investigated the brain activities of marmosets navigating narrow poles akin to natural tree branches, using electrocorticography (ECoG). Recorded data reveal that cortical Beta-oscillations correlate with body posture, while Gamma-oscillations are implicated in limb movements, highlighting the pivotal role of the MC in adaptive locomotion (Tia et al., 2021).

However, studies exploring the activity of single neurons during freely moving NHPs locomotion are still lacking, representing a critical gap. In-depth analysis of activations of single neurons in MC during freely locomotion could delineate the specific contributions of various motor and premotor areas and associated parieto-frontal circuits, functions that still remain elusive in the current literature (Drew et al., 2023; Drew & Marigold, 2015).

1.3.7. The Walking-Reaching Hypothesis

In 1989, Georgopoulos and Grillner postulated that the involvement of the MC in locomotor behavior, particularly in navigating challenging terrains and avoiding obstacles, underlies reaching behaviors observed in primates and other mammals. Their hypothesis suggested the possibility of a shared evolutionary origin between these two behaviors, indicating that reaching behaviors might have evolved from walking. Specifically, they proposed that the precise movements of the forelimbs, utilized for accurately placing them on the surface during locomotion, could have served as a foundation for directing the hand towards target objects. This implies that the neural substrates initially employed for precise limb positioning on the ground might have later been adapted for accurately guiding the hand towards target objects.

This idea has generated considerable interest in the literature, and this interest remains alive today, 35 years later. However, despite the interest, there is still a lack of clear evidence either confirming or refuting this hypothesis. Only recently, some studies have delved into the topic, contributing additional data to the discussion (Drew et al., 2023; Miri et al., 2017; Xing et al., 2019; Yakovenko & Drew, 2015).

In 2015, Yakovenko and colleagues designed a specific paradigm to address Georgopoulos's question. They conducted a study on cats, comparing the neural activity of single neurons recorded in Area 4 of the pericruciate cortex (homologous to M1) and electromyographic (EMG) activity. Cats were trained to perform two distinct behaviors: reaching for a lever with their front paw and walking on a treadmill while avoiding an obstacle. To facilitate a meaningful comparison of responses to the two behaviors, the researchers intentionally made the kinematics of reaching and walking very similar (a decision that may be subject

to debate). The results indicated comparable activity both in terms of EMG and single-neuron responses (see Figure 10). This study suggested that MC neurons contribute similarly to muscle activity during both reaching and stepping over obstacles supporting the hypothesis that the control circuits for reaching movements may have evolved from those originally utilized for modifying gait.

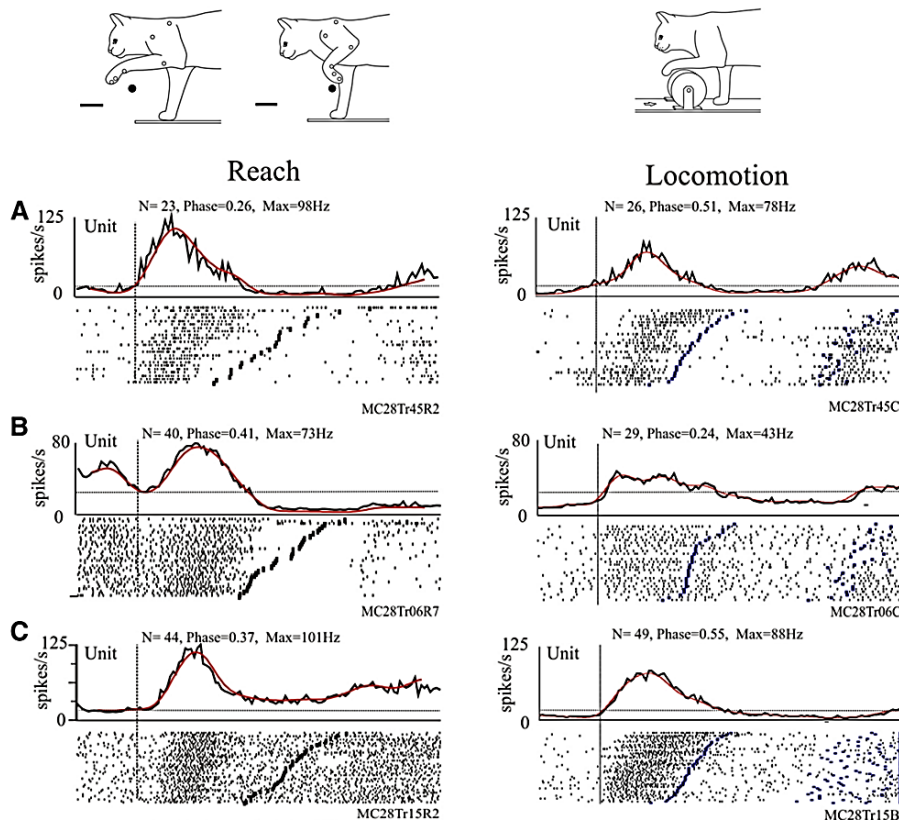


Figure 10 – Activity of three neurons (A-B-C) during reach (left) and locomotion (right). Raster and histograms for both behaviors are aligned with the onset of muscle activity in the contralateral forelimb flexor (CIB), wrist dorsiflexor extensor carpi radialis (ECR), and wrist/digit dorsiflexor extensor digitorum communis (EDC). The red line in histograms is for smoothing, and the staggered lines in the raster indicate the end of muscular activity for each behavior (Yakovenko & Drew, 2015).

Further support for this hypothesis is suggested by Xing and colleagues' 2019 study on NHPs, where they compared the dimensions of walking and reaching behaviors through a decoding operation. The authors juxtaposed the walking dimensions calculated in their study with literature data on reaching dimensions, concluding that a similar number of neural dimensions is needed to represent

these motor activities. This implies a common organization of neural activities involved in both behaviors. However, the authors acknowledge that this is a preliminary result requiring empirical confirmation. They underscore the limitations of their paradigm, which lacks data extracted from the same animal and systematically compared.

A different contribution to this debate comes from the study on mice by Miri and colleagues published in 2017. In this study, the authors compared walking with a precision pull task for which the mice were trained (it's worth noting that the authors do not explicitly reference Georgopoulos's hypothesis; the paradigm was not designed with that specific purpose). The study employed MC single neurons and EMG recordings, coupled with the use of optogenetic stimulation to selectively manipulate neural activity. Their results suggest that the influence of the MC on muscle activity is quicker and more widespread during precision pull tasks, whereas it is delayed and primarily affects flexor muscles during walking. These findings emphasize the behavior-specific role of the MC in modulating muscle activity, highlighting that MC activity varies depending on the specific behavior.

Given the current open questions in the literature, future studies with NHPs in naturalistic settings examining neural responses during reaching movements and limb positioning could shed light on the proposed similarities (Liang et al., 2023; Xing et al., 2019). The presented data suggests potential parallels in neural responses. It is plausible that the same neurons respond similarly to goal-directed actions (such as reaching or grasping) and limb placement during locomotion. Furthermore, given the varied characteristics observed in neurons of the MC (Maranesi et al., 2012; Rizzolatti & Luppino, 2001), similarities between reaching and walking may depend on the specific role these neurons play.

2. Aims of the Study

Building upon the findings and limitations of prior research, the aims of this study are multiple.

First, we aim to investigate neural activity within the Motor Cortex (MC) of Non-Human Primates (NHPs) during unconstrained locomotion. Utilizing wireless neural recording techniques, we aim to understand the characteristics and functional properties of MC neurons without the limitations imposed by traditional methodologies, like the treadmill.

Second, we aim to compare findings in NHPs with results from studies on other models, particularly the cat. This comparative analysis aims to reveal potential specie-specific variations in the neural mechanisms governing spontaneous and unconstrained movement, increasing our comprehension of the functional implications of MC activity.

Additionally, our study aims at contributing to scrutinize the hypothesis that precision walking and reaching share a common cortical control (“Walking-reaching” hypothesis, see section 1.3.7.). By examining neural correlates during unconstrained movement, we aim to provide data that could either support or refine this hypothesis, shedding light on the interplay between the MC and complex motor behaviors.

3. Materials and Methods

3.1. Ethical Statement

Every part of the experimental protocols, from animal handling to surgical and experimental procedures, comply with the European law on the humane care and use of laboratory animals (Directive 2010/63/EU) and with the Italian laws in force on the protection of animals used for scientific purposes (D.lgs 26/2014). They were approved by the Veterinarian Animal Care and Use Committee of the University of Parma and authorized by the Italian Ministry of Health.

3.2. Experimental Subjects and Surgical Procedures

Experimental protocols were carried out on two adults male *Macaca mulatta* (Mk1: 13 kg, 8 years; Mk2: 13 kg, 10 years). The recording sessions have been preceded by training through positive reinforcement that allowed to instruct the monkey to spontaneously sit in a primate chair and to be familiarized with the laboratory setting. The subjects were individually housed during the period of the experiments following veterinary advice, but with visual, auditory, and olfactory contacts with other conspecifics. Recorded animals were housed in 12h light and 12h dark regimen with lights off at 7 p.m.

After completing the training, each monkeys underwent two surgical interventions. All the procedures were performed under deep anesthesia and aseptic conditions: atropine (0.03 mg/kg) was administered to the macaques 15 minutes before anesthesia, which was induced with ketamine (Lobotor, 4.5 mg/kg) and medetomidine hydrochloride (Domitor, 0.05 mg/kg). Anesthesia was maintained with inhaled isoflurane (IsoFlo, 100% p/p). The first procedure involved

implanting the fixation system (headpost). In a second surgical procedure, four 32-channel Floating Microelectrode Arrays (FMAs) were implanted in Mk1 and six in Mk2 (see Figure 11). The procedure involved a craniotomy exposing a brain portion chosen via MRI. The implants aimed to record neural activity in the left premotor and motor cortices. Mk1's implant comprised four FMAs covering areas between the inferior arcuate sulcus and central sulcus, including F4, F5, and M1. Mk2's implant included six FMAs covering premotor areas F4 and F5 between the arcuate sulcus and central sulcus, along with a section of F2vr. Arrays were carefully positioned into the cortical tissue, with subsequent dura mater suturing and repositioning of the bone flap secured with dental cement and micro bone screws. The chamber was affixed to the skull with bone screws and dental cement, Omnetics connectors placed, and sealed with a protective cap. Following muscle and skin suturing, monkeys were awakened after pharmacological treatment, with three weeks recovery before neural recording sessions.

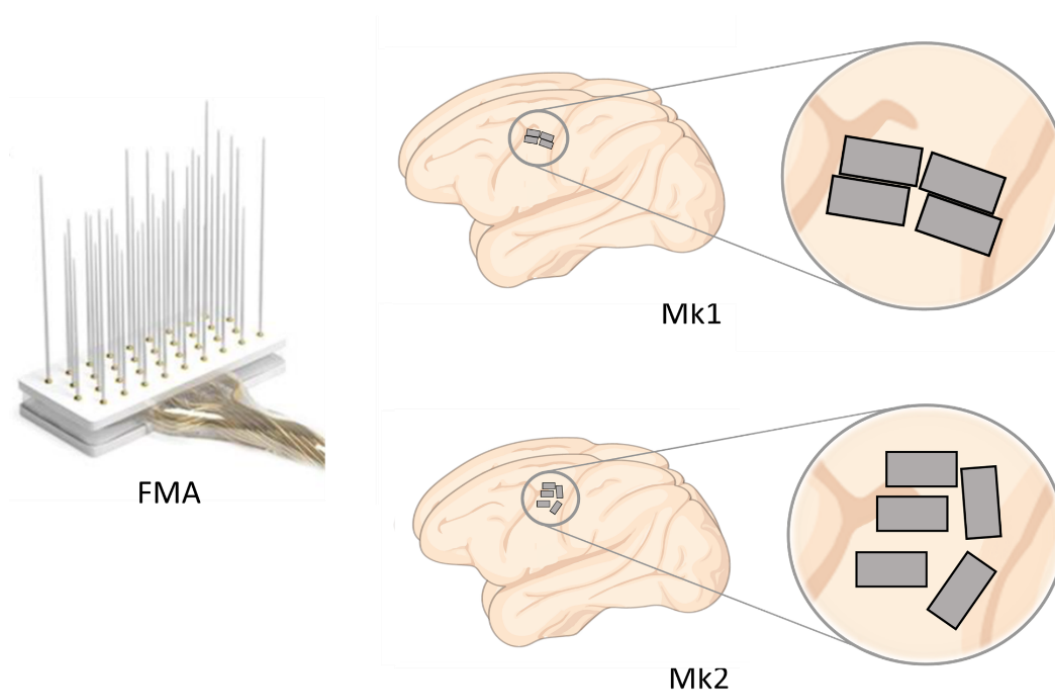


Figure 11 – Schematic representation of the FMAs implanted in the MC of the two monkeys.

3.3. Apparatus and Experimental Design: The NeuroEthoRoom (NER)

To allow the monkey to walk freely, recordings were conducted inside the NeuroEthoRoom (NER). The NER is a custom-made transparent plexiglass enclosure (Width: 208 cm; Height: 205 cm; Depth: 181 cm) equipped with a system of 8 color cameras for behavioral recordings of the monkey (see Figure 12). One of the four side walls of the NER consists of two large doors, allowing the experimenter to enter and prepare the environment before and after each session, or vice versa at the end of the session. Each door contains a smaller opening with a vertical sliding gate through which the monkey passes from its chair to the NER at the beginning of every session. In the NER, we used various enrichment items to create ecological conditions to elicit spontaneous species-specific behaviors.

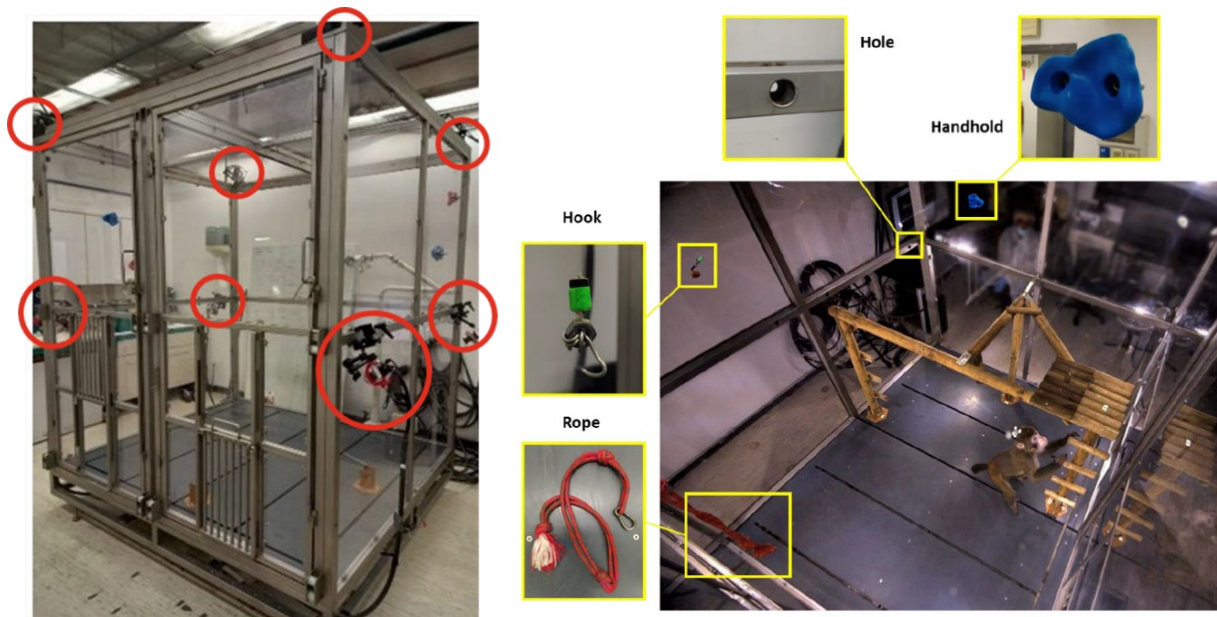


Figure 12 – On the left, an external view of the NER structure featuring 8 cameras (highlighted in red circles) positioned at the corners, along with access doors for experimenters and smaller entrances for primate access at the front. On the right, an internal image of the NER showcasing various enrichment elements such as holes, handholds, hooks, a wooden structure mounted on the floor, and a rope.

In particular, climbing holds were affixed to the walls, and a wooden structure through which the monkey could walk, rest, and climb. This design facilitated the examination of spontaneous walking on both the surfaces. Additionally, it allowed the study of control behaviors such as forelimb grasping and mouth-face movements. To stimulate foraging, the experimenter could introduce food from outside the NER through openings in the walls or hooks lowered from the ceiling, each connected to an externally operated nylon thread. Furthermore, transparent plexiglass walls facilitated experimenter-monkey interaction.

3.4. Behavioral Data Acquisition and Analysis

3.4.1. Video Acquisition

Data acquisition for the monkey behavior was accomplished with a system of eight high resolution synchronized cameras mounted at two different levels on movable arms attached to the four external corners of the NER (see Figure 12). Such cameras are Dual Gigabit Ethernet Machine vision cameras (mvBlueCOUGAR-XD, Matrix Vision) with a 1936x1214 resolution, set to 50Hz, and equipped with a global shutter with sensor size 1/2" format (5.86 μ m pixel), a manual C-Mount Lenses with 5 mm focal length (CCTV Lens, KowaOptical Products Co., Ltd) and LEDs ring lights. Each camera has two RJ-45 Gigabit Ethernet connectors with screw locking and two Industry standard 12-pin locking connectors to provide transmission of images and signals to the Windows computer, and to synchronize all cameras through a synchronization box connected to both cameras and computer. SIMI Motion Capture is the dedicated and commercially available software for 3D motion data acquisition and analysis used to capture and visualize the monkey's behavior.

3.4.2. Ethogram

The ethogram used for behavioral analyses was structured at multiple levels. Behaviors were categorized as points events and operationalized in detail in **Table 1 (Steps Classification)** and **Table 2 (Other Behaviors Classification)**.

To address the study's objectives, we defined "Walks" as sequences of steps, comprising three or more consecutive steps, with an average time difference of 0.7 seconds between each step (this average time was chosen based on behavioral observations, which demonstrated it to be the typical interval between steps in the walking patterns of monkeys in the NER). Walks that don't exhibit these characteristics have been excluded.

A step was considered at the initial contact of the forelimb to the ground (see Figure 13). For each walk, we annotated the *First step hand*, the *Central step(s) hand*, and the *Final step hand*. Starting from the *Steps Classification (Table 1-A)*, we indicated characteristics at multiple levels for each tracked step: *Initial Posture (1-B)*, the *Hand used (1-C)*, and the walking *Surface (1-D)*.

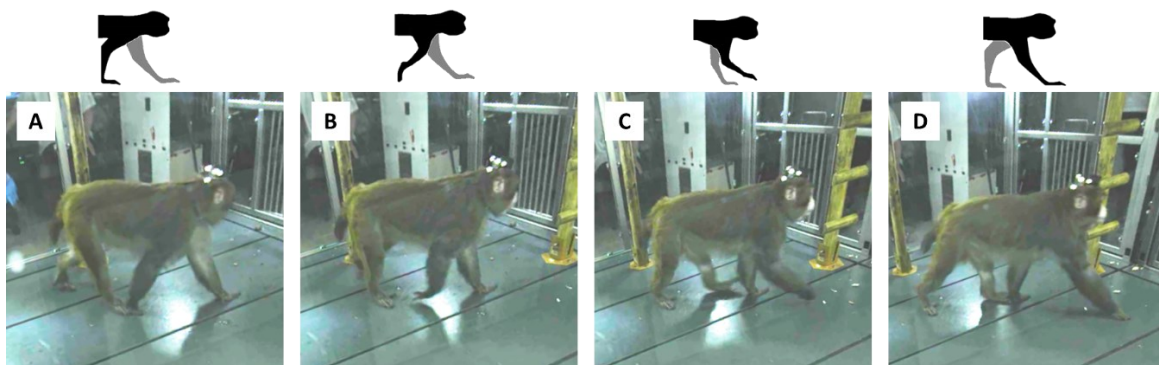


Figure 13 – The illustration shows a sequence of four frames capturing a single step taken with the right forelimb on the floor. The sequence begins from a quadrupedal position after the left limb move forward (A), follows the lifting of the right limb (B), its extension (C), and concludes with its contact with the ground (D). In the ethogram, this step is marked as a point event, specifically corresponding to image D, which represents the initial contact moment.

Table 1 – Steps Classification. The ethogram presents each behavior as a point event, accompanied by its operational description for scoring. Letters from B to D will be assigned to A to indicate specific aspects for each behavior.

STEPS CLASSIFICATION	
Behavior	Operational description
A) Step Category	
<i>First step hand</i>	The first step of a walk, the monkey places the first forelimb on the ground to start walk after performing another behavior.
	Point event: first frame in which the hand touches the floor.
<i>Central step hand</i>	The central steps of a walk, between the first and the last one.
	Point event: first frame in which the hand touches the floor.
<i>Final step hand</i>	The last step of a walk, followed by another behavior.
	Point event: first frame in which the hand touches the floor.
B) Initial Posture Category	
<i>Standing</i>	The first step of the walk is executed while standing (body weight on four limbs - or three).
<i>Sitting</i>	The first step of the walk is executed while the monkey is sitting (biceps femoris in contact with gastrocnemius), one forelimb extends and places itself to rise, supporting the body weight.
C) Hand Category	
<i>Right</i>	The step is executed with the right hand.
<i>Left</i>	The step is executed with the left hand.
D) Surface Category	
<i>Floor</i>	Monkey walks on the floor.
<i>Structure</i>	Monkey walks on the wooden structure or in the metal bar of the walls of NER.

In addition to the steps, other behaviors exhibited by the monkeys during the experiment were also recorded for use as controls. Similar to the classification of steps, detailed annotations were made for *Other Behaviors* as well: firstly, the behavior was tracked as a point event (**Table 2-A**); then, each behavior is assigned a *Posture* (**2-B**), *Hand* used (if applicable) (**2-C**), and the *Surface* (**2-D**) on which it was executed.

Table 2 – Other Behaviors Classification. The ethogram presents each State behavior as a point event, accompanied by its operational description for scoring. Letters from B to D will be assigned to A to indicate specific aspects for each behavior.

OTHER BEHAVIORS CLASSIFICATION	
Behavior	Operational description
A) State Category	
<i>Climb</i>	Monkey climbs up and down the wooden structure.
	Point event: first frame in which the hand touches the pole to climb/descend the wooden structure of the NER.
<i>Grasp food ground</i>	Monkey picks up food from the ground.
	Point event: first frame in which the hand reaches the food.
<i>Grasp food on air</i>	Monkey grabs airborne food hanging on a hook.
	Point event: first frame in which the hand reaches the food.
<i>Reward</i>	Monkey takes a liquid or solid reward directly into its mouth, given by the experimenter using a syringe or a stick through one of the holes in the NER.
	Point event: first frame in which the mouth reaches the syringe or food on stick.
<i>Rest</i>	Monkey sits or remains stationary in quadrupedal position and does not perform any other behaviors for at least 2 seconds.
	Point event: first frame in which the monkey stops for more than 2 seconds.
B) Posture Category	
<i>Standing</i>	The behavior is executed while standing (body weight on four limbs - or three).
<i>Sitting</i>	The behavior is executed while sitting (biceps femoris in contact with gastrocnemius).
C) Hand Category	
<i>Right</i>	The behavior is executed with the right hand.
<i>Left</i>	The behavior is executed with the left hand.
<i>Bimanual</i>	The behavior is executed with both forelimbs.
D) Surface Category	
<i>Floor</i>	The behavior is executed on the floor.
<i>Structure</i>	The behavior is executed on the wooden structure or in the metal bar of the walls of NER.

3.4.3. Behavioral Scoring

Before behavior scoring, all videos were edited in brightness, rotation and zoom using Shotcut, a free, open-source video editor software. Offline behavioral scoring was conducted using BORIS (Behavioural Observation Research Interactive Software (Friard & Gamba, 2016), a free, open-source event-logging software that allows to reproduce multiple recordings simultaneously also in frame-by-frame modality. Given the 50Hz set frame rate of the cameras, the maximum resolution for behavioral scoring was 20 milliseconds. The final output of BORIS provided the list of scored behaviors (the complete list is presented in **Table 1 and 2**) in order of appearance, each one with the precise timestamp at which they occurred.

3.5. Neural Data Acquisition and Analysis

3.5.1. Neural Activity Recordings

The neural recordings were conducted with 32-channel FMAs, with alternated electrodes of 4 and 2.5 mm, implanted in the MC of the left hemisphere. Each FMA was connected through an Omnetics connector to the recording system, a wireless 128 channel neural data logger (<http://deuterontech.com/>) synchronized along the whole session to the rest of the recording devices. The logger used a radio signal to communicate with the transceiver, updating the internal clock and allowing the synchronization of the neural recording with the video acquisition using a unique 50Hz digital signal generated by a LabView based software and transmitted via a BNC cable. For each channel, the neural signal was grounded and referenced using dedicated low impedance electrodes in each FMA and recorded at a conversion rate of 32 kHz with a bandpass filter ranged 2-7000Hz, thereby being able to sample single and multi-unit activity and Local Field Potentials. Neural signals were

amplified, digitized, and stored in a MicroSD memory card (64 GB) to prevent any possible transmission error. The device was powered by a small external battery connected with a short cable. After linking the logger device to the electrode arrays into the chamber, all the components were sealed within a cover screwed on top of the chamber (see Figure 14). In addition, the logger had a magnetic on-off switch, so that it could be switched on and off also when the device was sealed into the protective chamber, with no need to physically touch the animal or remove any component.



Figure 14 – On the left the recording chamber divided into its components: on the top the open recording chamber; below, the battery connected to the recording system (RatLog-128 by Deuteron Technologies). On the right, the chamber closed with its cover sewn around the components during recording.

3.5.2. Single Units' Extraction

All formal signal analyses were conducted offline on the whole dataset collected in the session. Spike sorting was performed with MountainSort, an open-access software with a fully automated spike sorting algorithm (Chung et al., 2017), setting a 3 standard deviation threshold of signal-to-noise ratio for each channel as a parameter for single units detection. Classification of units into single-units

or multi-units used a noise overlap threshold of 0.15. The noise overlap parameter varies between 0 and 1, and only units with values below the threshold were considered as single units, while all the remaining waveforms with higher noise overlap formed the multi-units' signal. In addition, visual inspection of the Inner Spike Interval distribution and the waveform shape was conducted to further verify the isolation of single units. Lastly, possible artefacts were removed by visual inspection and all the remaining waveforms that could not be classified as single units were included in the multi-unit's activity.

3.5.3. Walking-Related Neurons Analysis

After units' classification and behavioral scoring, we studied the firing of the recorded units in relation to steps. To explore the single neurons modulation during a step, we defined a temporal interval of 1.4 s, ranging from -0.7 s to +0.7 s around the step. This interval was determined based on the average time difference between steps derived from behavioral analysis (see paragraph 3.4.2.) to specifically capture neural activity corresponding to each step, excluding the influence of preceding and succeeding steps/behaviors. The 1.4 second activity was segmented into non-overlapping 200 ms windows. A one-way repeated measures ANOVA was employed to evaluate whether the temporal epochs (200 ms) exhibited significant differences ($\alpha < 0.001$), thereby revealing a modulation of single neurons in response to the step.

3.5.4. Surface Modulation Analysis

Once walking-related neurons were identified, we assessed which and how many single units were significantly modulated in terms of intensity and dynamics during steps performed on the wooden structure or on the floor. We employed a

sliding T-test for intensity differences and a correlation test for dynamic differences. For the sliding T-test, we chose a window from -0.8 s to +0.6 s, with sliding time windows of 200 ms jumping by 0.02 s (to create a 200 ms window with a central point at -0.7 s and +0.7 s). The significance criterion required at least 5 consecutive time windows with a p-value < 0.05. Regarding dynamic differences, the Pearson correlation coefficient was utilized to evaluate the linear relationship between a neuron's firing rates during different step phases. The analysis utilized a time window of -0.7 to +0.7 seconds, with a temporal resolution of 0.1 seconds, and significance was determined based on a p-value < 0.05.

3.5.5. Sequence-Dependent Activity Analysis

To address if the discharge of walking-related neurons remained constant across all steps of a walk or varied, we extracted walking sequences from our dataset, each comprising a right step for every category in our ethogram: a *First step Right*, a *Central step Right*, and a *Final step Right* (refer to **Table 1** in Section 3.4.2) - essentially, walks composed of 5 steps, initiated with the right hand. This analysis was conducted exclusively for Mk2, as the numbers did not allow for Mk1 (refer to Figure 15). Subsequently, we examined the dynamics of neural responses during each right step of these walks through visual inspection.

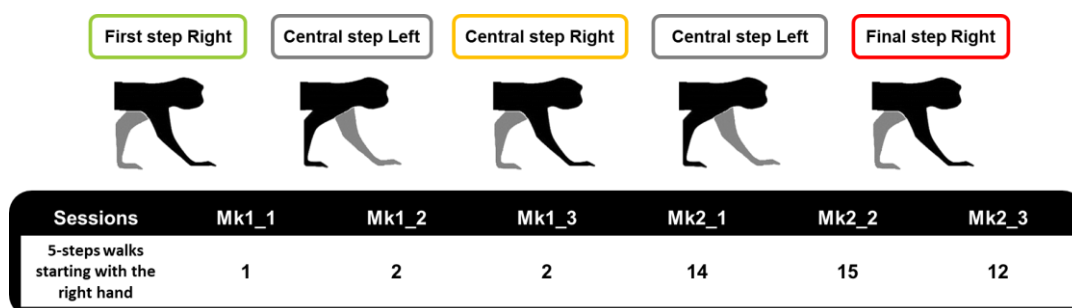


Figure 15 – Frequency of walks consisting of 5 steps starting with the right hand across the six sessions.

Afterwards, to identify potential significant differences in the mean firing rates of neurons involved in walking during the three right steps, a one-way ANOVA was applied to analyze single neurons. The single factor in the ANOVA represented the different steps. The target was to ascertain whether statistically significant differences existed among consecutive steps, as indicated by achieving significance in the ANOVA ($\alpha < 0.05$).

Subsequently, population-level analyses were conducted to explore the dynamics of neural responses within the population. Initially, the firing rate for each neuron during each step was calculated using a Gaussian filter with a standard deviation of 60 ms for smoothing. Following this, these activities were averaged within each step category to generate distinct curves for each neuron. A preliminary Principal Component Analysis (PCA) was then carried out on the data, focusing on the first 15 principal components (PCs). This analysis unveiled a common sequence-dependent) and a distinct sequence-independent) component that explain the neural dynamics of the steps. To quantify sequence-independent neural activity, another PCA was conducted on the data matrix [N bins (time) x N neurons walking-related] focusing on the first 15 PCs. This enabled the extraction of the initial two principal components (PCs), capturing over 80% of the variance. These components form what we refer to as the "Order-Independent plane," representing the "invariant" plane of neural activity for the steps. In this plane, activity remains consistent regardless of the step order. Following this, to quantify sequence-dependent neural activity, the temporal average around the peak activity of each neuron (200 ms) during the three steps was calculated. This resulted in a matrix [N (mean firing rate x order of steps) x N neurons walking-related]. PCA was once again applied to this matrix focusing on the first 15 PCs, extracting the initial two components that explained 80% of the variance. This constituted our "Order-

Dependent plane," maintaining separation between steps in the neural dynamics. Finally, the axes of the two planes were orthogonalized to create an orthogonal set of neural dimensions describing sequence-dependent and sequence-independent activity.

The same analyses were conducted also for the 7-step right walks of Mk2. However, due to limited numerical data, investigation at the level of single neurons was not possible, so population-level PCA analyses were conducted directly.

3.5.6. Walking-Reaching Analysis

To investigate the hypothesis that neuronal activity during walking may share similarities with reaching behavior, we conducted a series of analyses focused on neuronal responses during specific behaviors: step and grasp. The grasp behavior was chosen for comparison because, similarly to the step, it involves a reaching component before contact with the object.

Primarily, we examined the number of neurons walking-related that exhibited significant responses to right grasp: *Grasp food ground* and *Grasp food on air* (see **Table 2** in Section 3.4.2). Similar to the analysis of walking-related neurons, the assessment of significance in response to grasp was aligned with the moment of contact with food, using a one-way repeated measures ANOVA in a temporal interval of 2 s. This allowed us to explore any significant differences among 200-ms temporal epochs, with a significance level set at $\alpha < 0.001$.

Subsequently, we extended our investigation through population-level analyses, examining responses from both single and multi-units. The first phase involved calculating the firing rate of walking-related neurons during the right step, whose normalization and smoothing with a Gaussian window, yielded a matrix representing neuronal activity during the step. Similarly, neuronal activity

during the right grasp was analyzed and represented in a matrix. Next, an alignment of neuronal activities during the step and grasp was performed, followed by the computation of the Alignment Index (AI) to quantify the similarity between these activities. The AI is calculated using PCA performed on matrices of neuronal activity during the step and grasp. This index represents the temporal similarity between neural activities by measuring the fraction of common variance explained by the first 10 Principal Components (PCs). A higher AI indicates a greater temporal similarity between neuronal responses during the step and grasp. The matrices of neuronal activity were visualized through heatmaps, organizing neurons based on the time they reached peak activity during the step. Additionally, we explored the fraction of neurons exhibiting peak activity within the same 400 ms temporal window during the right step and right grasp. Finally, an analogous procedure was conducted for control behaviors, comparing the right step with "Reward" and "Rest" behaviors, and consistently calculating the AI.

3.5.7. Intra-Cortical Microstimulation (ICMS)

After the conclusion of the recording sessions, experiments involving Intracortical Microstimulation (ICMS) were conducted on the monkeys' implants. Monopolar, biphasic trains of cathodic square wave pulses were administered using a constant current stimulator (PlexStim, Plexon) with specific parameters: a total train duration of 500 ms, a single pulse width of 0.2 ms, and a pulse frequency of 200 Hz. Current intensity, either 100 or 150 μA , was monitored on an oscilloscope by measuring the voltage drop across a 10-k Ω resistor in series with the stimulating electrode. During these procedures, ICMS was administered at each site while the monkey was in a calm and relaxed state, situated in a primate chair without any head fixation. Induced movements were detected based on the consensus of two

experimenters observing the animal during pulse delivery. The stimulation protocol began with a current intensity of 100 μA for 500 ms, followed by an increase to 150 μA for another 500 ms. If each stimulation consistently elicited the same movement (typically three out of three consecutive stimulations with the same parameters), the site was considered associated with a specific effector. Upon completion of the stimulations, two maps corresponding to current intensity were generated, associating each site with the single or multiple effector movements evoked. To compare the functional properties of walking-related neurons and results obtained through ICMS, the maps corresponding to a current intensity of 150 μA were utilized. A χ^2 -test was applied to investigate the significant distribution of walking-related and walking-unrelated neurons among excitable and non-excitable sites. Furthermore, through the same test, the presence of a significant association between the channels of the FMAs where walking-related neurons were identified and those where motor responses of specific effectors were evoked via ICMS was evaluated.

4. Results

4.1. Behavioral Data

Spontaneous walking in the NER was observed across three sessions for each monkey, resulting in an overall dataset of six sessions (each typically lasting ~30-40 minutes). The monkeys performed 3181 steps, evenly distributed across the left ($n = 1593$) and right ($n = 1588$) forelimb, resulting in 382 walks. Despite the spontaneous nature of the walks, the monkeys balanced the choice of the initiating forelimb, with 193 and 189 walks that began with the left and right forelimb, respectively, suggesting the absence of any lateralization in initiating walking. Regarding the number of steps per walk, individual walks were relatively short, the majority lasted 13 steps or less (333 out of 382) and the most frequent duration was 4 steps (68), while longer walks (over 20 steps) were rare, totaling 26 instances (Figure 16).



Figure 16 – Distribution of walks and steps in the complete dataset. In the histogram, walks are categorized by frequency based on the number of steps and the initiating hand, while the pie chart shows the prevalence of right and left for both walks and steps.

Besides walking, monkeys exhibited also a wide range of *Other behaviors* (Figure 17-A). Grasping, comprehensive of *Grasp Food Ground* ($n = 193$) and *Grasp Food on Air* ($n = 110$), occurred frequently, constituting 46% of the total 658 recorded behaviors. They also often received a *Reward* ($n = 172$, 26%), or manage to *Climb* the wooden structure ($n = 98$, 15%), or simply remained at *Rest* ($n = 85$, 13%). However, the execution of these behaviors was linked to walking, e.g., the monkey often walked with the aim of grasping a food morsel or getting a reward. The final state of each walk (Figure 17-B) — specifically, behaviors occurring within 2 seconds after the last step — usually included *Climb* (15%), *Grasp Food Ground* (15%), *Grasp Food on Air* (21%), *Reward* (23%), and *Rest* (22%) and only 4% of the final states encompassed other rare behaviors, that could not be categorized within our ethogram. Finally, even the initial position could vary, with some walks starting while the monkey was standing (64%) and other while it was sitting (38%).

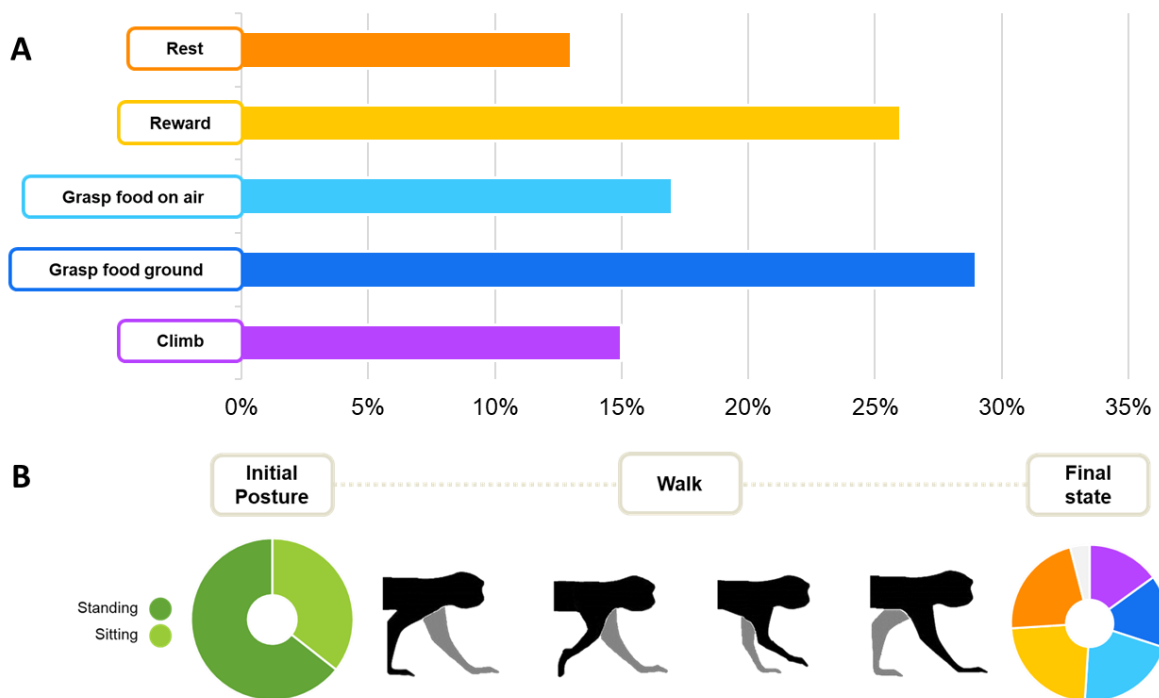


Figure 17 – Distribution of Other Behaviors across the entire dataset (A) and characteristics of the walks dataset (B), including posture (sitting or standing) and frequency of final state behaviors following walks.

4.2. Identification of Walking-Related Neurons

During the study, 255 single units were recorded in parallel to the behavior from the arrays implanted in the MC of the two monkeys (Figure 18). Out of them, the 41% ($n = 104$) showed significant response modulation during the execution of steps (one-way repeated measure ANOVA, $\alpha = 0.001$, see **Materials and Methods**).

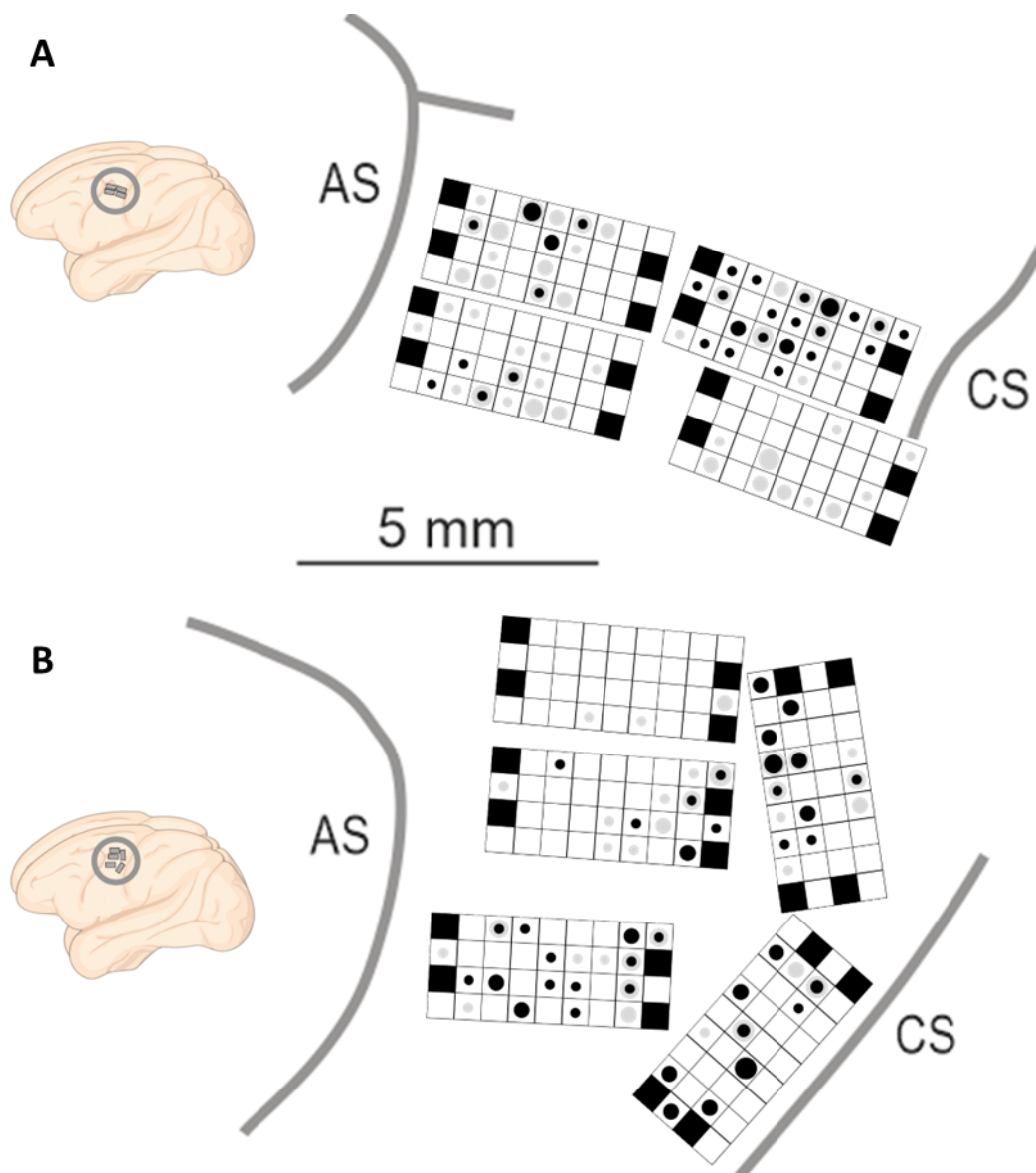


Figure 18 – Recorded neurons in the arrays implanted in the MC of the two monkeys: Mk1 (A) and Mk2 (B). Black dots represent channels that showed a relation to walking, while grey dots represent channels that are not related to walking. The size of the dot corresponds to the percentage of recorded neurons for each channel. Non-recording points are denoted by black squares.

Walking-related neurons displayed distinct firing patterns during specific phases of the step cycle (Figure 19). For instance, Neuron 1 (Figure 19-A) responded during the reaching phase as the forelimb approached the ground, whereas Neuron 2 (Figure 19-B) exhibited a ramping discharge leading up to the touch, subsequently interrupted. In contrast, Neuron 3 (Figure 19-C) specifically responded during the touch phase and Neuron 4 (Figure 19-D) displayed a post-touch response.

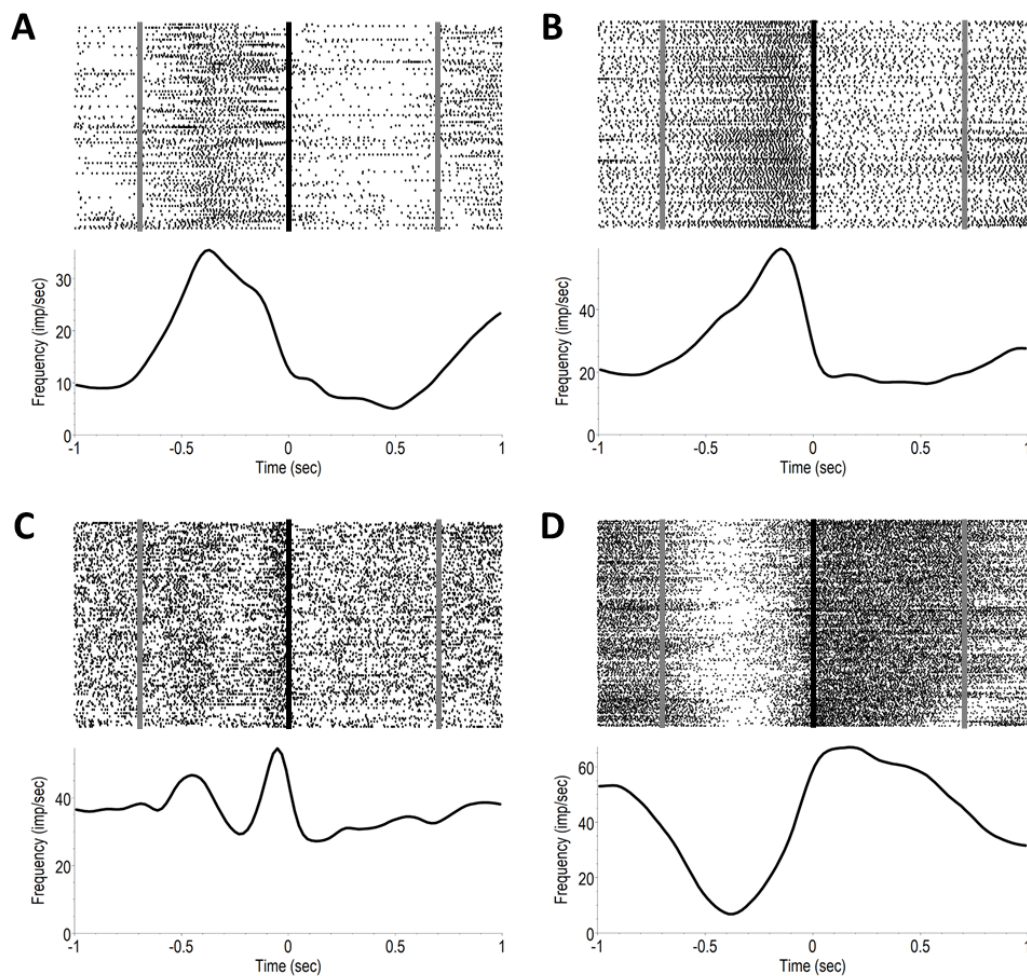


Figure 19 – Four examples of walking-related neurons respond in different phases of the step. A vertical line at 0 marks the right hand's ground contact, with lines at -0.7 and 0.7 indicating the approximate timing of left hand's ground contact.

Given the variability in single neuron activity, we aim to explore the relationship between the peak firing rate and the touch of the contralateral hand (specifically, the right hand for both monkeys) with the ground (Figure 20). The results indicate that single neuron variability aligns with diverse phases of the step cycle, consistent with previous findings (Foster et al., 2014). Afterward, for the subsequent analyses, we considered the neural activity aligned to the touch of the contralateral hand.

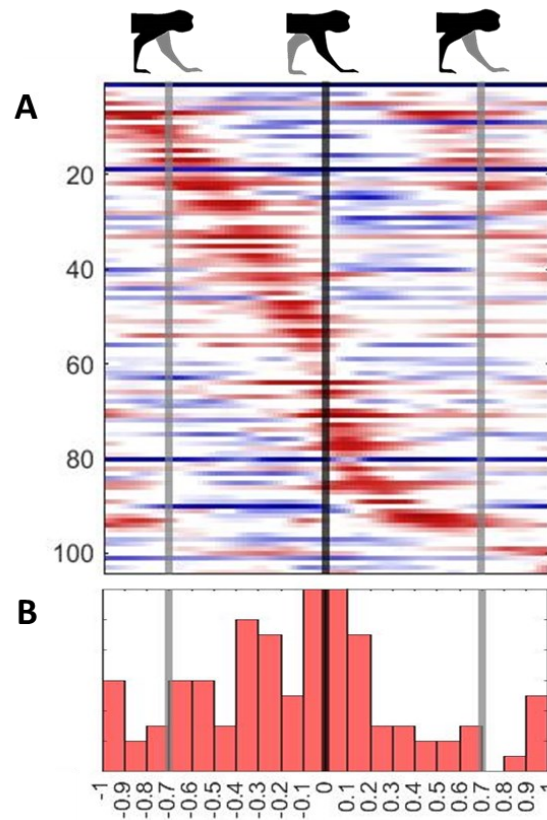


Figure 20 – Analysis of walking-related neuron responses aligned to the right step. Panel A presents heatmaps illustrating the soft-normalized firing rates of neurons. The color scale ranges from red (excited) to white (baseline) to blue (inhibited), reflecting the normalized firing rates. Panel B displays histograms of the positions of peak firing rates aligned to right steps. A vertical line at 0 marks the right hand's ground contact, with lines at -0.7 and 0.7 indicating the approximate timing of left hand's ground contact.

4.3. ICMS Data

To explore the causal role of single neuron responses to walking, at the conclusion of the experimental sessions we conducted Intra-Cortical Microstimulation (ICMS) under free-head conditions. The stimulations (see **Materials and Methods**) revealed that out of the total 97 channels where single units were recorded, 59 were electrically excitable, 27 of which were associated with walking-related responses while the remaining 32 were associated with movement of other body parts (Figure 21), with no significant difference in the incidence of excitability between walking-related and non-related sites (X^2 test, $p = 0.156$).

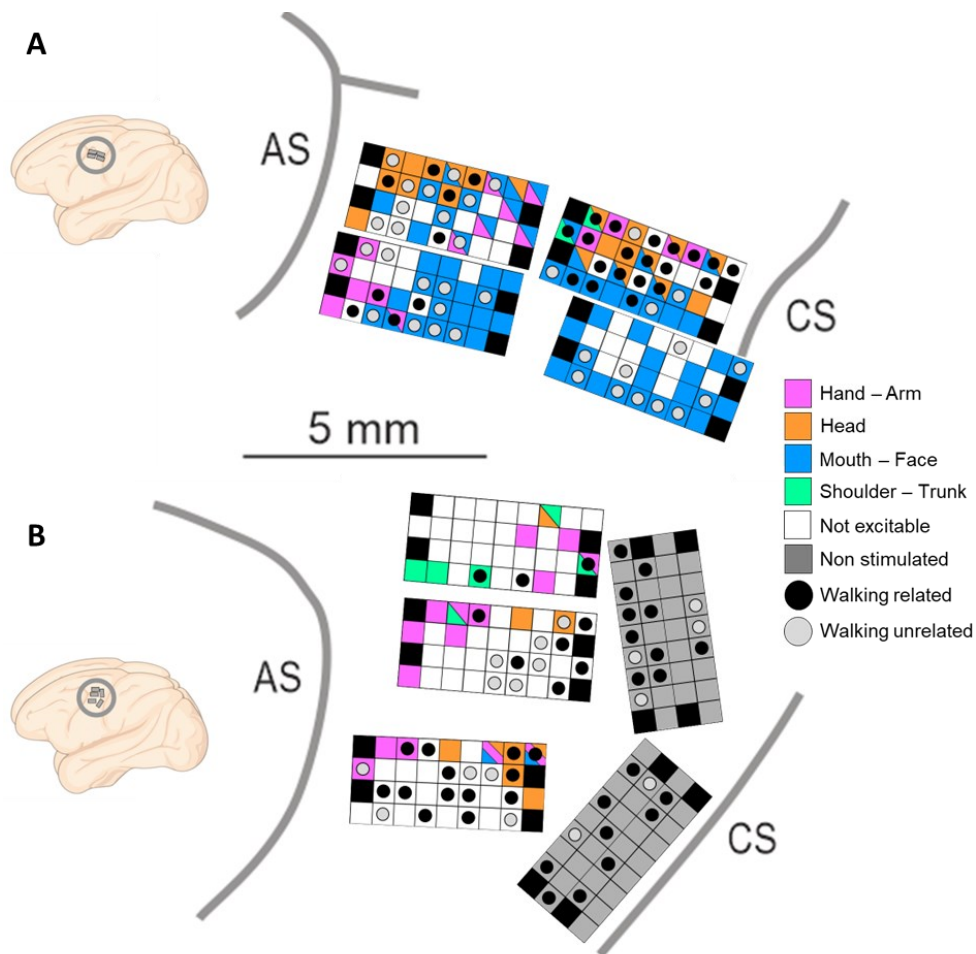


Figure 21 – Representation of ICMS results: Mk1 (A) and Mk2 (B). The channels were color-coded according to the legend in the figure to indicate the movements they evoked when stimulated. Black dots represent channels associated with walking, while grey dots represent channels not related to walking. Non-recording points are denoted by black squares.

Then, we asked whether the type of movement evoked through ICMS at the level of cortical sites had some association with the presence of neuronal responses to walking. We found that the sites whose stimulation caused Head (Table 3, $p = 0.0069$), or Shoulder/Trunk ($p = 0.0206$) movements were associated with more frequent neuronal responses to walking, whereas sites with ICMS-evoked responses to mouth/face were less frequently associated with walking neuronal activity ($p = 0.0014$). These findings suggest a potential link between the observed neural responses to walking and postural/proximal motor components.

Table 3 – Walking-related and unrelated neurons across excitable channels, categorized by the various evoked effectors.

	Hand-Arm	Not Hand-Arm	Head	Not Head	Mouth-Face	Not Mouth-Face	Shoulder-Trunk	Not Shoulder-Trunk
Walking-related channels	10	17	13	14	10	17	4	13
Walking-unrelated channels	5	27	5	27	25	7	0	32
Chi-Square Test	0.0599		0.0069		0.0014		0.0206 (Yates-corrected)	

In addition, ICMS served to validate the presumed placement of two Mk2 arrays, originally believed to be situated in M1. When initiating stimulation on channels closer to the premotor area within these arrays, we observed widespread responses elicited even at very low current intensities, down to using trains with parameters of 25 μA , 50 ms, which we identified as the threshold level. This observation supports the attribution of these two arrays to the territory of M1 and these sites were therefore not stimulated.

4.4. Neuronal Modulations Related to Walking Surface Characteristics

Since the monkey could move in the 3D volume of the cage by walking on the floor as well as on the wooden structure, in which walking requires a more intense use of the fingers to secure the grip rather than simply balancing the body weight on the palmar surface of the hand, we conducted an analysis to assess possible modulations of walking neurons based on the locomotion surface.

Hypothesizing increased responses for steps on the wooden structure, aligning with literature emphasizing the role of the MC in skilled locomotion (Armstrong, 1988), we initially compared response intensity to steps on the floor versus the wooden structure (sliding T-test, $\alpha < 0.05$, see **Materials and Methods**). Results indicated that 40 out of 104 walking-related neurons (38%) exhibited significantly different response in terms of discharge intensity and/or dynamic for steps on the floor versus the wooden structure (Figure 22-A). To further explore these differences, we evaluated the dynamics of the surface-modulated neurons in terms of phase variances in discharge during steps performed on the wooden structure or the floor (Pearson correlation, $\alpha < 0.05$, see **Materials and Methods**). Results indicated that among these neurons, 53% ($n = 21$) maintained the same discharge phase for steps on the floor and the wooden structure but with significant differences in firing rate (Figure 22-B), while 47% ($n = 19$) exhibited different responses in terms of both firing rate and phase (Figure 22-C).

In summary, our findings show diverse responses in walking-related neurons based on the surface. Contrary to expectations, there's no preference for challenging surfaces and 62% of neurons ($n = 64$) exhibit no surface-modulation, suggesting a broader role of the MC in temporal or postural aspects of locomotion, even in simple walks not requiring specific balance demands.

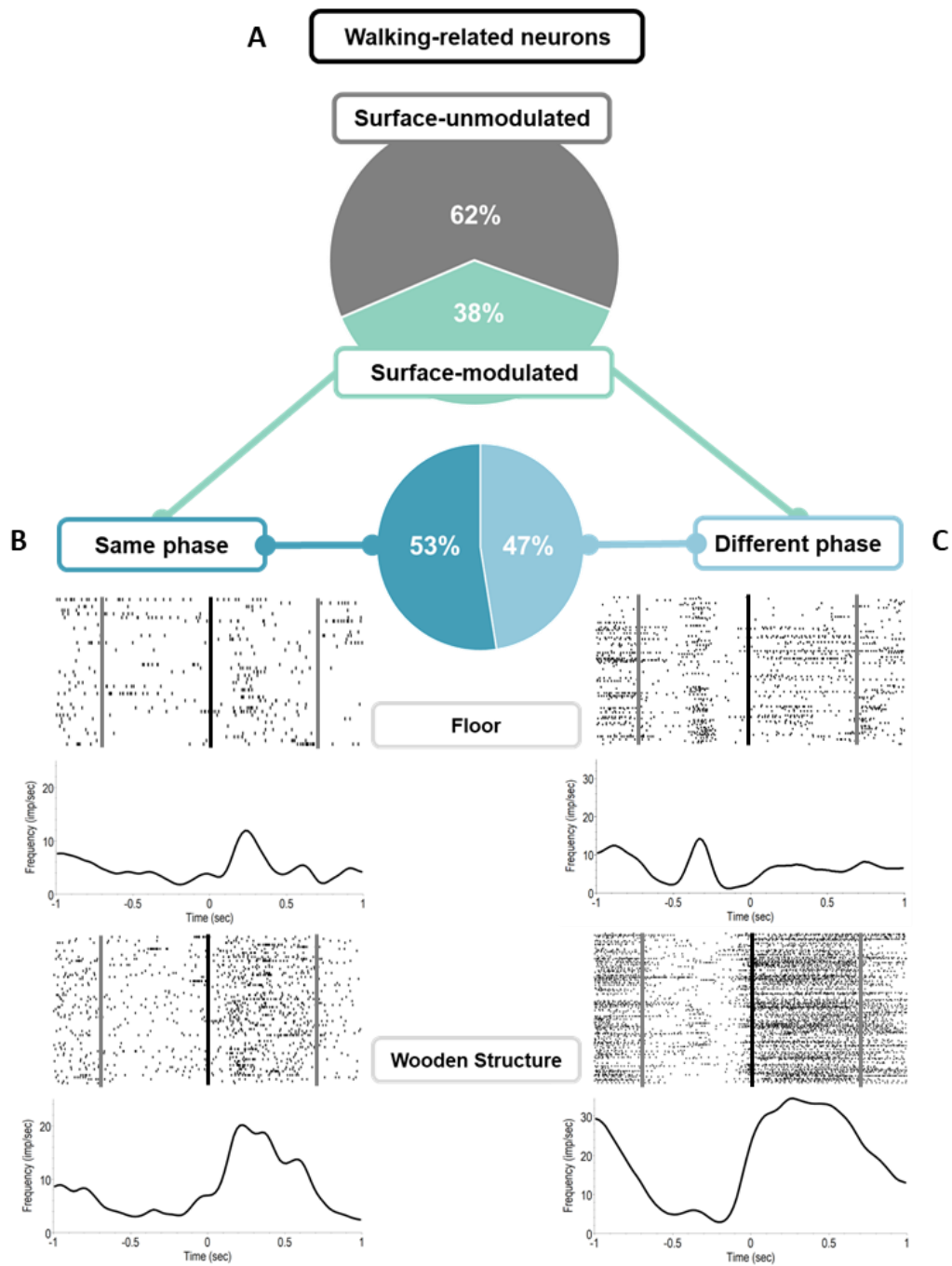


Figure 22 – Surface-modulated walking-related neurons. Neuron 5 (B) exhibits a modulation in firing rate based on the surface while maintaining the same discharge phase. In contrast, Neuron 6 (C) demonstrates a response in a different phase and intensity. A vertical line at 0 marks the right hand's ground contact, with lines at -0.7 and 0.7 indicating the approximate timing of left hand's ground contact.

4.5. Sequence-Dependent Walking-Related Activity

Literature suggested also that the MC may play a prominent role in the initiation of movement (Armstrong, 1986; Armstrong & Drew, 1984). To investigate if the discharge rates of walking-related neurons remained constant across all steps of a walk, we evaluated neural responses to 3 consecutive steps performed with the right hand (extracting walks composed of 5 steps that start with the right hand, see **Materials and Methods**). This allowed us to create a pool of walks, each comprising a right step for every category in our ethogram: a *First step Right*, a *Central step Right*, and a *Final step Right*.

Examining the neural responses to these steps (one-way ANOVA, $\alpha < 0.05$, see **Materials and Methods**), we identified four distinct response categories among walking-related neurons (Figure 23-A). First, we found that most neurons ($n = 41$, 62%) confirmed the null hypothesis discharging in a non-significantly different way during all the steps within a walk (Figure 23-B). Second, a sizeable fraction of the walking-related neurons ($n = 10$, 15%) has a higher response to the First step compared to the others (Figure 23-C); a third group of cells ($n = 5$, 8%) exhibits a greater response to the Central step (Figure 23-D), whereas, another group ($n = 10$, 15%) shows a bigger response to the Final step (Figure 23-E).

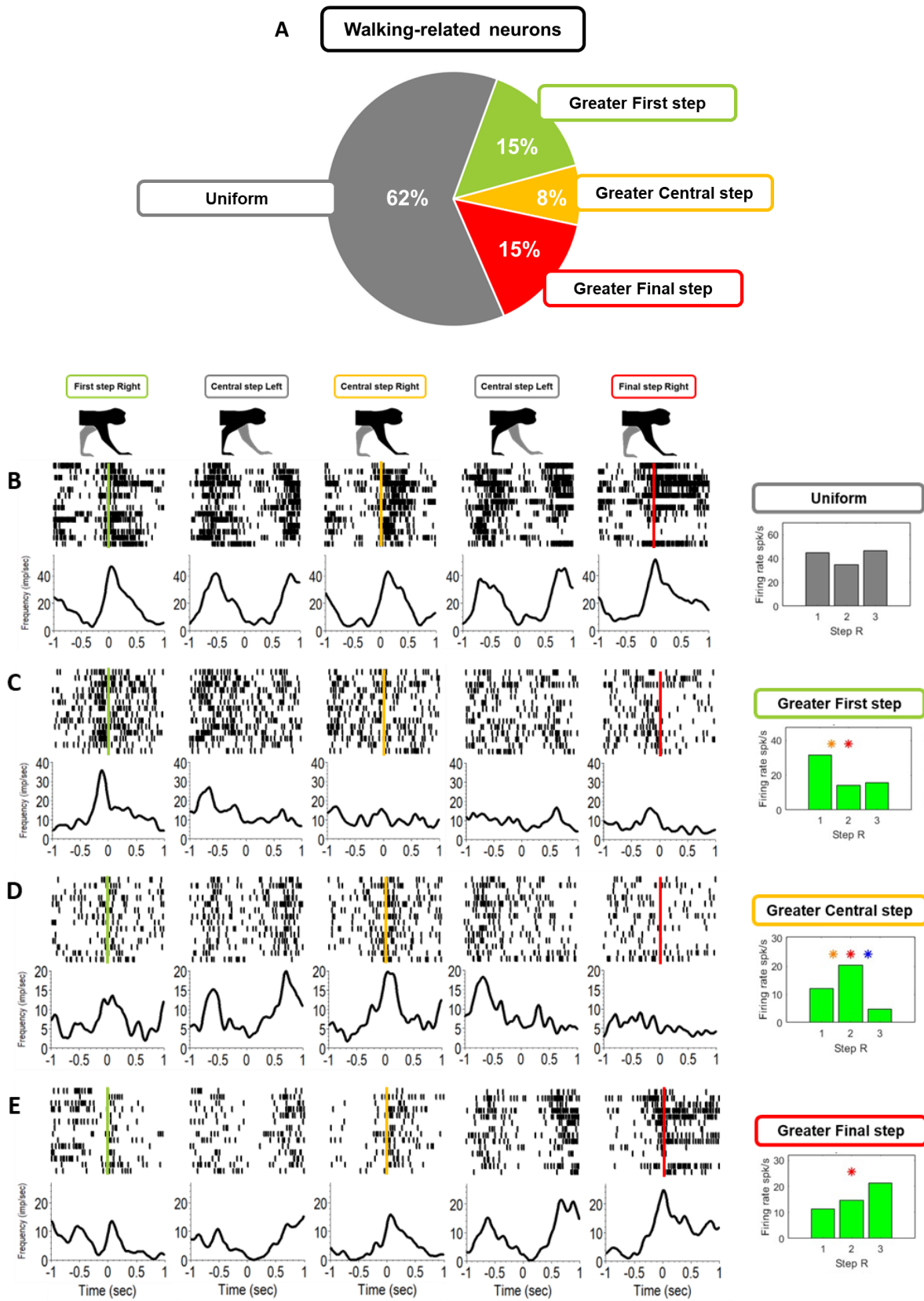


Figure 23 – Neural dynamics in a 5-steps walk. A vertical line at 0 marks the right hand's ground contact.

Collectively, this result indicates that a high percentage of neurons show no significant differences in discharge between one step and another within the same walk, coherently with the hypothesis that they encode a specific motor synergy related to a discrete walking phase. On the other hand, a still substantial percentage (38%) modulates its activity based on the order of steps.

Considering these data, we hypothesized the presence of shared and a distinct neural activity that could explain the dynamics of walking-related responses and the differences across step categories. To investigate this, we conducted population-level analyses. A preliminary analysis (PCA, see **Materials and Methods**) revealed three components that explained the neural dynamics of each right step (Figure 24). Observing these components, we noted that they exhibited a shared circular trajectory and a distinct component among the steps. This supports the hypothesis of a common sequence-independent and a distinct sequence-dependent component that explains the neural dynamics during the steps.

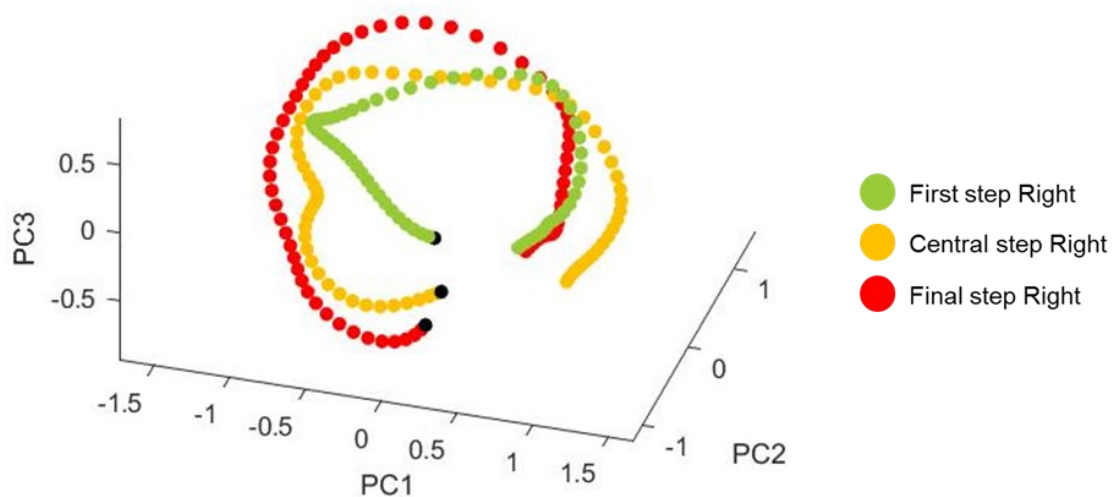


Figure 24 – Three-dimensional scatter plot representing the projection of data onto three principal components (PC1, PC2, PC3) obtained from PCA. The points on the graph correspond to different steps of a walk (First, Central, and Final), identified by distinct colors. Black points represent the starting points of each step.

To quantify sequence-independent/dependent neural activity, successive analyses were conducted (PCA, see **Materials and Methods**), allowing us to extract four principal components: *Order Independent 1*; *Order Independent 2*; *Order Dependent 1* and *Order Dependent 2*. The intersection of the two independent components forms the *Order-Independent plane*, representing the invariant neural activity plane for the steps, while the intersection of the two dependent components forms the *Order-Dependent plane*, maintaining the separation between steps. These four components collectively explain 55% of the step activity variance (Figure 25-A). The independent components explain approximately 42% of the variance, indicating a substantial common foundation among different steps. Meanwhile, the dependent components collectively account for about 12%, indicating a relevant portion of the dynamics that can be attributed to different mechanisms. As observed, despite the predominant role of the Order-Independent plane in explaining the majority of the variance, an important residual persists, seemingly attributed to variables associated with other characteristics of walking, such as the order of steps.

Projecting the dynamics of the steps onto the planes formed by their respective components (Figure 25-B), we observe that in the Order-Independent plane there is a consistent circular dynamic among the steps, suggesting that these steps share common features in their neural trajectories. On the other hand, in the Order-Dependent plane, separate dynamics are illustrated for each step. The absence of a consistent pattern in the Order Dependent plane suggests that the neural trajectories associated with each step are distinct and vary in a manner may dependent on the order of execution.

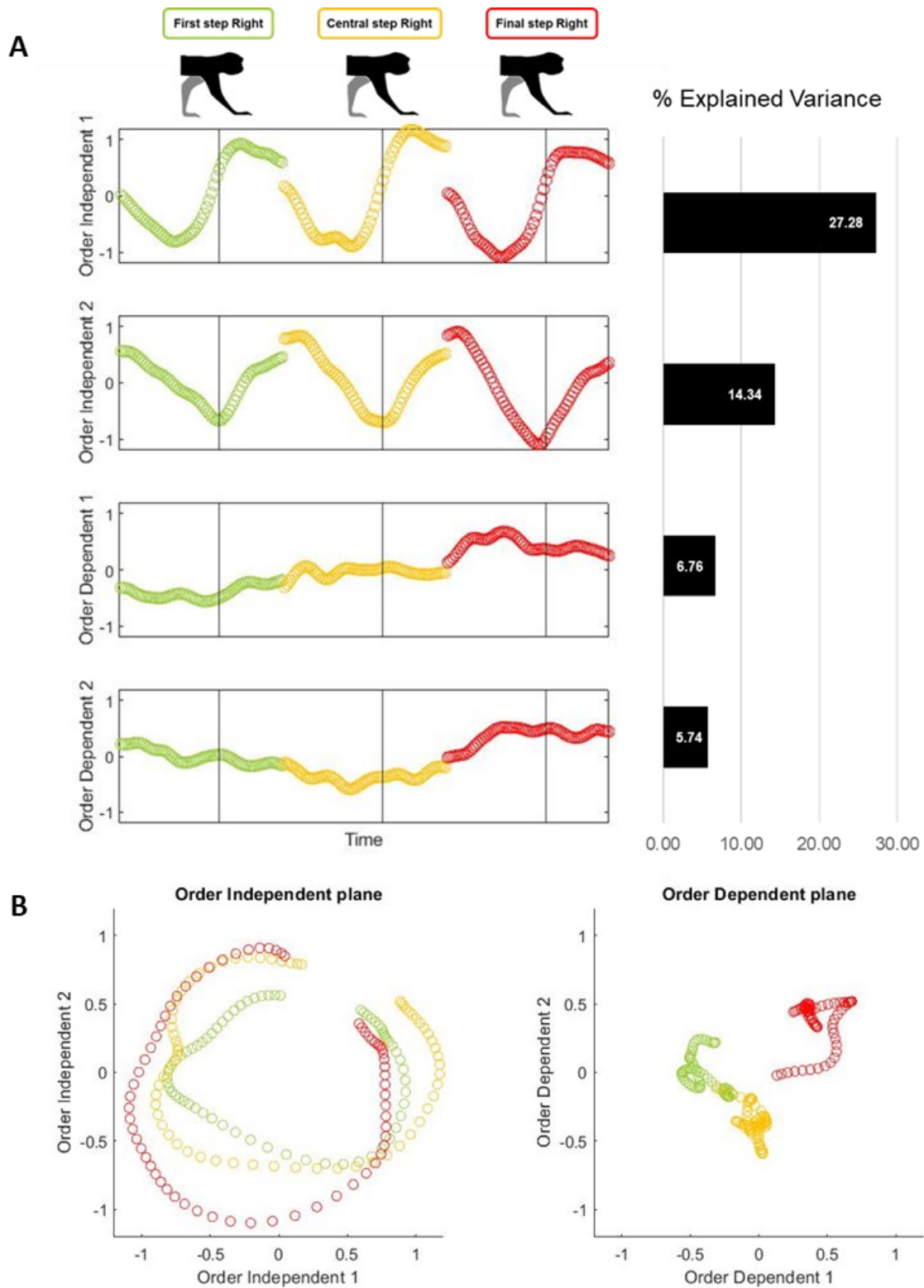


Figure 25 – Population analysis for the 5-step walks. A) Visualization of the dynamics in the four components during the three right steps of the walk, along with an overview of the percentage of explained variance attributed to each dimension. B) Projection planes of neural modes during a walk in Order-Independent and Order-Dependent plane. Each color-coded point corresponds to a specific walking step: green for First Step; yellow for Central Step and red for Final Step.

Starting from these results, we questioned whether the diverse dynamics among the steps observed in the Order-Dependent plane could be explained in terms of behavioral correlates. Examining the behavioral aspects of the considered walks (Figure 26), we observe that the First and the Central step do not coincide with other behaviors, while in approximately 30% of cases, other behaviors occur within the 600 ms window around the touch of the Final step. Regarding posture, the First step presents different postures (80% sitting and 20% standing), while the other two steps are standing. The limited consistency of these factors in explaining the observed differences between steps suggests that there is no specific connection between other behaviors and posture concerning the detected results. Instead, the issue might be associated with the temporal order or progression from the beginning to the end of the walk.

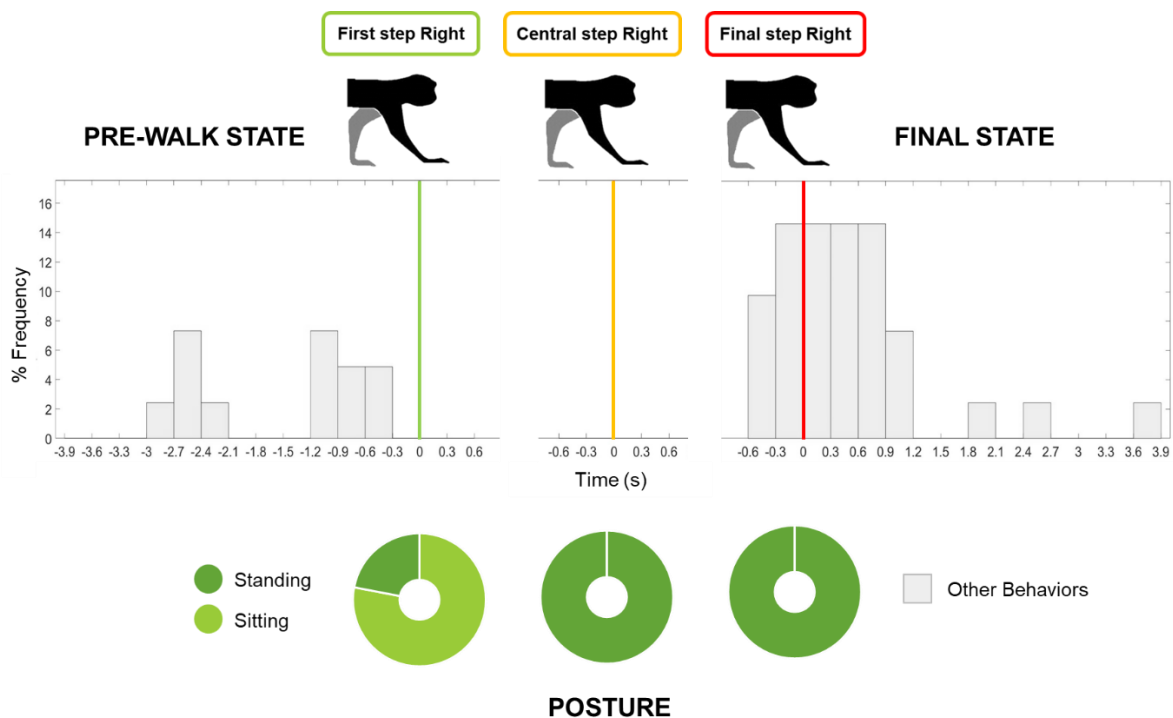


Figure 26 – Behavioral aspects analysis of the 5-step considered walks.

Then, if visible behavioral components could not explain the observed differences in the dynamics of various steps, the alternative explanation is the presence of a coding for the order/sequence of steps. To provide additional insights into the obtained results, we replicated the same population-level analyses with walks consisting of 7 steps (again for Mk2) that initiate with the right hand (Figure 27).

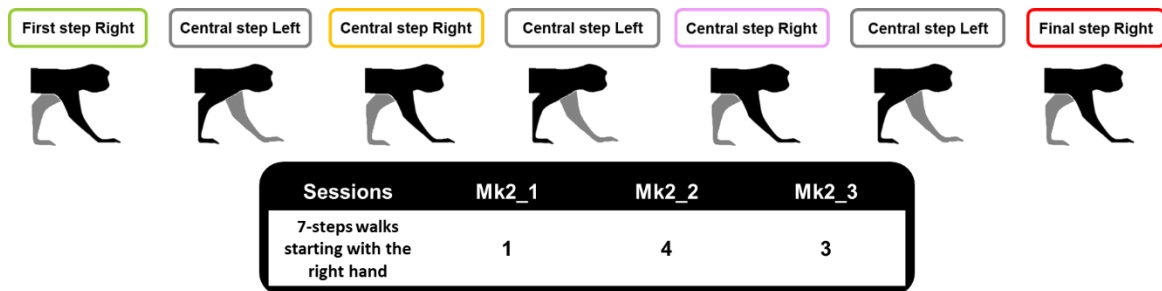


Figure 27 – The frequency of walks consisting of 7 steps starting with the right hand across the 3 sessions.

The results of the same analyses, when applied to the 7-step walks, are proportionally comparable to those conducted for the 5-step walks (Figure 26). The combination of the four components explains a total of 37% of the variance. The independent components explain approximately 26% of the variance, while the dependent components collectively account for about 11% (Figure 28-A). An interesting observation arises in the construction of the Order-Dependent plane, where we notice an overlap of the two Central right steps (Figure 28-B). This suggests that the differences in the dynamics of the steps we observed may not be attributed to a sequential issue but rather to the distinction between the First step of the walk, the intermediate (Central) steps, and the Final step. This data could support the hypothesis of the greater involvement of the MC during the voluntary transition to different phases of walking, or between walking and other behaviors (or vice versa).

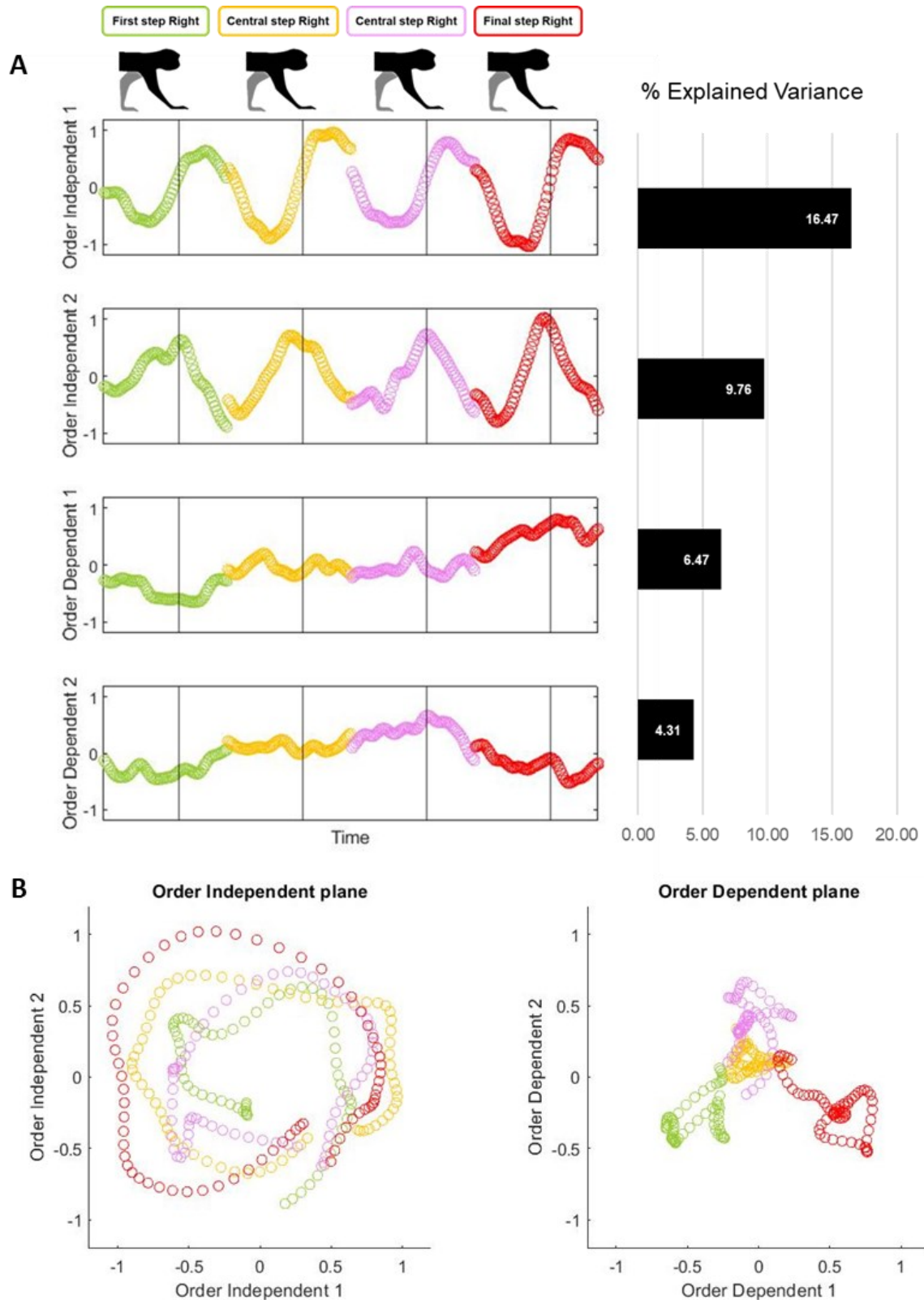


Figure 28 – Population analysis for the 7-step walks. A) Visualization of the dynamics in the four components during the four right steps of the walk, along with an overview of the percentage of explained variance attributed to each dimension. B) Projection planes of neural modes during a walk in Order-Independent and Order-Depended plane. Each color-coded point corresponds to a specific walking step: green for First Step; yellow for the first Central Step; purple for the second Central Step and red for Final Step.

4.6. Evidence for Walking-Reaching Hypothesis

To investigate the hypothesis that neuronal activity during walking may share similarities with activity during reaching behavior (Georgopoulos & Grillner, 1989), we conducted a series of analyses focused on neuronal responses during specific behaviors: step and grasp. The grasp behavior was chosen for comparison because, similarly to the step, it involves a reaching component before contact with the object.

Primarily, we examined if single walking-related units exhibited significant responses to contralateral grasp (*Grasp food ground* and *Grasp food on air*). The result (one-way ANOVA, $\alpha < 0.001$, see **Materials and Methods**) demonstrate that out of the 104 walking-related neurons, 49% (51 neurons) exhibited a significant response to at least one of two grasping behaviors performed with the right hand (Figure 29-A). The responses of walking-related neurons detected during grasp behaviors exhibited variability. For example, we identified neurons exhibiting peaks in the same touch phase during both step and grasp with different discharges (Figure 29-B); neurons activating in different phases during reaching, distinctively for the two behaviors (Figure 29-C) and neurons that activate at different moments in the touch phase (Figure 29-D). These single neurons results reveal varied discharge frequencies in MC's neurons during reaching and locomotion, with some showing higher peaks in reaching, while others exhibited similar peak activity for both tasks. These findings, consistent with a previous study (Yakovenko & Drew, 2015), emphasize that many MC neurons contribute similarly to muscle activity in both behaviors. This implies a shared neural basis and supports the idea that the same neurons are involved in regulating different motor activities.

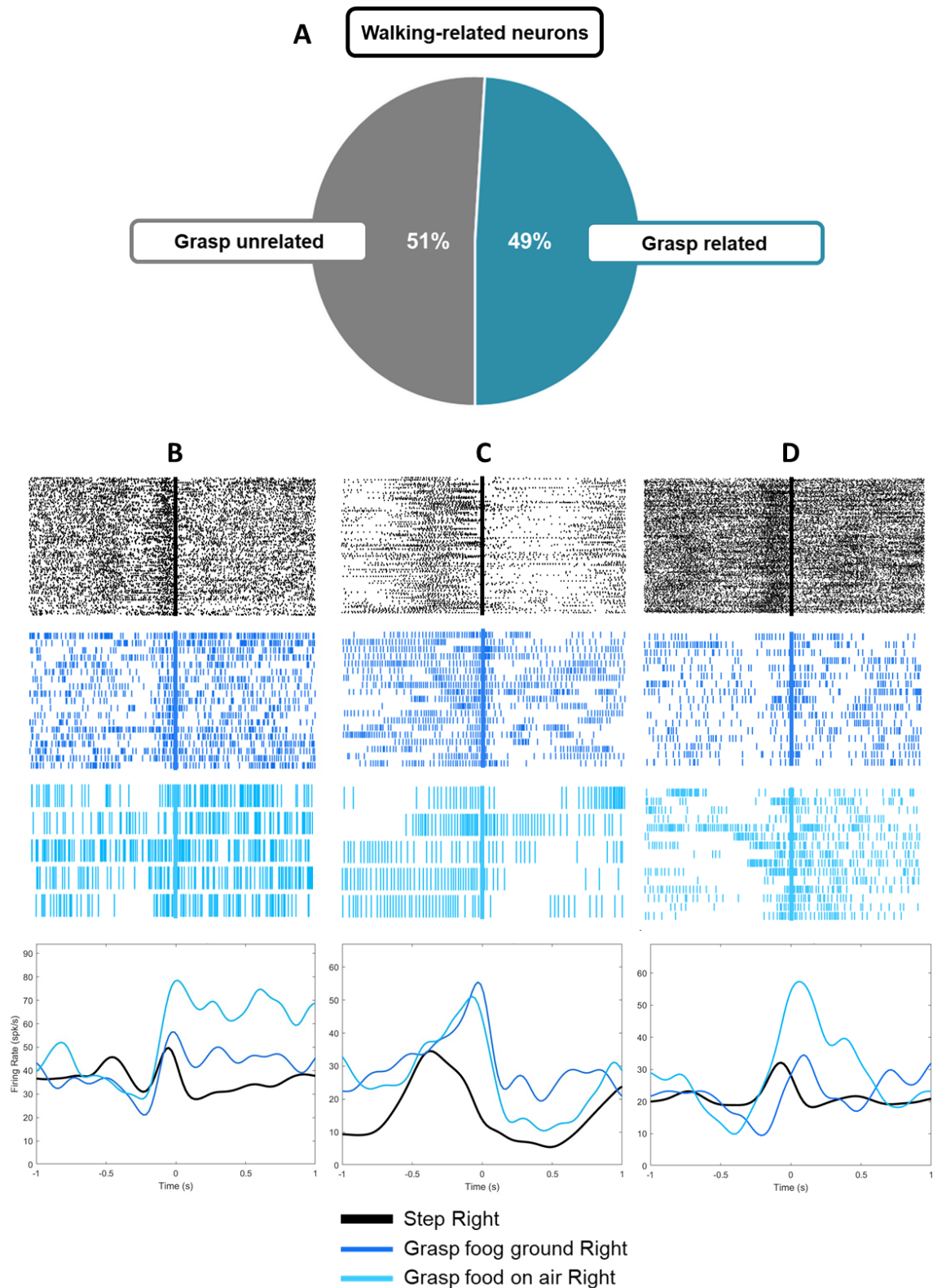


Figure 29 – Neurons related to walking also exhibit significance in grasping. A) Results of significance for walking and grasping performed with the right hand through ANOVA. B-C-D) Examples of neurons that respond similarly to both step and grasp behaviors performed with the right hand.

To enhance our comprehension at a broader level, our investigation was extended through population-level analyses. This involved examining responses from both single and multi-units. Response of walking-related neurons were computed during both the right step and right grasp, along with control events related to mouth behaviors and relaxation (respectively *Reward* and *Rest*). Subsequently, the activities were aligned to the right step (Figure 30-A), demonstrating a similar distribution for step and grasp compared to control behaviors. To assess the similarity, the Alignment Index (AI) was applied through PCA on matrices of neuronal activity (see **Materials and Methods**). This index signifies the temporal similarity between neural activities, with a higher AI indicating increased temporal similarity in neuronal responses. The analysis revealed an AI of 0.17 between neural responses during the right step and right grasp, suggesting a notable similarity between the two behaviors. Conversely, lower AIs for control behaviors, 0.05 for *Reward* and 0.04 for *Rest*, indicated reduced similarity compared to walking (Figure 30-B). These findings support the hypothesis of a more pronounced functional connection between neuronal activity during walking and reaching compared to control behaviors.

Moreover, to identify the specific phase responsible for the observed similarity between step and grasp, we examined the fraction of neurons displaying peak activity within the same 400 ms temporal window during the right step and right grasp (Figure 30-C). The analysis indicates that the majority of neurons elevate their activity peaks in the phase preceding the touch, suggesting that the similarity in responses between the two behaviors can be attributed to the reaching phase.

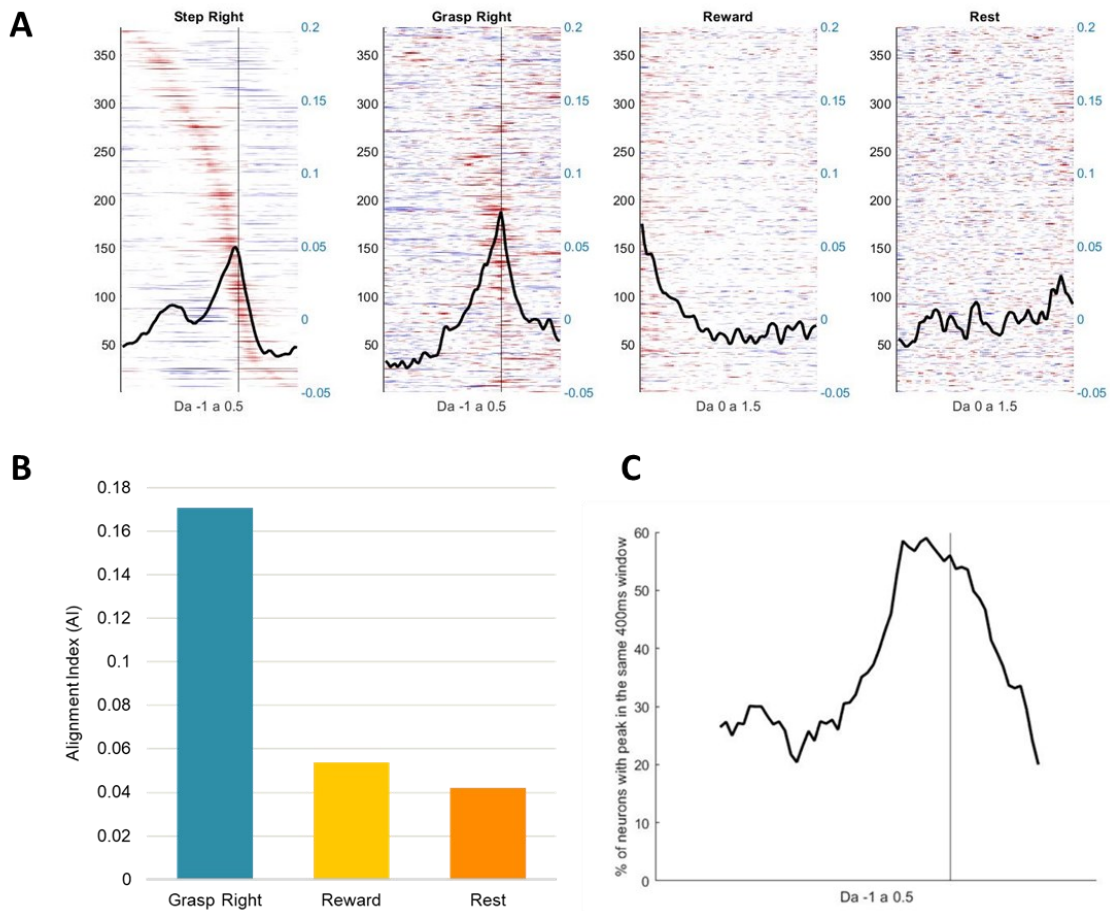


Figure 30 – A) Matrices of walking-related neurons during contralateral step, grasp and control behaviors (reward and rest), ordered based on the time they reach peak activity during contralateral step. The color scale ranges from red (excited) to white (baseline) to blue (inhibited), reflecting the normalized firing rates. The black vertical line in the "Step" heatmap indicates the hand's ground contact, while the black vertical line in the "Grasp" heatmap indicates the touch of the food with the hand. The "Step" and "Grasp" behaviors are compared in the interval from -1 to 0.5, while control behaviors "Rest" and "Reward" are examined in the interval from 0 to 1.5 to avoid overlap with the preceding step. B) Values of the Alignment Index (AI) comparing the step for grasp and control behaviors. C) Percentage of neurons exhibiting activity peaks in specific temporal windows (400 ms) during step and grasp events.

5. Discussion

Walking represents one of the most fundamental forms of movement, but a comprehensive understanding of its neural correlates remains elusive. In the context of mammalian locomotion control, the literature has emphasized the role of subcortical areas, with significant attention directed towards Central Pattern Generators (CPGs) in the spinal cord, which have been central figures in the locomotion debate for over a century (Brown, 1911; Grillner & El Manira, 2020). Until now, the contributions of the supraspinal regions to walking control have been largely understated. Despite established hypothesis regarding the Motor Cortex's (MC) role in "*skilled locomotion*" (Armstrong, 1988) or precise limb placement during obstacle avoidance and balance maintenance (Courtine et al., 2005; Drew et al., 1996; Drew et al., 2002; Georgopoulos & Grillner, 1989), clear proof is still lacking. Several studies aimed to validate these hypotheses, but they face significant limitations. First, the models predominantly rely on data from cats (Adkins et al., 1971; Armstrong & Marple-Horvat, 1996; Beloozerova & Sirota, 1993; Drew, 1988; Drew et al., 1996; Prilutsky et al., 2005), lacking evidence from translational models such as non-human primates (NHPs). Moreover, all studies use experimental settings involving treadmill use, preventing free locomotion (Fitzsimmons et al., 2009; Foster et al., 2014; Liang et al., 2023; Schwarz et al., 2014; Xing et al., 2019; Yin et al., 2014).

This study aimed to overcome the limitations of prior research by examining neural responses in the MC of NHPs during spontaneous walking on different surfaces. Our experimental 3D setup allowed for voluntary movement, gait selection, and control over walking sequence duration. This approach enables us

to provide a more comprehensive understanding of cortical mechanisms involved in natural locomotion.

Our results demonstrated the presence of walking-related neurons in the MC, specifically identified in the PMv and M1 areas. Concerning M1, the data confirm previous findings in studies on cats, supporting the hypothesis of a shared evolutionary schema in movement regulation across mammalian species (Drew et al., 2008; Drew et al., 2023; Georgopoulos & Grillner, 1989). On the other hand, our findings in the PMv yield interesting implications from various perspectives. Firstly, the PMv contribution to locomotion suggests a wider role of this area than the previously hypothesized one, essentially focused on the control of distal aspect of goal-directed object grasping (Bonini et al., 2012; Hoshi & Tanji, 2007; Maranesi et al., 2012; Murata et al., 1997). This result, combined with Foster's (2014) findings, which identified similar neurons in PMd, could open new ways for studying the circuits within these PMC areas. Furthermore, the observation that the prevalence of walking-related neurons identified in the PMv significantly correlates with sites associated with head and shoulder-trunk movements may indicate a closer connection between the control of walking and the representation of these body parts. This suggests that the PMv, classically deemed to be involved in the control of goal-directed, distal motor actions, could encode axial or proximal components of locomotor movement, such as those related to posture.

Responses of walking-related neurons exhibited variability in relation to the encoding of the step cycle, displaying preferences for different phases: before, during, or after forelimb contact (in accordance with: Foster et al., 2014). This suggest that the MC encoding involves specific muscle control in the cyclic formation of joint angles during walking, as well as the coding of posture and weight distribution.

To assess the MC's role in precise limb placement and balance maintenance in challenging terrains (Armstrong, 1988; Drew et al., 2008; Drew et al., 1996; Hermer-Vazquez et al., 2004; Lajoie & Drew, 2007), we compared neural responses during spontaneous walking on both the floor and the wooden structure. Contrary to expectations, the majority of neurons remained invariant to the surface, indicating an equal contribution of the MC in both situations. On the other hand, our data also revealed a substantial portion of walking-related neurons exhibiting surface-modulation, suggesting that the walking environment might influence neuronal activity. Nevertheless, despite the increased demands for balance control during walking on the wooden structure, suggesting increased of MC involvement, our analyses did not reveal a consistent neural preference for this surface. We observed neurons performing differently for the two surfaces, changing either in discharge and/or phase. Possible explanations for these results could include variations in sensory feedback or kinematics resulting from the interaction between the forelimb and different surfaces. Neurons exhibiting different responses may be sensitive to specific surface-related sensory inputs or show varied responses based on different muscles involved in diverse types of locomotion. Additionally, it is important to consider that a critical factor could also be the size of our experimental setup, which imposes continuous directional changes and may require ongoing contributions from the MC, even during walks on the floor, mirroring the demands observed on the wooden structure.

Another aspect our study aimed to assess is the differential activation of the MC during different steps within the same walking sequence. We began with the assumption that this area may contribute mostly to the initiation of walking, subsequently delegating the management of locomotion to subcortical structures (Armstrong, 1986; Armstrong & Drew, 1984). Indeed, we demonstrated that the

recorded walking-related neurons exhibited modulation based on what we hypothesize to be the sequence of steps in a walk. While a large percentage of neurons remained stable in discharge frequency across different steps, suggesting that the MC contributes equally to each step, our analyses revealed a considerable group of neurons demonstrating significant differences in neural discharge between consecutive steps, suggesting a differentiated involvement of the MC. These neurons were organized into three groups, exhibiting respectively a higher discharge for the first, last, or, in fewer cases, the central step(s) of the walk. Qualitative behavioral analysis of the walks did not reveal any predominant factors that could explain the dynamic variation observed in neural responses during consecutive steps. This led us to consider that the diversity of observed dynamics might depend on the order of execution of the consecutive step. In accordance with literature, the distinct dynamics observed in the first and last steps may, respectively, reflect the need to prepare the motor system for initial contact and the necessity to stabilize movement after the conclusion of walking. Preparation for contact in the first step may require rapid and precise adaptations to anchor to the supporting surface when bearing body weight, while the last step may involve strategies to stop the movement, recuperate stability and prepare to perform another behavior. Consequently, the identified modulation could indicate the monitoring of the walking phase, with a precise encoding of the initiation and termination phases, while maintaining sustained activity for all the walk process that enables the guidance of appropriate corrective movements when intervention is necessary. This hypothesis aligns with the observation suggesting that the MC monitors walking and is recruited when changes in gait patterns are required (Capogrosso et al., 2016; Foster et al., 2014; Nakajima et al., 2019).

An additional aim of our study was to contribute to the 'Walking-reaching' hypothesis (Georgopoulos & Grillner, 1989), which posits a shared neural substrate between walking and reaching. This hypothesis suggests that reaching may have evolved from walking in animals using their forelimbs for both terrestrial locomotion and reaching-grasping behavior, as observed in primates. Despite various studies exploring this topic (Miri et al., 2017; Xing et al., 2019; Yakovenko & Drew, 2015), diverse results have been obtained, and there is currently no clear consensus in the literature. In our study, we aimed to investigate the relationship between these two behaviors by comparing neural responses during walking with those during grasping, specifically assessing the similarity in reaching phases. In contrast to the study by Yakovenko (2015), where behaviors were controlled in their execution, our behaviors were studied in their natural form, without imposing stereotyped execution modes in grasp (performed from two different postures: in the air and on the floor) and in step (considered both on the wooden structure and the floor). Our results unveiled that approximately half of the walking-related neurons significantly activate during grasping, identifying the moment of maximum similarity during the reaching phase. In our findings, many cells exhibited a higher peak discharge frequency during reaching compared to locomotion, while others displayed similar peak activity for both tasks. These results align with the findings of Yakovenko (2015), which appears to be the only study in the literature that has systematically compared these two behaviors similarly to what we have done. In their study, Yakovenko and colleagues, having access to EMG data, observed that MC neurons contribute in a remarkably similar manner to muscle activity during both reaching and walking. This suggests a shared neural substrate for these behaviors, implying that the same neurons are involved in the regulation of distinct motor activities. Although we cannot infer

neuron-muscle connections as we did not record EMG data, our results seem to support this hypothesis by demonstrating different activations that could indicate a coding of patterns and phase relationships between neurons and muscular activity for different behaviors. Additionally, our data becomes even more interesting since the monkey was not instructed to avoid obstacles during walking, as in the case of Yakovenko, who demonstrated that the MC plans the sequential activation of muscle activity during reaching and walking with obstacle avoidance. Conversely, the similarities we found pertain to natural walking and grasping behaviors without the presence of challenges, making our findings potentially even more supportive of the proposal by Georgopoulos and Grillner (1989) that corticospinal control circuits for reaching movements share a substrate with those implicated in locomotion and may have evolved from them. From our overall results, we can infer that the contribution of the MC is not limited to initiating walking or skilled walks requiring balance and precise placement. Instead, the walking-neurons are recruited during all performed steps, behaving similarly to goal-directed reaching behaviors, without distinctions based on the characteristic of walking. This result confirms that primate brain regions controlling upper limb goal-directed actions are also involved in walking, suggesting shared neural mechanisms for fine motor skills and locomotion. This insight may enhance our understanding of the evolutionary and functional aspects of the primate brain, contributing to neurobiology and evolutionary biology fields and offering a basis for comparative studies among species, revealing universal principles in motor control network organization (Drew et al., 2023).

6. Conclusion and Future Directions

Our study contributes to the investigation of neural correlates during spontaneous locomotion in NHPs, providing valuable insights into the role of the MC during walking. Further analyses could be conducted to consolidate and expand upon our results. For example, a limitation of our experimental design is the frequent need to change direction, which prevents the study of long straight walks. Subsequent studies could design a setup to focus on longer walks that do not require directional changes to confirm our findings regarding the order of steps. This would involve assessing whether what we observed concerning different activations for the first and last steps holds true for longer walks as well. Moreover, an in-depth analysis of neural responses to different surfaces could help identify specific environmental conditions that modulate MC responses. For these purposes, a focused kinematic study is essential. This might involve more systematic analyses of movement patterns, trajectories, joint angles, and limb coordination during various steps. Kinematic data could also extend the investigation into the relationship between "Walking" and "Reaching." Following studies could compare these two behaviors across various dimensions, including environmental variations or adjusting speeds. Finally, for comparative perspectives, it would be interesting to construct a paradigm that allows for the study of walking in a cross-sectional manner, comparing the results with other mammals, especially cats, which have been extensively studied in locomotion. This could potentially demonstrate a shared substrate for locomotion behavior, enhancing translational knowledge for the study of motor circuits.

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