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Neuroethological investigation of the macaque's ventral premotor cortex

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Abstract (English)

Neurophysiological studies in non-human primates are essential to understand human brain functions and dysfunctions. However, highly controlled laboratory settings hinder the possibility to generalize the findings to unconstrained, close-to-natural situations and strongly limit the range of investigable behaviors. Here, we addressed these issues by leveraging ethological and neurophysiological techniques to simultaneously record behavioral and neural data in monkeys from a transparent plastic enclosure (NeuroEthoRoom, NER) with a two-step approach: first, in a head-restrained laboratory setting, and then during free behavior. We recorded ventral premotor (PMv) neurons during both conditions. Generalization analyses revealed that the neuronal functional properties measured in the head-restrained condition only partially explain the firing statistics of the same neurons during free behavior. By contrast, during the second part of the session it was possible to readout a larger variety of spontaneous behavior from PMv activity. Our findings demonstrate that a two-step approach can open up new avenues to the study of social and emotional domains in freely behaving primates.

Abstract (Italiano)

Gli studi neurofisiologici nei primati non-umani sono essenziali per capire le funzioni e le disfunzioni del cervello umano. Ciononostante le condizioni sperimentali altamente controllate possono ostacolare la possibilità di generalizzare le scoperte a situazioni più vicine all'ambiente naturale, limitando inoltre il range di possibili comportamenti da studiare. In questa tesi verranno approfondite queste problematiche andando ad utilizzare tecniche etologiche e neurofisiologiche per registrare contemporaneamente dati comportamentali e neurali in macachi all'interno di una gabbia trasparente (NeuroEthoRoom, NER) con un approccio a due fasi: prima in una condizione laboratoriale canonica, e poi durante il comportamento libero. Abbiamo registrato neuroni dalla corteccia premotoria ventrale (PMv) in entrambe le condizioni. Con le analisi di generalizzazione è stato trovato che le proprietà funzionali neuronali misurate durante la condizione laboratoriale canonica spiegano solo parzialmente l'attivazione degli stessi neuroni durante il comportamento libero. Durante la seconda parte della sessione, quindi durante il comportamento libero, è stato possibile trovare una più grande varietà di comportamenti spontanei dall'attività della PMv. I risultati dimostrano come questo approccio in due fasi possa aprire la strada allo studio del comportamento sociale ed emotivo in primati in condizioni di libertà.

1. Introduction

Classical studies in neurophysiology allowed to understand brain functioning and the relationship between neuronal activity and behaviors. Being phylogenetically close to humans, non-human primates' neural activity has been subject to a wide interest. In particular, studies on the motor system revealed empirically that the direct control of movement is a function of the so-called agranular frontal motor areas, but the extended cortical motor system encompasses a larger set of frontal as well as parietal areas, in turn connected to a variety of sensory areas (Matelli and Luppino, 2001; Rizzolatti and Matelli, 2003).

The primary motor and premotor cortex are anatomically and functionally different. Neurons of the primary motor cortex mainly encode the parameters and details of simple movements, while premotor cortex neurons have been found to be involved in higher order motor and even non-motor functions. In particular, premotor cortex neurons contribute to sensory-motor transformations of objects physical properties in the appropriate sequence of acts to attain specific goal with object-directed actions (Rizzolatti and Luppino, 2001; Rizzolatti et al., 1988a; Gentilucci et al. 1988; Rizzolatti et al., 1988b), play a role in action recognition (Gallese et al., 1996; Umiltà et al., 2001) and decision making (Pardo-Vazquez et al., 2008). The ventral portion of the premotor cortex, subdivided in areas F4 and F5, encodes goal-directed actions regardless of the specific sequence of single movements required to attain the action's goal (Umiltà et al., 2008), as well as defensive actions directed to objects intruding in the subject's peripersonal space (Graziano et al., 2002; 2005).

Premotor areas are part of a series of parieto-frontal circuits that maps a variety of sensory information with specific motor plans (Rizzolatti and Luppino, 2001), and according to classical studies they can be schematically organized into parallel circuits for different forelimb motor actions. For example, F5 is richly connected with the anterior intraparietal area AIP, forming a circuit that transforms the shape, size and orientation of graspable objects in the correct hand and wrist posture required to grasp and manipulate it, the so-called "grasping circuit". Area F4 is connected to VIP and underlies the so-called "reaching circuit", using spatial information about objects to define the motor pattern to reach or avoid them, mostly in a body-centered reference frame.

Traditional neurophysiological studies on non-human primates revolved around laboratory setup and testing conditions in which the monkey was sitting in a primate-chair with only limited movement possibilities, typically with the head restrained with dedicated cranial implants because neural recording systems so far included tethered connections from the implanted electrodes and the amplifiers and recording apparatus. Furthermore, the traditional setups provided highly

controlled condition with the opportunity to control several potentially confounding variables, such as eye movement, muscle activity, stimuli presentation and reaction times, as the monkey learned to respond to cues in a standardized, stereotyped, and thus highly replicable way.

Thanks to recent technological advances, there is the possibility to capitalize on wireless neural recording systems to sample the activity from naturally behaving animals. In the last few years, an increasing number of publications on different animal models showed the feasibility of recording neural activity in freely moving animals: initially, in small mammals such as bats (Yartsev and Ulanovsky, 2013; Omer et al., 2018), but also marmosets (Roy and Wang, 2012) and, most recently, macaques (Berger et al, 2020; Mao et al, 2021).

Until now, wireless technology generated new important findings in domains that would have been impossible to tackle with conventional tethered systems, such as spatial navigation and complex motor and social behavior. However, the physiology of primates' motor system during unrestrained behavior has still not been investigated, likely because of the great complexity and variability of the movements that primates can perform using their entire behavioral repertoire. A small, but apparently increasing, number of studies have done the first steps in this direction, providing promising results (Berger et al., 2020; Nourizonoz et al., 2020).

1.1 Neurophysiological studies on the cortical control of behaviors

Anatomically, the non-human primates' motor cortex is composed of cytoarchitectonically different motor areas (Matelli et al., 1991). The primary motor cortex is represented by area F1; the ventral premotor cortex is constituted by areas F4 and F5, whereas the dorsal premotor cortex includes areas F2 and F7; the mesial portion of the premotor cortex is subdivided into areas F3 and F6, corresponding to the supplementary and pre-supplementary motor areas, respectively (Tanji et al., 1996).

Specifically, the ventral premotor cortex (PMv) lies on the cortical surface lateral to the spur of the arcuate sulci, whereas the dorsal premotor cortex (PMd) occupies the dorsal portion. Within these two sectors, F5 and F4 represent the rostral and caudal halves of the PMv, whereas F7 and F2 constitute the rostral and caudal halves of the PMd (Figure 1).

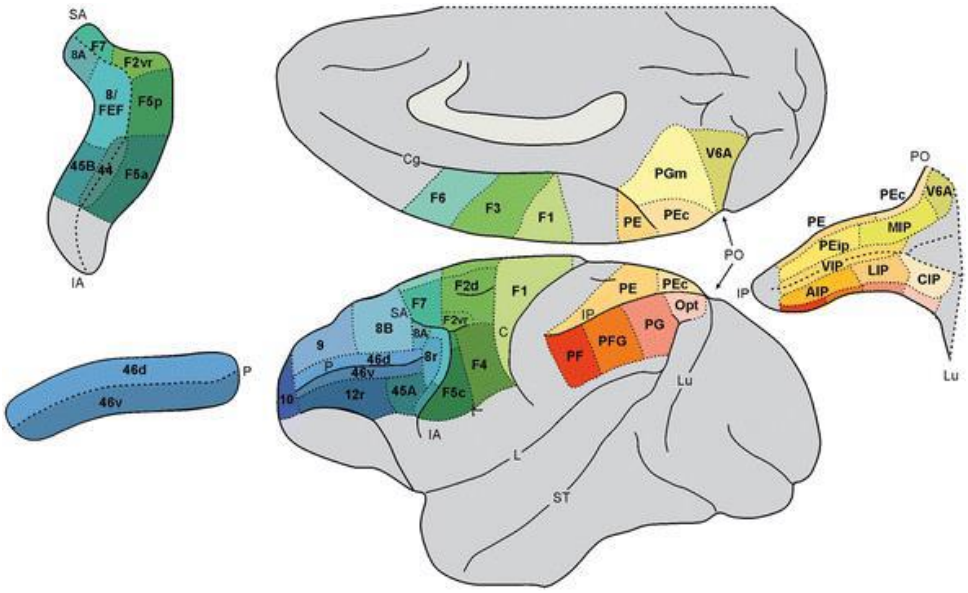

Figure 1. Cytoarchitectonic organization of the extended fronto-parietal motor system in the macaque. The prefrontal cortex is subdivided according to Carmichael and Price (1994), the caudo-ventral part of prefrontal cortex according to Gerbella et al. (2007). Agranular frontal cortex is subdivided according to Matelli et al. (1991) and Belmalih et al. (2009). Parietal areas are classified according to Pandya and Seltzer (1982). Modified by Gerbella et al. (2017).

The traditional view of these areas maintains that distal movements were represented in Broadmann Area 4 (BA4), which is the primary motor cortex (F1), while proximal and axial movements in BA6, which is the lateral and dorsal part of the premotor cortex (Woolsey et al., 1952). Further studies on PMv have shown that distal movements are represented also in BA6 (Rizzolatti et al., 1981a), and neurons in the rostral part of BA6 are activated with tactile stimulation of distal parts of the body (Rizzolatti et al., 1981b).

In a paper published in 1988, Gentilucci and coworkers demonstrated that distal movements are represented both near the central sulcus and near the arcuate sulcus, while proximal movements are represented mostly in between the two, both in F4 and in the rostral portion of F1. The representations of different effectors are partially overlapping in the ventral premotor cortex, while in F1 they are more clearly segregated.

Electrical stimulations with short trains of pulses delivered to the ventral premotor cortex evokes arm, mouth, neck and face movements, even at the level of the same cortical site (Maranesi et al., 2012), thus revealing a more complex organization of movement representations than the primary motor cortex, where electrical stimulations recruits, instead, more often a single joint. Long-train intracortical microstimulation (ICMS) was used to better understand the cortical organization of motor maps (Graziano et al., 2002; Graziano et al., 2005). The results highlight a map of actions whose duration fits with the duration of the stimulation train, revealing ethologically relevant behaviors such as “reach to grasp” or “hand to mouth” (Figure 2) (Graziano and Aflalo, 2007),

distributed along the ventro-dorsal extent of the premotor cortex, and suggesting a previously neglected organization principle.

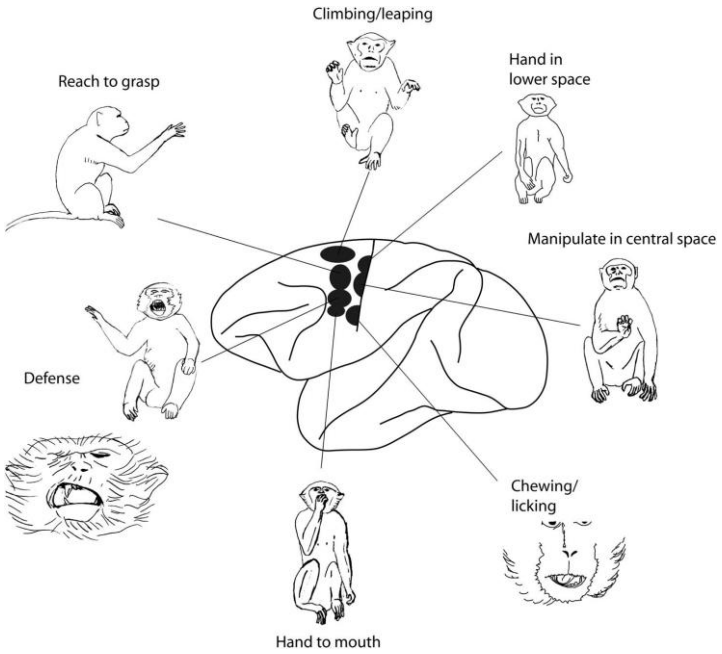

Figure 2. Action zones in the motor cortex of the macaque. Thanks to intracortical stimulations, behaviorally relevant pattern were evoked. Each image shows the final posture of the monkey at the end of the stimulation-evoked movement (Graziano and Aflalo, 2007).

Anatomically, the BA6 can be divided in two sectors, depending on the connections with different brain regions (Rizzolatti et al., 2014; Borra et al., 2017). The parieto-dependent motor areas are the posterior ones, from F1 to F5, which are linked with parietal regions; the prefronto-dependent motor areas are the anterior ones, namely F6 and F7, which are mainly connected with the prefrontal cortex (Albertini et al., 2020). Parieto-dependent areas receive sensory inputs from different parietal areas and use them for sensory-motor, the coding of potential motor actions, action recognition and peripersonal space representation transformations (Schaffelhofer and Sherberger 2016; Borra et al., 2017; Gerbella et al., 2017); F1, F3 and part of F2, in order to perform sensorimotor transformation, mainly exploit somatosensory information, while F4, F5, F6 and the rostro-ventral part of F2 use both somatosensory and visual information. Prefronto-dependent areas receive information from the prefrontal cortex (Gerbella et al., 2013; Caminiti et al., 2017), contributing to the encoding of the context and the motivation underlying action selection, specifying when and whether the potential motor actions have to be performed (Rizzolatti and Luppino, 2001). As previously declared, PMv is involved in the execution of sensorimotor tasks and this is consistent with its connectivity since it receives inputs from parietal (Borra et al., 2008) and prefrontal (Lu et al., 1994) cortices and send outputs to motor areas (Luppino et al., 1993). The

ventral premotor cortex neurons contribute to the decision making processes, by leveraging multimodal stimuli including somatosensory (Romo et al., 2004), visual (Pardo-Vazquez et al., 2008) and auditory (Lemus et al., 2009) signals used to perform a variety of discrimination tasks, where neurons become active both in response to sensory stimulation and during the entire decision process.

1.1.1 Functional properties of PMv neurons

The rostral part of PMv, namely area F5, is divided in three sectors, F5p, F5a, and F5c, each characterized by rather distinct functional properties. In general, F5 hosts a representation of the hand and the mouth, the former located in the most rostral and dorsal portion, the second in the ventral portion, with a rather large territory of overlap between the two (Maranesi et al., 2012).

Classical textbook studies identified in F5 three types of neurons: purely motor, canonical and mirror neurons. Since the pioneering studies on this area, it emerged that neurons encode the goal of the actions, not single movements; indeed, F5 neurons could respond when the monkey grasped food morsels regardless of the hand used (ipsilateral or contralateral) and of the direction of the reaching movement toward them (Rizzolatti et al., 1988). Moreover, F5 grasping neurons could discharge even independently from the effector used (e.g. the hand or the mouth), and from the specific extension or flexion movement of the hand, such as, for example, when monkey grasped an object with different types of pliers implying an opposite sequence of muscle activation (Umiltà et al., 2008). Some F5 neurons encode not only the general motor goal (e.g. grasping), but also the specific grip type, such as whole hand , precision grip or finger prehension (Bonini et al., 2012).

In addition to purely motor neurons, area F5 hosts two other different neuronal categories: canonical neurons, mostly located in F5p/a, and mirror neurons, in F5c.

According to the original definition, canonical neurons exemplify the “canonical” visuomotor transformation for grasping objects: they respond to the visual presentation of three-dimensional stimuli as well as during the preparation and execution of a reaching-grasping actions directed to them (Murata et al., 1997). Importantly, they were assumed to keep the same visuomotor selectivity from the visual presentation of the target to the end of the grasping action (Murata et al., 2000), thereby contributing to transform the intrinsic physical features of an object (size, shape, weight) into the appropriate motor repertoire required for grasping it. Subsequent multiareal chronic recording studies emphasized the fact that the stability of the visuomotor selectivity is neither an important nor a frequent properties of visuomotor neurons in AIP and F5: instead, the

population activity in area AIP provide a readout of the visual similarities across a variety of three dimensional objects, whereas F5 turns these features into a neuronal representation of the most remarkable similarities in the hand shape required for grasping them, indicating that the visuomotor transformation requires the mutual contribution of these two nodes of the network as well as of the primary motor cortex, which finally reflect just the hand posture required to perform the action (Schaffelhofer and Scherberger, 2016).

Regarding mirror neurons, according to the classical definition, they are cells originally discovered in area F5c firing during the execution of an action (in the light and in the dark) and during the observation of the same (or a similar) action performed by another agent, but not during the simple sight of a graspable object or of the motion of a non biological effector or of a tool action. Based on these findings, it was proposed they constitute a neural mechanism for mapping observed action onto their corresponding motor representation, thereby playing a role in action recognition (Di Pellegrino et al., 1992; Gallese et al., 1996; Rizzolatti et al., 1996). Subsequent studies have stress the same coding principle generally applied to other visuomotor neurons in area F5, that is, mirror neurons would code one's own or others' action goal, both when the observed action is not fully visible (Umiltà et al., 2001), or when it can be only heard in its acoustic consequences (Kohler et al., 2002), or even when it can be just recollected from contextual (Bonini et al., 2010) or arbitrarily learned (Bonini et al., 2014b; Maranesi et al., 2015) information previously associated with a given motor goal.

More recent studies, however, have challenged this categorical subdivision of F5 neuronal population into segregated classes: in fact, by simultaneously recording without any prior selection bias several individual neurons with linear probes and testing all of them with a larger set of tasks suitable to reveal motor, canonical and mirror properties, it has been shown that many neurons can show an highly diversified mixture of motor and visual responses to both objects and others' action (Bonini et al., 2014a).

The premotor area F4 contains a representation of head, trunk, arm and mouth movements (Gentilucci et al., 1988) as also confirmed by intracortical microstimulation studies (Maranesi et al., 2012). Proximal, axials and arm movements tend to be represented dorsally, whereas mouth movements are represented mostly in its ventral part. In F4 three classes of neurons have been originally identified: somatosensory neurons, visual neurons and bimodal neurons. Somatosensory neurons of area F4 respond to tactile stimulations delivered on the face, neck, arm or hands (Rizzolatti et al., 1981). Visual neurons encode visual stimuli typically moved close to the monkey's body. Bimodal neurons respond to both tactile stimuli applied to specific body parts and to visual stimuli (Fogassi et al., 1996), moved around the subject in its peripersonal space (Gentilucci et al.,

1983), typically with a clear spatial relationship between the somatosensory and the visual receptive field. Indeed, visuo-tactile neurons with the tactile receptive field (RF) on the arm or hand have the visual RF usually anchored to the tactile RF, which means that the visual RF depends on the position of the limb rather than on the retinal position of the stimulus, suggesting that F4 neurons operate in body-centered coordinates (Graziano et al., 1994). Specifically, Fogassi and coworkers found that the visual RFs locations are usually independent from the gaze position and they remain constant regardless the eye movements (Fogassi et al., 1996): in this latter study, only 10% of the recorded neurons had a retinocentric visual RF. In addition, some F4 neurons are trimodal, as they encode the peripersonal space even based on the noise signaling the specific position of an object around the monkey's body (Graziano et al., 1999), suggesting that F4 is encoding the nearby space with different modalities. Finally, in F4 a small part of neurons shows complex properties: for example, they discharge when visual stimuli withdraw from the subject, but also during reaching movements, particularly by fast arm extension towards an object (Gentilucci et al., 1988).

1.1.2 Fronto-parietal circuits

The cortical motor areas are involved in different fronto-parietal circuits, working in parallel and performing specific sensorimotor transformations (Rizzolatti et al., 2002). Anterior motor areas, F6 and F7, are connected to the prefrontal cortex, while posterior motor areas (F1-F5) to parietal regions. Each of the motor areas dependent from a parietal area receives specific sets of sensory inputs. Area F5 is mutually connected with AIP and, together, they form the core of a grasping circuit in which area V6A (Fattori et al., 2001; Breveglieri et al., 2019) and F2vr (Raos et al., 2004) represent a more dorsal component, together with the mesial presupplementary motor area F6 (Lanzilotto et al., 2017; Livi et., 2019; Albertini et al., 2020). These cortical nodes use the physical properties of an object to select and activate an appropriate motor plan to interact with it, with different specificities in the coding principles of the various nodes (Gerbella et al., 2017). The reaching circuit includes F4 and VIP, which transforms extrinsic characteristics of an object, such as their position in space, in the correct motor plan to reach them (Matelli and Luppino, 2001; Rizzolatti and Matelli, 2003), together with the dorsal premotor area F2 and its parietal targets in the inferior and superior parietal lobule (Rozzi et al., 2006; Gamberini et al., 2009; Passarelli et al., 2011).

Similarly to F4, VIP contains neurons that respond to visual stimulation, bimodal neurons and trimodal neurons (Duhamel et al., 1991; Colby et al., 1993; Schlack et al., 2005). The F4-VIP circuit

might be involved in defensive mechanisms and of the encoding of peripersonal space (Cléry et al., 2015). In an intracortical microstimulation study, VIP has been found to produce defensive-like movements, as previously shown for F4 (Cooke and Graziano, 2004). If VIP is stimulated, the monkey blinks, retract its head, lifts its upper lip and makes different arm movements (Graziano et al., 2003). Specifically, VIP may play a role in constructing a head-centered representation of the environment, whereas F4 may be more involved in generating defensive and avoidance actions.

1.1.3 Strengths and weaknesses of non-human primate neurophysiology

Classical neurophysiological studies on non-human primates allowed us to get fundamental insights on the neural underpinnings of motor and cognitive functions of the premotor system. However, existing approaches suffered from several limitations, mostly arising from tethered neural recordings that make unavoidable the physical-restrain of monkeys. Most of the studies presented data from head-restrained monkeys sitting in the primate chair, limiting the whole-body movements and allowing only partial upper limbs actions. The monkeys could not perform complex behaviors, nor explore the environment or freely interact with other subjects. Macaques remain a crucial model to study in detail how neuronal systems work, and to understand the mechanisms underlying perceptual, motor, and cognitive functions more accurately than in any possible human study (Roelfsema and Treue, 2014; Buffalo et al., 2019).

Until few years ago, the use of the primate chair was imposed by the need to manage and protect the tethered systems necessary to record neural activity, especially in acute experiments. The tethered neural recording approach requires the subject to remain still and not to move in order to implant and/or connect the electrodes in the brain via cables to the amplifiers and recording devices, preventing the neural signal to be influenced by the mechanical noise and artifacts potentially caused by the huge movements of the monkeys, and allowing to control eye movement during visual and motor tasks.

Due to these limitations, monkeys can perform tasks involving simple and repeated actions and behaviors, which provide the methodological advantage of observing controlled and repeatable movements, even if they are likely different in many respects from natural behavior (Jackson et al., 2007). Studies with restrained macaques offer controlled results with only few and specific variables, limiting confounding factors and resulting in a considerable internal validity.

Complex behaviors cannot be investigated under constrained conditions, making necessary the use of different methodologies allowing freely moving subjects. With small animals, it is possible to

record neural activity with tethered systems while they freely move in arenas, allowing for example the discovery of place cells in rodents (O'Keefe, 1976). Conversely, it was not possible to record neural activity in macaques with tethered systems in order to study complex behaviors because of the need of large and three-dimensional environments.

In the last few years, thanks to the implementation of new, miniaturized technologies, neurophysiological studies have been possible to perform with wireless recording systems. The subjects are free to move in setups that are more naturalistic and can react to different types of stimuli. Neural activity is recorded while the animal performs complex and articulated behaviors in a wider range of activities, making it more difficult to elaborate data and to control possible confounds, but leading to a more ecologically relevant approach, questions and answers, adherent to the naturalistic behavior of the investigated subjects. This new methodology is a great step toward a neuroethological approach, which leads to the solution of relating naturalistic behavior to neural recordings in order to better understand brain function (Datta et al., 2019).

1.2 A neuroethological approach for the study of the neural basis of animal behavior

In a recent review, Testard and colleagues (2021) argued that the neuroethological approach is necessary to pursue more ecologically relevant questions. There is the opportunity to exploit new technologies and move on to unrestrained wireless recordings of single neuron activity in different animal models. The technology used must be carefully adjusted to the animal's ecological niche and biology, in order to focus on more ethologically relevant questions.

In smaller animals, wireless technology has been already widely used for different topics, making it possible to deepen our knowledge in the underlying of complex behaviors and conditions.

The unconstrained paradigm led to great findings in spatial cognition, as the modulation of place-cells, grid-cells and head-direction cells in three dimensions, for example in rodents (Grieves et al., 2020) and bats (Yartev and Ulanovsky, 2013; Finkelstein et al., 2015). Wireless technology helped expand the use of different animal models, showing spatial cognition not only in bats (Genzel and Yarstev, 2021), but also in fishes (Vinepinsky et al., 2020) and birds (Ben-Yishay et al., 2021).

In the last few years, new methodological papers have been published, which allowed the study of the spatial encoding in non-human primates. Nourizonoz and colleagues (2020) presented the

"Etholoop", a multi-camera closed-loop tracking system capable of providing simultaneous close-up views of mouse-lemurs in a large arena. They combined it with markerless pose estimation to identify specific poses online and reinforce those behaviors either through automatic classical conditioning or optogenetic stimulation of the reward system (Figure 3). Finally, they demonstrated the existence of place-cell-like activity in the hippocampus of freely moving primates.

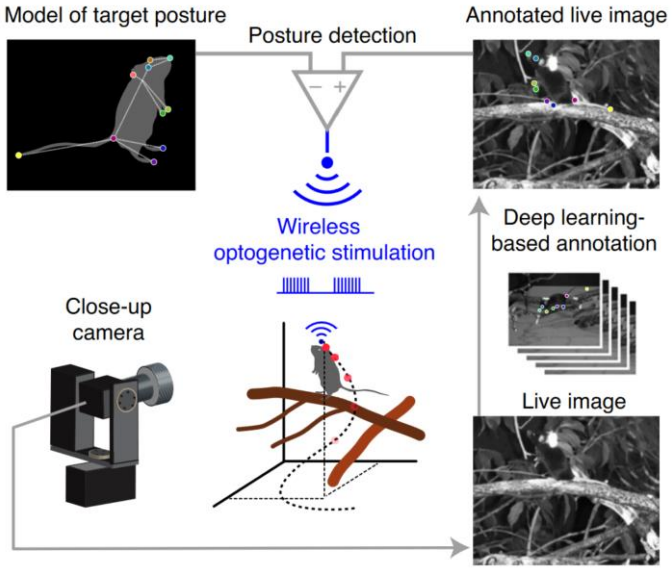

Figure 3. Schematic representation of the wireless optogenetic stimulation setup with Etholoop. Multiple infrared cameras and a close-up camera take images from different viewing angles. The close-up camera provides high-resolution live images of the animal; the body parts are tracked and then classified in real time using a pre-trained deep-learning network. A behavioral event can be detected when the tracked posture of the animal is matching a geometric model of the posture. This detection triggered wireless the optogenetic stimulation (Nourizonoz et al., 2020).

Mao and colleagues (2021) recorded wireless hippocampal activity and tracked behaviors in freely moving macaques, demonstrating that spatial representations are dominated by facing location and allocentric direction, mostly in head-, rather than gaze-centered, coordinates. Their findings revealed that the macaque hippocampal formation represents three-dimensional space using a multiplexed code during free exploration.

Another growing topic that is addressable with these new methodologies is the neurobiological study of social behavior. Typically, in non-human primates species-specific social behaviors were neglected, mainly due to the physical constraints imposed to accommodate neural recording equipment. In the field of social behavior, an important step forward have been the dyadic social decision-making tasks, but still in laboratory and constrained conditions. Such distinctly unnatural experimental conditions may fail to engage the full neural circuitry that mediates social interactions (Testard et al., 2021). In the last years, thanks to wireless recording technologies, social behavior

has been studied in freely moving animals such as rodents (Kingsbury et al., 2019), bats (Zhang and Yarstev, 2019) and birds (Hoffmann et al., 2019).

The next step is to use wireless recording techniques to study more complex and complete social interactions in primates, which are not displayed under classical, constrained laboratory conditions. For instance, marmosets, which are arboreal and highly social New World monkeys, make a wide use of vocalizations to communicate with each other; however, when marmosets are restrained in a primate chair, their vocal behavior is strongly inhibited. With a wireless multi-channel single-unit recording technique, it was possible to study marmoset vocalizations (Roy and Wang, 2012), demonstrating the applicability of wireless neural recordings in a social context.

An important topic is the study of the motor system, which has been difficult to investigate with this new technology. Freely moving animals perform a large variety of whole-body behaviors, which were not possible to study previously. Until now, the motor system has been recorded in animals that could not perform their entire pattern of behaviors. In particular, non-human primates' premotor cortex has been recorded while monkeys were performing simple and stereotyped movements.

1.2.1 Wireless technologies for the study of the motor system

There are still few studies on the motor system carried out in freely moving non-human primates. In a pioneering experiment, Jackson and coworkers (2007) compared the data collected by using a wireless system with those obtained by using a tethered system. They recorded from the macaques' primary motor cortex neurons in different conditions: performing a torque-tracking task in a primate-chair, laboratory setting; freely behaving in the home cage; and sleeping. In each of the awake conditions (but not during sleep), they found a positive correlation between neural activations and EMG activity, with results obtained with the tethered and wireless methodologies partially overlapping. However, the adoption of a wireless recording system in unrestrained conditions is important to extend the data obtained under classical restrained experimental conditions.

Wireless technology was used to investigate how monkeys plan limb movements to reach a distant goal, which could not be studied in restrained animals. Berger and colleagues (2020) developed an experimental environment called "Reach Cage" that was equipped with a visuo-haptic interaction system, where trained macaques performed controlled visually-guided reaching movements to a target with instructed delay; thanks to the unconstrained setup, the targets could be within

immediate monkey's reach or beyond the immediately reachable space. Combining markerless limbs motion estimation and, simultaneously, single unit activity from PRR (parietal reach region), PMd and the primary motor cortex, they demonstrated that premotor and parietal cortical activity contain information not only about the position of targets located in the peripersonal space but also of "walk-and-reach" targets located far away from the subject during movement planning. This paradigm allowed studying whole-body freely moving macaques but at the same time, it allowed the monkey to perform structured tasks (Figure 4).

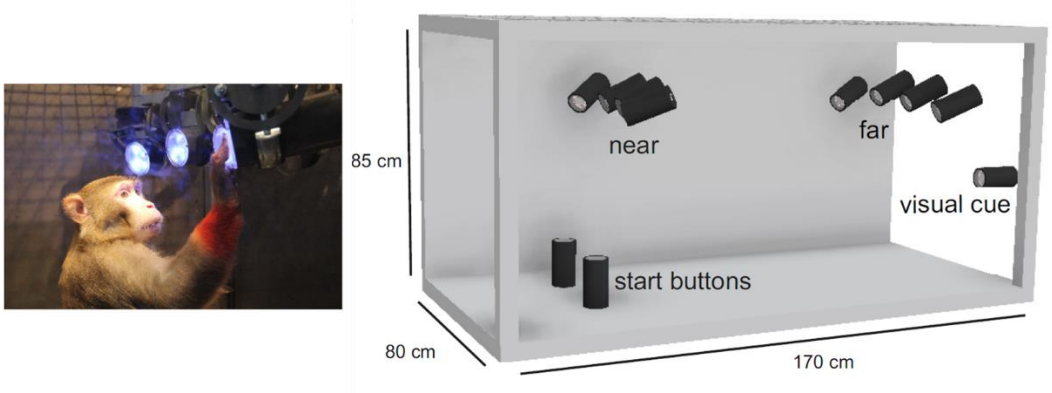

Figure 4. On the left, a monkey is performing a visually guided task in the Reach Cage. On the right, a sketch of the Reach Cage with two start positions and eight targets, divided in two rows (near and far). In this setup, the neural activity of the subject is recorded wireless from three different brain areas (Berger et al., 2020).

Further studies regarding the motor system will help elucidating how the neural activity is involved in the control and processing of whole-body behaviors. A deeper look in this field will promote the development of new approaches to neuroprosthetics (Aflalo et al., 2015; Bouton et al., 2016; Capogrosso et al., 2016; Wagner et al., 2018), with an ecological valid comprehension of the brain-behavior relationship, capable to open up new translational perspective and research avenues.

2. Aims

The non-human primate motor system has been deeply investigated and offered great discoveries about how complex the movement control can be. However, the limitations due to the traditional setup allowed the study of stereotyped and simple actions. To truly understand, how the motor system works, neural activity should be recorded while the monkey is able to perform its whole range of behaviors in situation as much as possible similar to the unconstrained ones in which the brain evolved.

Thus, we implemented the new wireless technology to study whole-body movements and complex behaviors in freely moving macaques. As a reference point, we studied neural activity first in a traditional neurophysiological setting, with the monkey's head restrained and the repetition of the same behaviors of interest in a rather stereotyped but reproducible way. Then, the same neurons have been tested while the monkey moved around in a large enclosure enriched with a variety of items aimed at eliciting the larger variety of spontaneous behaviors, most of which of the same type as those tested in the primate chair.

We developed different analytic approaches to explore the relationship between neuronal activity tested in these different context, paving the way to a new ecologically-relevant approach to the investigation of the neural underpinnings of primates' behavior.

3. Materials and Methods

Two rhesus macaques (*Macaca mulatta*) were trained to autonomously enter in a primate chair and to be transported in the laboratory. Both monkeys were chronically implanted with a titanium head post (Crist Instruments, Hagerstown, MD, USA) and microelectrode arrays for neural recordings (Microprobes for Life Science, Gaithersburg, MD, USA). They were first trained to have the head fixed and passively receive various type of stimulations, to perform forelimb and mouth motor actions under conventional, well-controlled laboratory conditions at the center of a large Plexiglas enclosure (W x H x D, 205 x 205 x 180 cm): the NeuroEthoRoom (NER). Then, after a first part of the recording session in the primate chair (Chair condition), they were introduced in the NER for recording of neuronal activity during free movement (NER condition). The NER was enriched prior to each freely moving session with different devices, to stimulate a variety of spontaneous behaviors monitored with a system of 8 fixed 50 Hz cameras.

3.1 Ethical statement

All experimental protocols in this study were approved by the Veterinarian Animal Care and Use Committee of the University of Parma (Prot. 52/OPBA/2018) and authorized by the Italian Ministry of Health (Aut. Min. 802/2018-PR), in compliance with the European law on the humane care and use of laboratory animals (Directive 2010/63/EU), and reported according to the ARRIVE guidelines (du Sert et al., 2020).

3.2 Subjects

The subjects in our study were two male rhesus macaques (*Macaca mulatta*: W, 8 years, 13 kg; R, 10 years, 13.5 kg), non-genetically modified, acquired from a European distributor (RC Hartelust, Tilburg, Holland). The findings presented on the first animal have been replicated in a second subject, according to the requirement of the 3Rs international standards for neurophysiological studies. The experimenters and the technical staff received a FELASA (Federation of European Laboratory Animal Science Associations) certificate of competence specific for the work with non-human primates before the beginning of the study, and they were in charge of monitoring animals' behavior and health state daily.

3.3 Housing and husbandry

The facilities in which the animals were housed provide cage sizes exceeding the requirements of Italian and European regulations (at least 4m^3 per animal; Tecniplast, Italy). The subjects used for this project were initially housed in pair, and subsequently single-housed based on veterinary advice and consultation with independent expert primatologists, because of incompatibility and risk of serious aggressive episodes due to occurred instability in the social dominance rank between the two. The environmental temperature was maintained in the range 24-27 °C, with a humidity at 30-40%. The light/dark cycle was electronically maintained with a 12/12-hour cycle with artificial illumination, in addition to large windows providing natural sunlight daily. The diet was mainly based on pellet of different flavors (Mucedola Srl, Italy), in addition to food enrichments, fruits and vegetables administered by the experimenters during the training sessions. Water was administered by the experimenters in compliance with the experimental protocols. Sawdust and bark were provided as bedding in different parts of the cage, which was daily cleaned and sanitized. The subjects of our study had daily access to an enriched environment including wooden structures and various toys (Calapai et al., 2017).

3.4 Training procedures

The two subjects were trained for several months once a day. During the first period (about 12 months), the monkeys were trained with positive reinforcement methods to sit in the primate-chair, by rewarding every correct behavior with fruit morsels or fruit juice (Laule et al., 2003; Schapiro et al., 2003). Both monkeys learned to individually and voluntarily sit in the primate-chair, and to be transported in the laboratory, reducing the distress typically associated to these procedures when carried out with coercive methods such as pole and rigid collars.

Once in the laboratory, the monkey gradually learnt to stay with the head fixed for an increasing amount of time (from a few minutes to about 1 hour). Once this first part of the training was achieved, the monkey was trained to perform different actions while head-fixed in the primate-chair, such as grasping fruit morsels, objects or a rope with either the right or left hand, observing visual stimuli and the experimenter's actions. Thanks to the training with positive reinforcements, the macaque accepted calmly every part of the task and the experimenter manipulations.

One important step for the training was teaching the monkey to voluntarily enter in the NER and, most importantly, to spontaneously exit from it to go back in the primate-chair, to be moved in its home cage. In the NER, the subject was free to move and it did not require any specific training,

because the aim was to investigate naturalistic and spontaneous behaviors in comparison with those tested in the primate chair.

3.5 Surgical procedures

To perform controlled tasks in a classical primate-chair settings with the monkey's head fixed, a titanium head-post was implanted (Crist Instruments, Hagerstown, MD, USA) under general anesthesia and aseptic conditions, followed by postsurgical pain medications. For both the head-post implantation and the intracortical electrodes implantation, animals were prepared for the anesthesia with atropine administration (0.03 mg/kg) 15 minutes prior to the induction of anesthesia. Next, anesthesia was induced with ketamine (Lobotor, 4.5 mg/kg) and medetomidine hydrochloride (Domitor, 0.05 mg/kg), and maintained via inhaled isofluorane (IsoFlo, 100% p/p). Hydration was maintained by administration of saline solution (5 ml/kg/h). The animals were prophylactically treated with phenobarbital (Gardenale, 2 mg/kg) for seizure prevention after probe implantation, starting from 1 week before the surgery and until 4 weeks post-surgery (fading-in/out the drug over 2 weeks). Additionally, they were administered with antibiotics (Rubrocillina, Benzylpenicillin Benzatin 25000 U.I./ml + Dihydrostreptomycin 100 mg/ml), dexamethasone (Soldesam, 0.15 mg/kg) and ketoprofen (Vet-Ketofen, 3 mg/kg) for a variable number of days depending on the procedure, as prescribed by the veterinary.

Electrodes arrays were implanted by estimating the location and extent of the craniotomy with MRI-based reconstruction of the monkey skull. To identify the site of insertion, anatomical references were used, particularly the superior and inferior arcuate sulci and the central sulcus were taken as fundamental landmarks (Figure 5). Neuronal recordings were performed by means of chronically implanted floating microelectrode arrays, each with 32 recording channels (FMA, Microprobes for Life Science, Gaithersburg, MD, USA). The small size and great flexibility of the FMAs make them ideal for the implantation of a large number of arrays simultaneously in the same animal, allowing a variety of cortical locations to be targeted at the same time. Our customized FMAs design includes two alternated electrode lengths (2.5 and 4 mm), which allow us to explore different depths of the cortex (Figure 5A).

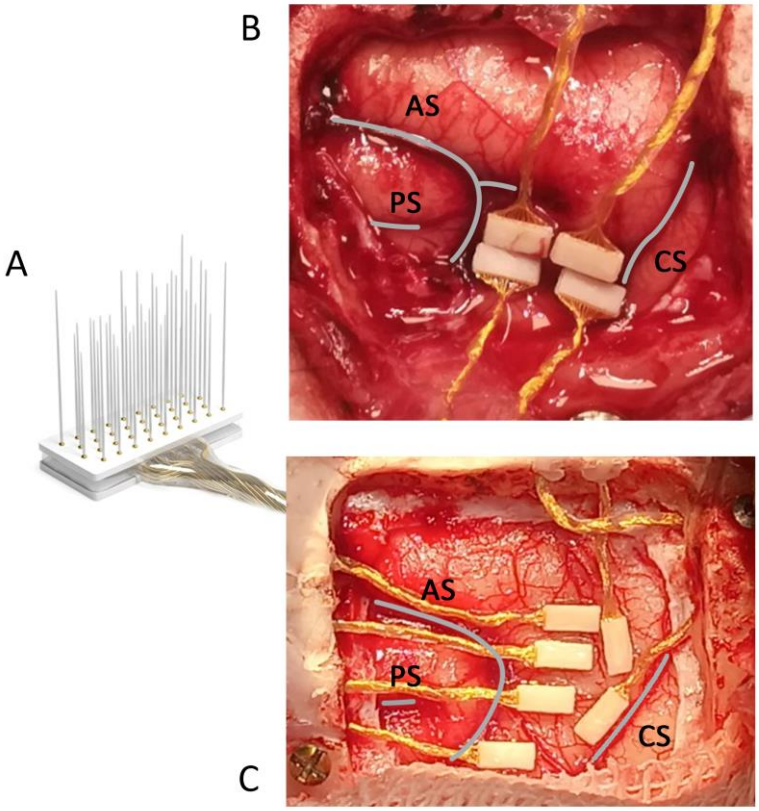

Figure 5. Floating microelectrode arrays (FMAs) implanted in macaques, W and R. (A) Schematic representation of a FMA with 36-channels; (B) Image of microelectrode arrays placement in monkey W and, (C) monkey R. Anatomical landmark descriptions: CS - central sulcus; AS - arcuate sulcus and PS - principal sulcus.

In monkey W, we implanted 4 FMAs in the ventral premotor cortex (Figure 5B), while monkey R received 6 FMAs (Figure 5C).

To protect the implant, we built a customized cranial device in plastic material (PEEK) with head-caps of different sizes and shapes for the different stages of the experiment. We designed three configurations: 1) home-cage configuration (to be used without the recording system); 2) primate-chair configuration; 3) freely-moving animal configuration (Figure 6).

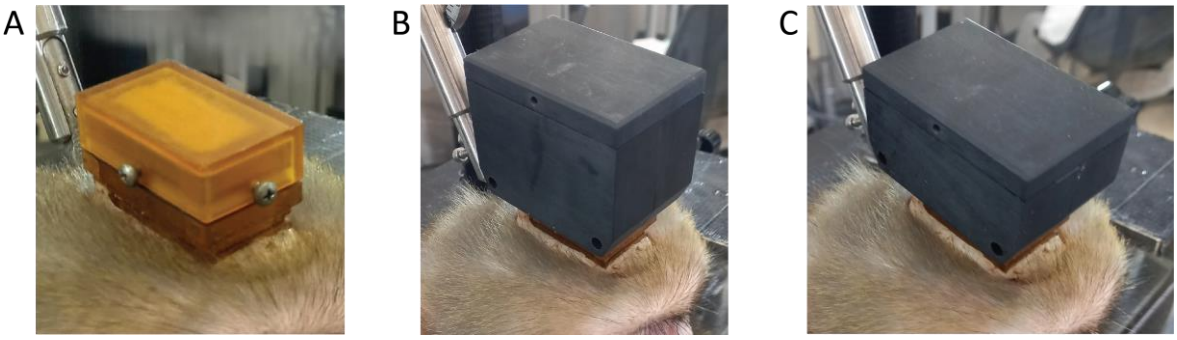

Figure 6. Implant system design. A) Home-cage configuration; B) Primate-chair configuration; and C) Freely-moving animal configuration.

The main difference between the configurations is the size of the head-caps. The home-cage configuration is the smallest because it does not include the recording system. The primate-chair configuration is the one used during the first stage of the recording sessions: it has the biggest head-cap so that it could contain the recording system and a large battery in order to keep recording for long periods of time. Note that even if in a chair setting using a data logging system is not necessary, with this approach we could merge the entire dataflow, from the head-fixed to the freely-moving condition, in order to apply filtering, spike sorting and all the offline data processing procedures on the whole recording session collected with the same system and no drift in the signal-to-noise ratio. The freely-moving animal configuration is big enough to contain the recording system and a small battery, but the size is limited to the minimum to prevent accidental crashes while the monkey moves.

3.6 Recording techniques

We simultaneously recorded from 128 channels with a wireless data logging system (Deuteron technologies, RatLog-128), which allowed us to set a band-pass filter (2 - 7000 Hz) at a conversion rate of 32000 Hz for each channel, thereby recording both LFP and single/multi-unit activity signals. Data are amplified, digitized and stored locally, in a MicroSD memory card (64 GB): no wireless data transmission occurs, with the exception of the clock signal, thus preventing any possible transmission error. The device is powered by an external battery (3.7V) connected via a short cable. Once the logger device was linked to the electrode arrays into the chamber via micro-omnetics connectors, all the components were sealed with a head-cap (Figure 7).

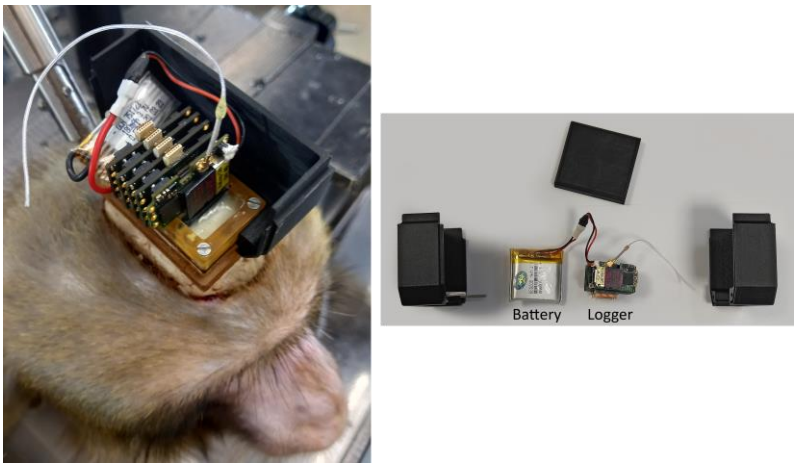

Figure 7. The recording chamber divided in its components. On the left, the recording chamber open; on the right, the battery connected with the recording system (RatLog-128 from Deuteron technologies) between different parts of the headcap.

The logger communicates with a computer by means of a transceiver, as described in Genzel and Yartsev (2021).

In particular, the video of the recording sessions was recorded through a system of eight high-resolution cameras placed around the NER (four in the upper corners and four at a middle height), connected with a dedicated, commercially available software for 3D motion data acquisition and analysis (Simi Reality Motion Systems GmbH, Unterschleissheim, Germany). We used Dual Gigabit Ethernet Machine vision cameras (mvBlueCOUGAR-XD, Matrix Vision) with a resolution of 1936×1214 at up to 164 frames per second, set to 50Hz. The cameras were equipped with a global shutter with sensor size 1/2" format (5.86µm pixel), a manual C-Mount Lenses with 5 mm focal length (CCTV Lens, KowaOptical Products Co., Ldt) and LEDs ring lights. Each camera was connected through a synchronization box connected to both cameras and computer.

To synchronize the recordings, a 50 Hz pulse signal of 5 V generated by a LabView-based program was sent simultaneously to the recording devices, namely, the logger – where it was stored on the SD card, and the 8 cameras, whose frame grabber was triggered by that signal through the Simi Motion software.

3.7 Apparatus and behavioral paradigm

All the experiments took place in the NeuroEthoRoom (NER, Figure 8). A system of 8 color cameras enabled us to record the macaque behavior at 50 Hz throughout the sessions.

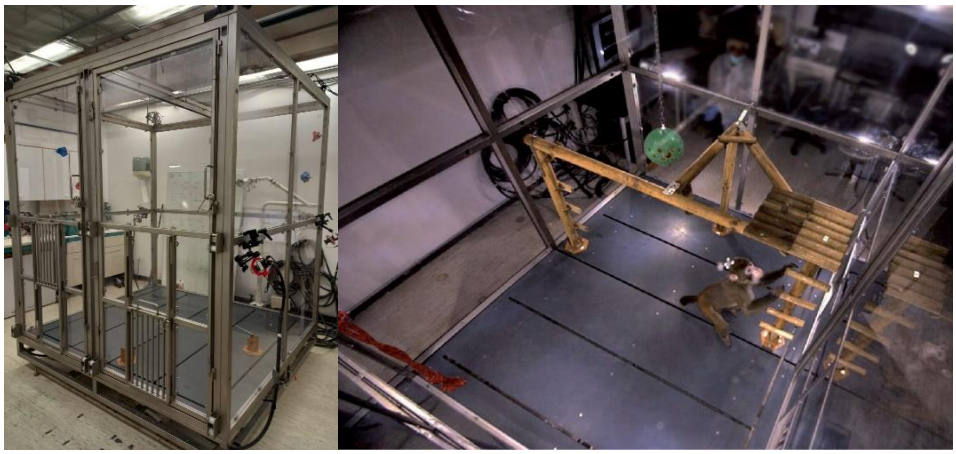

Figure 8. The NeuroEthoRoom (NER), a large plexiglas enclosure where the sessions are recorded. On the left, a view from the outside, when the enclosure is empty; on the right, a view from the inside, while the monkey is freely moving around the enriched cage.

The experimental session was divided in two steps: during the first, the monkey was in the primate chair with the head fixed at the center of the NER; during the second one, the monkey was left free

to move in the NER, enriched with a wooden structure, a rope and different foraging opportunities (fruit morsels placed into holes dug in the wooden structure, the walls of the NER, or on the floor).

3.7.1 First step: Primate-chair condition

During the first stage, we studied the motor properties of the premotor cortex neurons with classical methodologies while the monkey was head-fixed in the primate chair. The monkey performs different types of actions for at least 10 trials, such as reaching and grasping fruit pieces, and receives liquid rewards by means of a syringe.

Using a behavioral scoring software (BORIS, Behavioral Observation Research Interactive Software; Friard and Gamba, 2016), we were able to score the monkey and experimenter's actions with a specifically compiled ethogram (Table 1), allowing us to define specific behavioral occurrences synchronized with the neural recordings.

Table 1. Ethogram of behaviors in the primate-chair condition

Behavior code	**Behavior type**	**Description**
grasp near R	Point event	Monkey grasps food pieces with the right hand when the food is presented in front of him - Start when hand touches food
grasp near L	Point event	Monkey grasps food pieces with the left hand when the food is presented in front of him - Start when hand touches food
grasp far R	Point event	Monkey grasps food pieces with the right hand when the food is presented far from him and then brought near - Start when hand touches food
grasp far L	Point event	Monkey grasps food pieces with the left hand when the food is presented far from him and then brought near - Start when hand touches food
failed grasp	Point event	Monkey tries to grasp food pieces with left or right hand but fails - Start when hand touches food
solid reward	Point event	Monkey receives passively solid (fruit pieces, raisins, ...) reward directly in the mouth - start when the food touches the mouth - Only if the experimenter gives it
liquid reward	Point event	Monkey receives passively liquid reward with syringe (directly in the mouth) - start when the mouth touches the syringe
finger prehension 0° R	Point event	Monkey grasps carabiner with the right hand - Start when hand closes around the carabiner
finger prehension 0° L	Point event	Monkey grasps carabiner with the left hand - Start when hand closes around the carabiner
finger prehension 90° R	Point event	Monkey grasps a rope with the right hand - Start when hand closes around the rope
finger prehension 90° L	Point event	Monkey grasps a rope with the left hand - Start when hand closes around the rope
active food to the mouth R	Point event	Monkey actively places the food into the mouth with the right hand - start when the right hand reaches the mouth.

active food to the mouth L	Point event	Monkey actively places the food into the mouth with the left hand - start when the left hand reaches the mouth.
grasp experimenter	Point event	Experimenter grasps food pieces with the left or right hand - Start when fingers stop closing
active food to the mouth experimenter	Point event	Experimenter actively places the food into the mouth with the left or right hand - start when the distance between the mouth and the food is at its minimum
undefined	Point event	Monkey does movements not better explained in the ethogram
food presentation	Point event	Monkey sees the food hidden behind the vertical panel. Start when the panel is under the horizontal panel

Before starting the second stage, the monkey was carried out of the cage while still in the primate chair. The NER was prepared for the subsequent part of the session, and a wooden structure, a rope and several pieces of fruit were positioned inside it. The subject went directly from the primate chair to the enclosure. During this stage, the monkey could move around and behave naturally. While the monkey was in the NER, the experimenter gave it liquid and solid rewards through different holes in the Plexiglas walls.

3.7.2 Second step: Freely-moving condition

In this condition, the monkey was free to move around and manipulate objects and food items in the environment, while its neuronal activity was recorded. We used a specific ethogram to identify onset/offset of critical time point corresponding with a variety of possible behaviors that the monkey performed in the NER (Table 2).

To score the behavior based on the ethogram, independent observers watched the videos of the experimental sessions within the NER, one for each subject. Point events and state events were distinguished, the former indicating instantaneous events, while the latter indicating events with a certain duration. For example, the grasping actions are considered as point events because they could be localized in time based on the contact between monkey's hand and the target, while resting was defined as state event because it has a relevant and highly variable duration. Next, the observers categorized in general terms the most important actions performed during the sessions, for example: walk, grasp food, grasp to climb, rest, yawn … (Table 2). Considering the brain area of interest, we decided to differentiate left- and right-hand actions.

Table 2. Ethogram of behaviors in freely-moving condition

Behavior code	Behavior type	Description
liquid reward	Point event	Monkey receives passively liquid reward with syringe (directly in the mouth) - start when the mouth touches the syringe.
solid reward	Point event	Monkey receives passively solid (fruit pieces, raisins, ...) reward directly in the mouth - start when the food touches the mouth - Only if the experimenter gives it.
grasp food R	Point event	Monkey grasps food pieces with the right hand - Start when hand touches food. Grasp in a hole: start when the finger enter the hole. If bimanual add a comment.
grasp food L	Point event	Monkey grasps food pieces with the left hand - Start when hand touches food. Grasp in a hole: start when the finger enter the hole. If bimanual add a comment.
failed grasp	Point event	Monkey tries to grasp food pieces with left or right hand but fails - Start when hand touches food. Failed grasp in a hole: start when the finger enter the hole.
active food to the mouth R	Point event	Monkey actively places the food into the mouth with the right hand - start when the right hand reaches the mouth. If bimanual or hand-mouth interaction add a comment.
active food to the mouth L	Point event	Monkey actively places the food into the mouth with the left hand - start when the left hand reaches the mouth. If bimanual or hand-mouth add a comment.
grasp food with mouth	Point event	Monkey eats food with its mouth (it does not pick it up with hands) - Start when mouth and food get in contact.
walk	State event	Monkey moves from one location to another (not climbing) - Context independent - Start at the first step, minimum two steps with each hand. Stop when last limb touches the floor/structure and the monkey does not move for, at least, 2 seconds.
rest	State event	Monkey stands still: in this moment monkey is not walking - Start when the rear-end touches the ground. Stop when the rear-end gets up. – Minimum: 2 s
scratching	Point event	Start when hand touches the body the first time.
yawn	Point event	Start when mouth starts opening.
threat	Point event	Start when monkey starts moving the mouth.
grasp thread R	Point event	Monkey grasps nylon thread with the right hand - Start when hand touches the nylon thread. If bimanual add a comment.
grasp thread L	Point event	Monkey grasps nylon thread with the left hand - Start when hand touches the nylon thread. If bimanual add a comment.
grasp climb R	Point event	Monkey grasps something with the right hand for climbing – Start when hand touches the object. If bimanual add a comment.
grasp climb L	Point event	Monkey grasps something with the left hand for climbing – Start when hand touches the object. If bimanual add a comment.
step hand R	Point event	Monkey step with right hand – Start when the right hand touches the floor/structure. Only those steps included in the walk behavior, all steps not included in the walk has to be considered as undefined.
step hand L	Point event	Monkey step with left hand – Start when the left hand touches the floor/structure. Only those steps included in the walk behavior, all steps not included in the walk has to be considered as undefined.
grasp solid reward R	Point event	Monkey grasps food given by the experimenter with the right hand.
grasp solid reward L	Point event	Monkey grasps food given by the experimenter with the left hand.
undefined	Point event	Monkey does movements not better explained in the ethogram.
autogrooming	State event	Monkey does autogrooming - Start at the first touch, Stop the first time the monkey stops touching itself.

grasp for grooming R	Point event	Monkey grasps itself with the right hand for grooming - Start when the fingers are closed.
grasp for grooming L	Point event	Monkey grasps itself with the left hand for grooming - Start when the fingers are closed.
power step R	Point event	Monkey grasps the wooden structure or the cage for walking. Start when the hand touches the wooden structure/cage. Only those power steps included in the walk behavior, all power steps not included in the walk has to be considered as undefined.
power step L	Point event	Monkey grasps the wooden structure or the cage for walking. Start when the hand touches the wooden structure/cage. Only those power steps included in the walk behavior, all power steps not included in the walk has to be considered as undefined.

Behavioral analysis was performed by means of offline analysis of the video-recorded sessions.

To increase the accuracy and reliability of behavioral scoring, the video recordings were analyzed by different observers independently and then it was calculated the inter-rater reliability using the Cohen's kappa statistic ($k>0.7$; Cohen, 1960). Finally, the output for each session contained all the behavioral events with the exact timestamp in which they happened in case of point events.

3.7.3 Peripersonal space testing

As part of the broader project in which this study was carried out, we also designed a methodology to investigate the visuo-tactile properties of the ventral premotor neurons, in both the conditions previously described. The results and experiments are not presented here as the data analysis is still in progress.

Ventral premotor cortex neurons are known to respond to visuo-tactile stimulations in the peripersonal space of head-restrained monkeys in the primate chair (Fogassi et al., 1996), but it is still matter of debate how these neurons would respond in a freely-moving condition, when the monkey approaches or avoid stimuli rather than being passively administered with various types of moving stimuli with limited motor possibilities.

Thus, during the first part of a series of sessions, we studied the visuo-tactile and motor properties of the premotor cortex neurons with classical methodologies while the monkey was head-fixed in the primate chair. As part of the training procedures, the experimenter trained the monkey to remain still while applying somatosensory stimuli to specific body parts. The experimenter used a spherical marker (automatically tracked by the multicamera system) attached to the tip of a stick to touch the subject's face and upper body, whilst the monkey was blinded by opaque goggles to avoid visual confounds in the tactile-related neural activity. Then, the goggles were removed and the experimenter used a longer stick with a black plastic ball on its end (6 cm diameter, tracked by

the multicamera system) to stimulate the whole visual field with different movement patterns both in the subject's peripersonal and extrapersonal space. The tactile stimulation lasted 20-minutes and subsequent visual stimulation additional 30 minutes.

A dedicated, commercially available software for 3D motion data acquisition and analysis (Simi Reality Motion Systems GmbH, Unterschleissheim, Germany) was used to load, visualize and preliminary extract the 3D position of the retroreflective markers. Thus, we were able to track the 3D position of the markers used for the tactile and visual stimulation (gray dots in Figure 9).

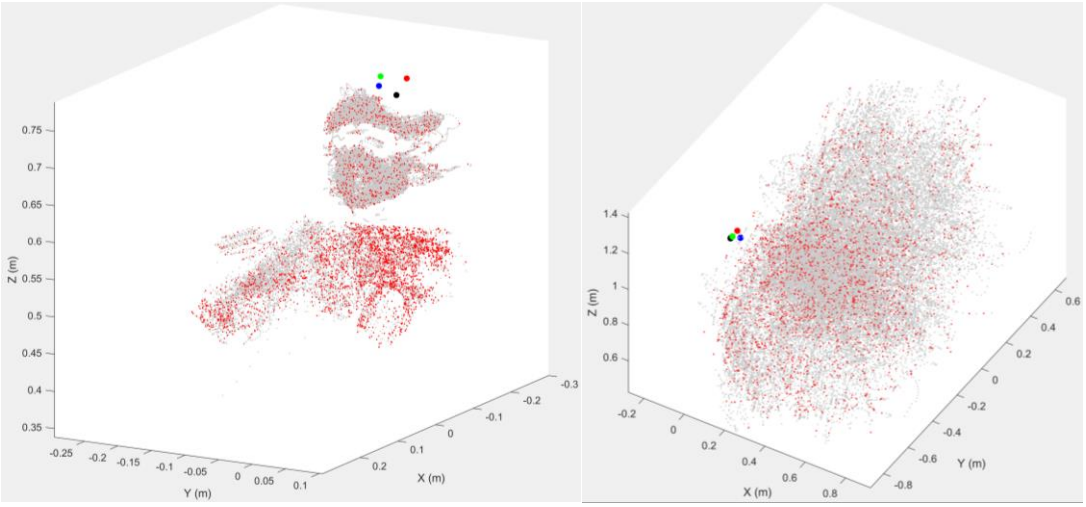

Figure 9. 3D tracking position of the marker in grey, colored in red when the neuron fires. On the left, the marker positions during a tactile stimulation; on the right, the marker positions during a visual stimulation: the four colored dots represent the markers anchored to the monkey's headpost.

Subsequently, during the second step of the session we could track the monkey's position in the NER through a customized head-post cover equipped with 4 retroreflective markers (Figure 10A). Thanks to the SIMI system, we could study the exact monkey's head position and how it moved throughout the session (Figure 10B). Therefore, we could analyze how the neurons responding for visuo-tactile stimulations were modulated during unconstrained behavior.

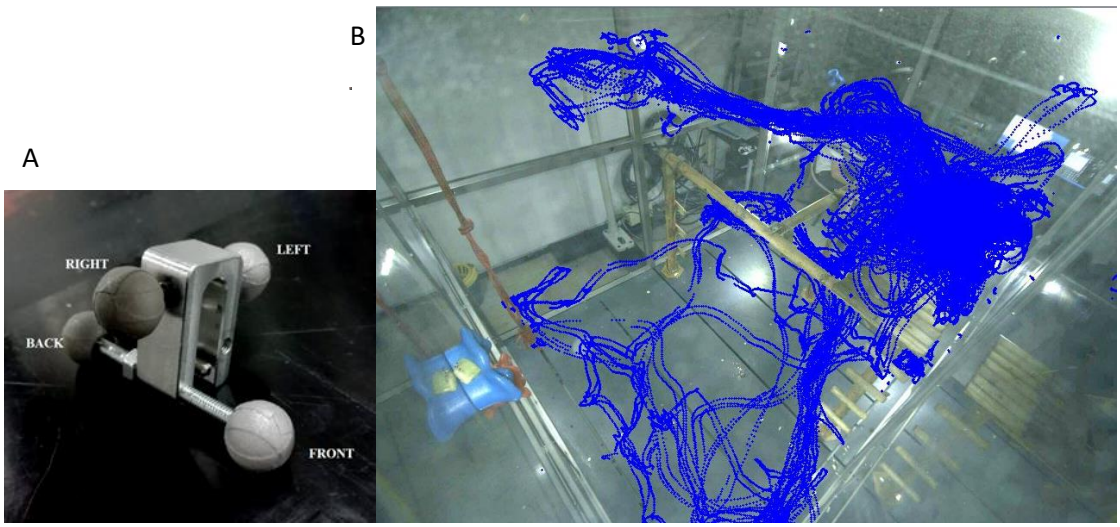

Figure 10. Retroreflective markers were used to track the animal positions throughout the freely moving session. (A) The 4-markers cover used on the head post in order to track the head position and all the angles. (B) The tracked position of the head of the monkey in a 30-minutes session.

This methodology will use multi-channels arrays with continuous acquisition throughout the session to investigate how the peripersonal space coding evolves from a primate-chair condition to a freely-moving condition, assuming that the body-place coding of single neurons characterized in head-restrained condition may vary considerably when the monkey can actively move in the environment, varying the distance of the center of a body-centered receptive field from the objects (or even other subjects) in the environment.

3.8 Neural activity analyses

To obtain the single-unit and multi-unit signals, we used an off-line fully automated spike sorting software (Mountainsort, Chung et al., 2017). In brief, the timepoints of waveforms' negative peaks were first detected using a -3 standard deviation threshold of each channel's signal-to-noise ratio. Next, for any given channel, the waveforms around the detected timepoints were clustered using a nonparametric, density-based algorithm termed ISO-SPLIT, whose only assumptions are that each cluster density function is unimodal and that any two distinct clusters may be separated by a hyperplane. To distinguish single- from multiunit waveforms we used the noise overlap, a parameter that can vary between 0 and 1, with units with a value below 0.1 considered as single. Single unit isolation was further verified using standard criteria (ISI distribution, refractory period > 1 ms, and absence of cross-correlated firing with time-lag of ≈ 0 relative to other isolated units to avoid oversampling); possible artifacts were removed, and all the remaining waveforms that could not be classified as single units formed the multi-unit activity.

3.8.1 Behavioral Analysis

We synchronized scored behavioral events with the neural activity for the whole session.

For each neuron and trial, we calculated the mean firing rate in 200ms time windows, ranging from the -1100/-900ms window to the +900/+1100ms windows, where 0 corresponded to the onset of the behavioral event. We performed this on seven randomly chosen trials and assessed statistical significance by applying one tailed t-tests between the firing rate values in each window and the neuron's average firing rate in the whole testing condition. Then, we repeated the above procedure 100 times by subsampling the seven trials without replacement, and obtained a 100 x 110 matrix of p-values (where 110 is the total number of time windows). For each window, we calculated the average p-value across iterations with the Bonferroni geometric function (Vovk and Wang, 2020, excluding p-values equals to 0 or 1).

The Bonferroni geometric function is:

$$F_K^{BG}(p_1, \dots, p_K) := 2\min\{K\min(p_1, \dots, p_K), e\tilde{p}\}$$

where $\tilde{p}$ is the geometric mean of $p_1, \dots, p_K$.

This function implies that the p-value mean is equal to the minimum between two mean types, which are the Bonferroni mean and the geometric mean. As a result, we obtained the p-value mean through the session.

We considered a neuron significantly modulating for that behavior if we found at least five consecutive bins with a p-value mean under 0.05.

3.8.2 Burst Analysis

Firing rates were computed by binning the spiking activity of each neuron in 20-ms time bins, smoothing with a 100-ms Gaussian kernel, for the chair-condition and the NER-condition separately.

For each neuron, we calculated the smoothed firing rate for the two tested conditions, chair and NER. We calculated the 95th percentile for each neuron for chair and NER conditions separately. We defined burst as the time interval where the firing rate exceeds the 95th percentile for at least 15 consecutive bins (300 ms).

For each neuron we matched bursts with behaviors, looking for behaviors from 500 ms before the start of the burst to 500 ms after the stop of the burst. If more than one behavior was matched to

the burst, we chose the one nearest to the mean time between start and stop of the burst. We defined “empty” a burst, where there were no behaviors matched.

For each neuron and for each behavior, we calculated the percentage of trials that have a burst matching that particular behavior, relative to its total occurrences. Then, we used these percentages across all neurons to calculate pairwise correlations between different behaviors, both intra- and inter-conditions.

4. Results

We isolated 121 single neurons (n=60 in W; n=61 in R) with highly restrictive criteria (see Methods) in recordings carried out from 128 electrodes of all the chronic arrays implanted in monkey W (Figure 11A), and from 128 electrodes of the chronic arrays B, C, D and E implanted in monkey R (Figure 11B). Neuronal activity was recorded in a series of naturalistic tasks performed with the monkey's head fixed in the primate-chair (Chair condition), and next while the monkey freely moved in the NER (NER condition).

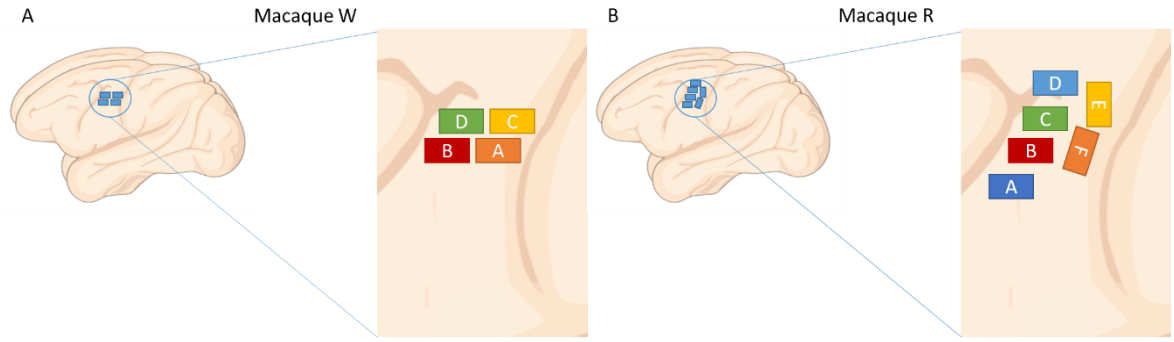

Figure 11. Schematic representations of the insertion sites in the premotor cortex. (A) Left hemisphere of the first macaque, W; (B) Left hemisphere of the second macaque, R.

We analyzed one session per monkey (W and R), and scored the behavioral events as described above (see Methods). Table 3 illustrates the relative number of behavioral events classified in the chair and NER conditions, while Figures 12, 13, 14 and 15 show how the behavioral occurrences are distributed along the sessions' timelines.

Table 3. Behavioral events in chair and NER conditions

Chair Behaviors	Occurrences		NER Behaviors	Occurrences	
	W	R		W	R
active food to the mouth R	21	23	active food to the mouth R	24	28
active food to the mouth L	24	23	active food to the mouth L	77	46
solid reward	11	14	solid reward	0	17
liquid reward	50	63	liquid reward	8	21
failed grasp	6	14	failed grasp	1	15
grasp near R	11	8	grasp food R	16	23
grasp near L	12	12	grasp food L	66	33
grasp far R	9	11	grasp thread R	11	6
grasp far L	10	8	grasp thread L	18	5
finger prehension 0° R	15	12	grasp climb R	12	10
finger prehension 0° L	10	13	grasp climb L	12	10
finger prehension 90° R	0	10	grasp solid reward R	2	0
finger prehension 90° L	0	12	grasp solid reward L	0	0

food presentation	42	47	autogrooming	1	0
grasp Exp	16	15	grasp groom R	1	0
active food to the mouth Exp	15	13	grasp groom L	21	0
			step hand R	96	77
			step hand L	94	55
			power step R	60	34
			power step L	72	38
			scratch	1	3
			yawn	21	0
			threat	0	0

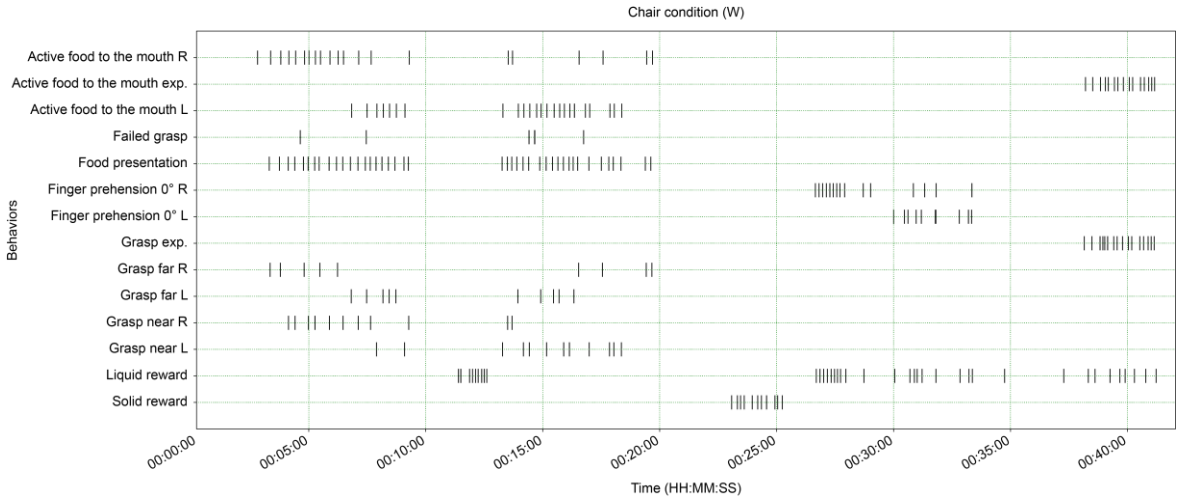

Figure 12. Example timeline of behavioral events during the Chair condition for the first subject (W).

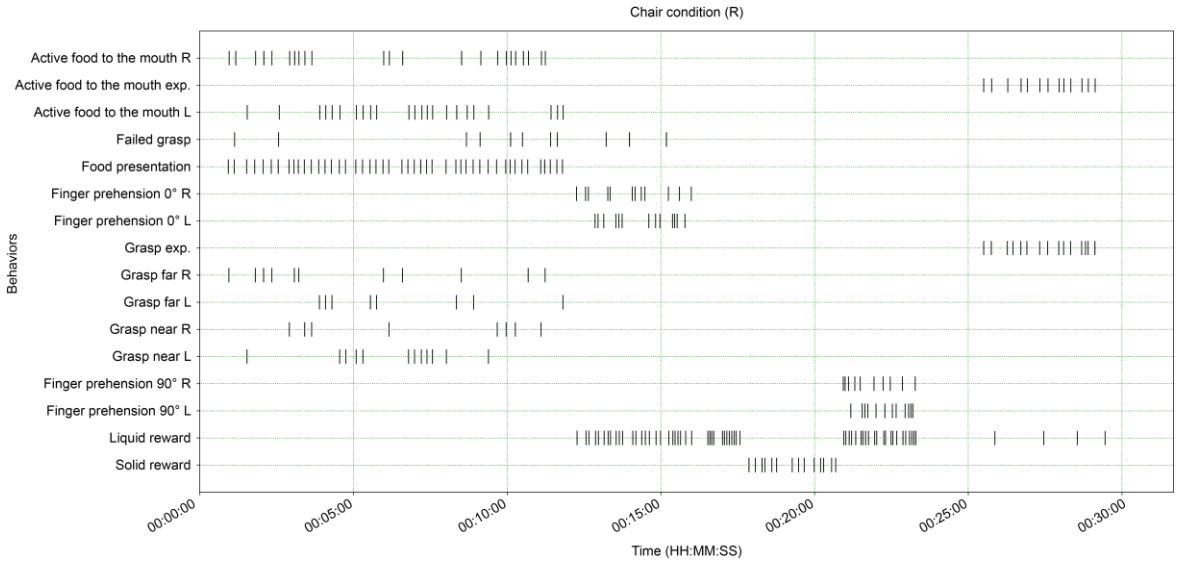

Figure 13. Example timeline of behavioral events during the Chair condition for the second subject (R).

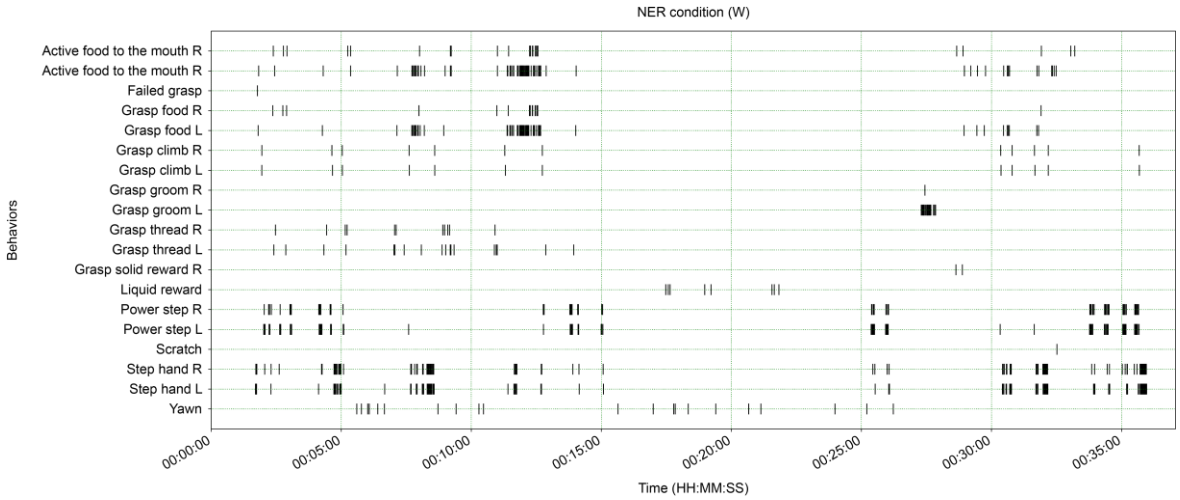

Figure 14. Example timeline of behavioral events during the NER condition for the first subject (W).

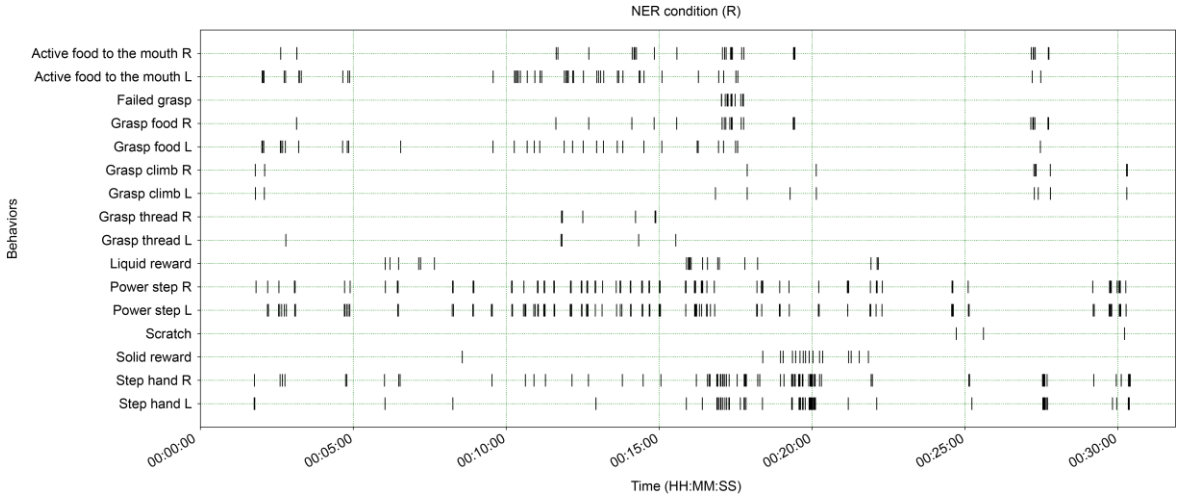

Figure 15. Example timeline of behavioral events during the NER condition for the second subject (R).

4.1 Single neuron functional properties in relation to behaviors

We studied the neural activity with relation to the various behaviors, both in the Chair condition and in the NER condition, scored along the entire session as described above (see Methods).

For each neuron and each behavior, we performed a one-tail sliding t-test (200ms windows advanced in steps of 20ms) between the neuron's firing rate in a 2-seconds interval centered on the behavior and its average firing rate in the condition to which the behavior belongs, as described in Methods.

4.1.1 Chair condition

First, we adopted a classical neurophysiological approach and investigated the response of individual units to specific behaviors and testing conditions (chair vs NER). We found 53 out of 121 (44%) neurons (25/60, 42% in monkey W; 28/61, 46% in monkey R) with a significant modulation of their discharge during at least one behavior, whereas the other fraction did not appear to be responsive for any of the scored behaviors. The single-units modulated for at least one behavior in the chair condition have been found in all the recorded arrays of both macaques.

Figure 16 shows an example of a “mouth” neuron modulated during bringing food to the mouth regardless of the (left or right) hand used, and mouth behaviors, such as receiving passively pieces of fruit and fruit juice directly into the mouth and subsequent mouth behavior (such as chewing and swallowing). Figure 17 shows an example of a “hand” neuron that, instead, discharge specifically during manual actions with the left hand. Figure 18 shows an example of another neuron discharging during both hand-and-mouth behaviors, including the two main types of grip tested in the Chair Condition.

In the chair condition, we found a total of 53 neurons responding to mouth behaviors, or to manual behaviors or both. In particular, 43 (23 in W and 20 in R) single units responded for at least one mouth behavior and 39 (14 in W and 25 in R) to at least one manual behavior; more in general, 53% of them responded to both manual and mouth actions. In summary, we found a sizeable number of neurons responding for the scored behavioral events as shown in Figure 19, except from actions performed by the experimenter, which did not produce any modulation in the recorded neurons.

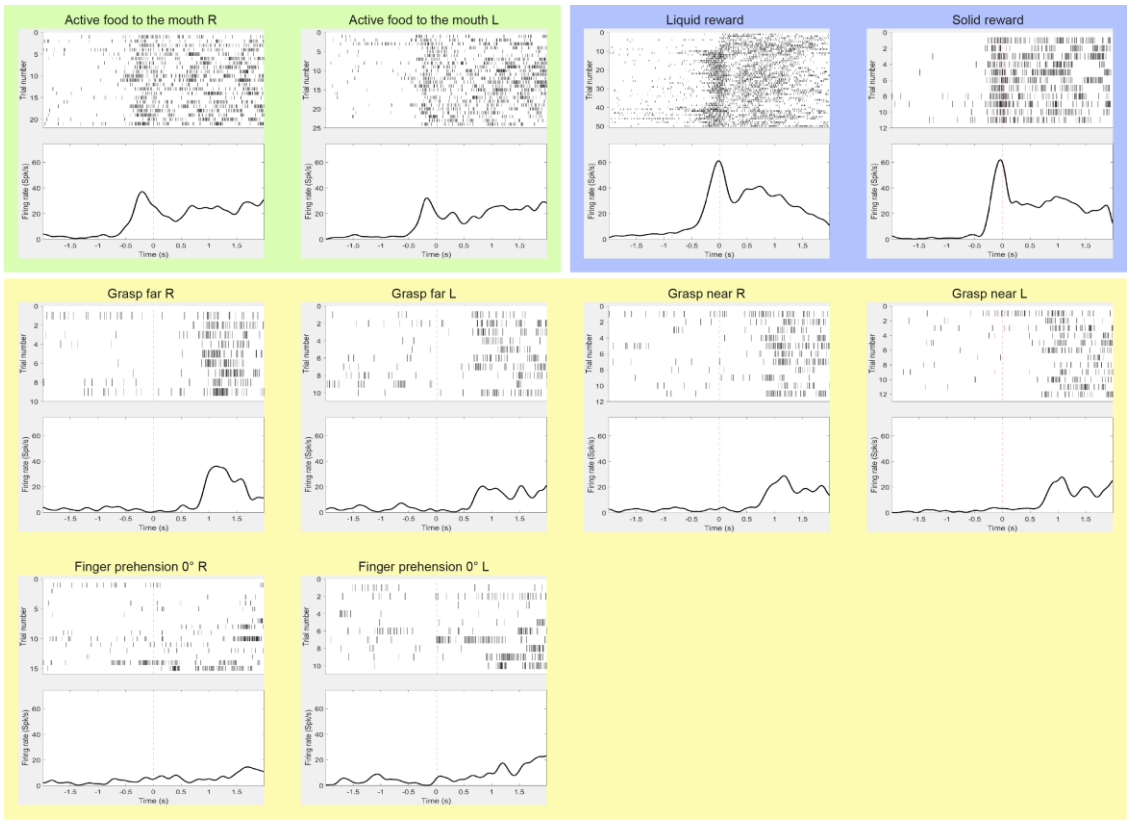

Figure 16. Single-unit from the first subject responding for hand-and-mouth and mouth actions during the primate-chair condition. Significant modulations found in: "Active food to the mouth R", "Active food to the mouth L", "Liquid reward", and "Food reward". We reported mouth actions in green, when the monkey was actively bringing food to the mouth; mouth actions in blue when the monkey was passively receiving food or liquid rewards, and manual actions in yellow.

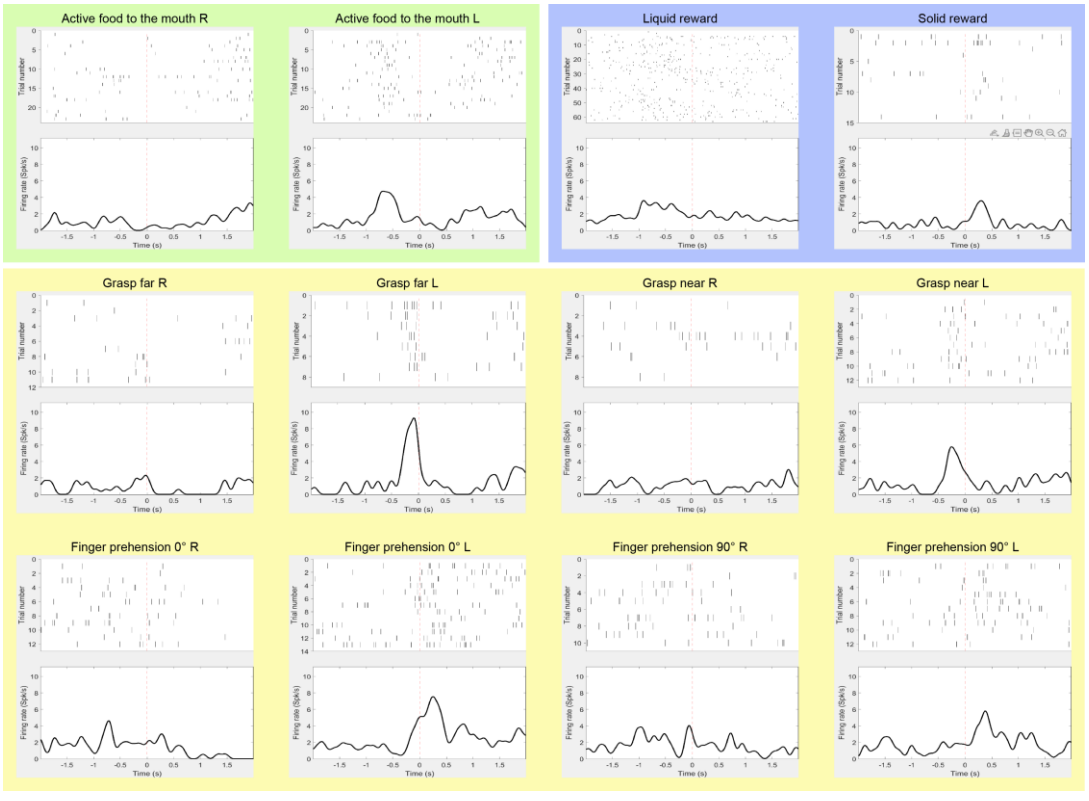

Figure 17. Single-unit from the second subject responding for manual actions during the primate-chair condition. Significant modulations found in: "Grasp far L", and "Finger prehension 0° L".

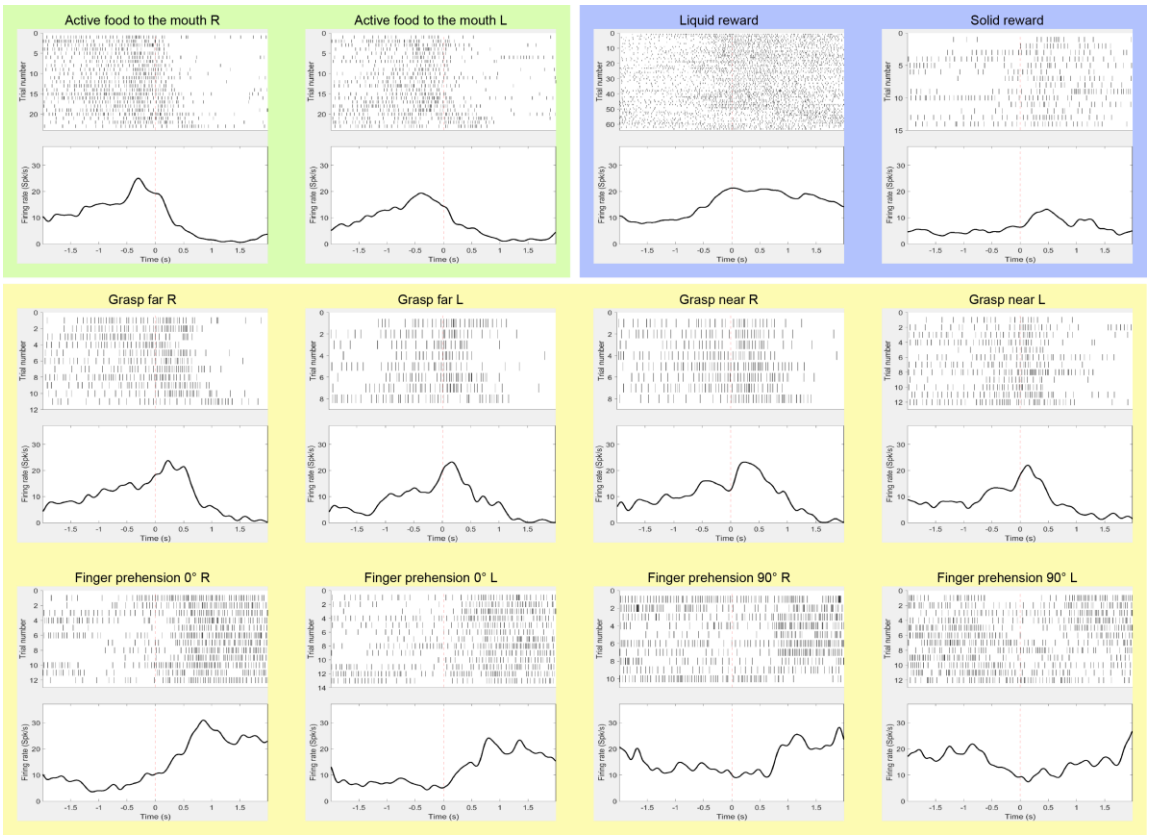

Figure 18. Single-unit from the second subject responding for hand-and-mouth, mouth and manual actions during the primate-chair condition. Significant modulations found in each behavior displayed.

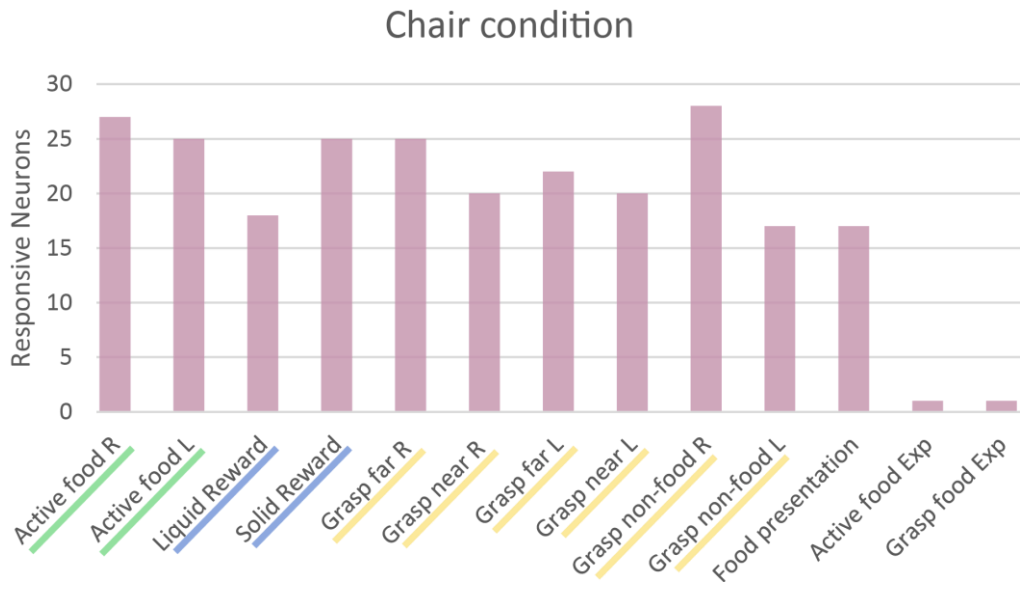

Figure 19. Total number of neurons for both monkeys, which respond to different behaviors in the chair-condition. We put together "Finger prehension 0°" and "Finger prehension 90°" as the category Grasp non-food. We underlined mouth actions in green, when the monkey was actively bringing food to the mouth; mouth actions in blue when the monkey was passively receiving food or liquid rewards, and manual actions in yellow.

4.1.2 NER condition

By analyzing single neurons' activity during specific behaviors of the freely moving monkey we found 31 out of 121 neurons (13/60, 22% in monkey W; 18/61, 30% in monkey R) with the same ethological approach described above to study single neurons' activity in the chair condition. As for units tested in head-restrained condition, also when tested in the NER no anatomical segregation of the recorded neurons was observed in the two animals.

Figure 20, 21 and 22 show the same three neurons as in Figures 16, 17 and 18 aligned to the behavioral events of interest detected in the freely-moving session. The neuron in Figure 20 was significantly active for hand-mouth and mouth behaviors during both chair (light gray) and NER (black) conditions, while the neuron showed in Figure 21, was responsive for manual actions only during the Chair condition and it did not exhibit the same pattern during the NER condition. In Figure 22, the example unit responded for different types of hand and mouth behaviors in both conditions, but with a clearer response while the subject was in the primate-chair.

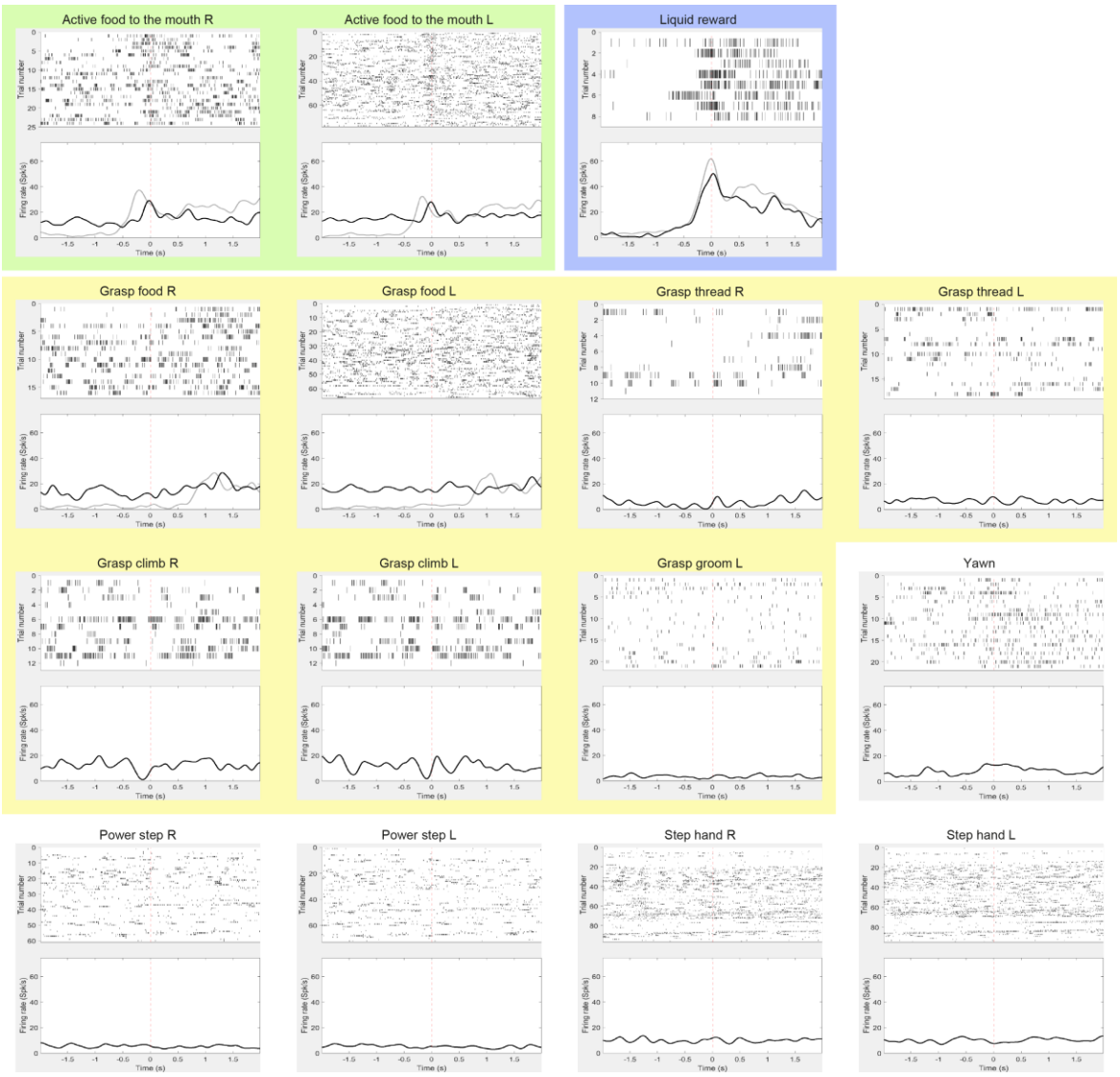

Figure 20. Same single-unit shown in Figure 16 for primate-chair condition, responding for mouth motor actions during the freely-moving condition. Significant modulations found in: "Active food to the mouth R", "Active food to the mouth L", and "Liquid reward". The average firing rate during the primate-chair condition is shown in gray for hand-mouth behaviors ("Active food" on the same event in the NER condition), mouth action ("Liquid reward" on the same event in the NER condition) and manual behaviors ("Grasp near" on the similar event "Grasp food" in the NER condition).

Figure 21. Same single-unit shown in Figure 17 for primate-chair condition, where it responded for manual actions. During the NER condition, the "Grasp food" behavior is no more clear, while we found an interesting modulation in "Power step" and "Step hand". The average firing rate during the primate-chair condition is shown in gray.

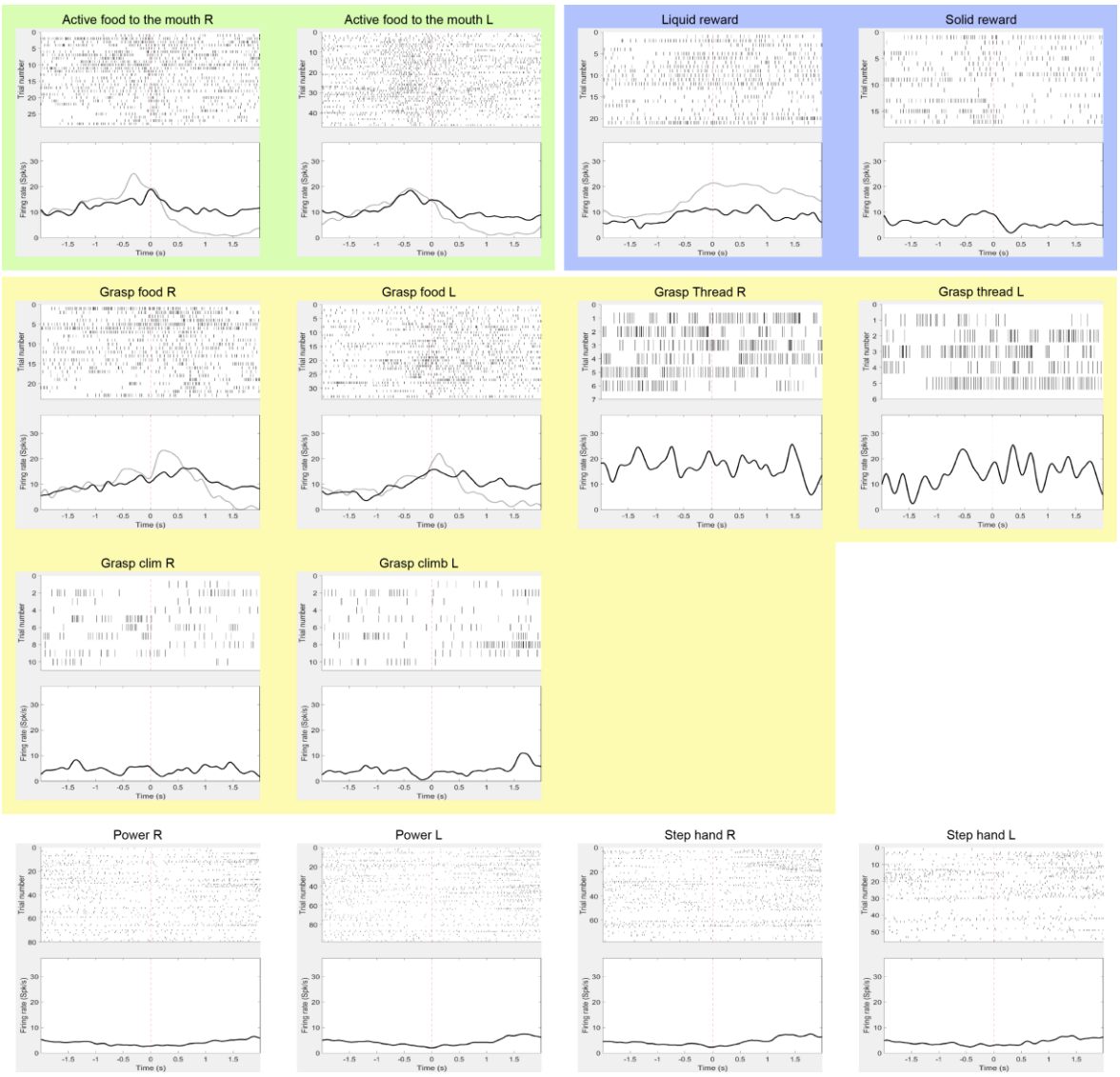

Figure 22. Same single-unit shown in Figure 18 for primate-chair condition, where it responded for both manual, mouth and hand-mouth actions. During the NER condition, the neuron is still significantly responding for "Grasp food", "Active food" and "Liquid reward", even if it is worth notice how the average firing rate shows a slightly different pattern. The average firing rate during the primate-chair condition is shown in gray.

It is clear, based on the example neurons presented above, that there is a broad correspondence between the neuronal responses investigated with the monkey's head fixed in the primate chair and during its free behaviors in the NER, as also shown by the lower number of neurons modulated during the NER relative to the Chair condition (Figure 23).

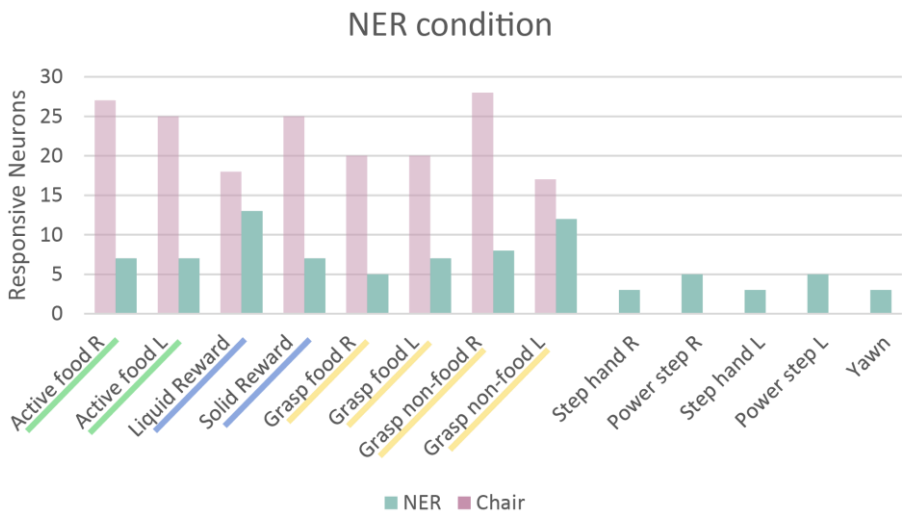

Figure 23. Total number of neurons for both monkeys, which respond to different behaviors in the NER-condition. We put together "Grasp climb" and "Grasp groom" as the category Grasp non-food, and we presented behavioral events that occurred only for one of the subjects. For some behavioral events, we reported with a light pink the correspondent bar of the same event during the Chair condition as described in Figure 19.

Thus, we more specifically aimed at assessing the possible match between the neuronal properties in the two contexts, with the null-hypothesis that – if the neuronal response and the firing rate/behavior relationship is captured in a reliable and ecologically-relevant manner by testing neurons in head restrained conditions - then the relationship between single neuron's tuning and behavior should be the same also when tested in the NER, which is the context more closer to the one in which we need to clarify the brain-behavior relationship.

4.1.3 Comparison between conditions

In total, we found 58 single-units modulating for at least one scored behavioral event in the two sessions together. The same neuron could be active exclusively in one of the two context, either the chair (n=27) or the NER (n=5), whereas a set of neurons became active in both contexts (n=26; Figure 24).

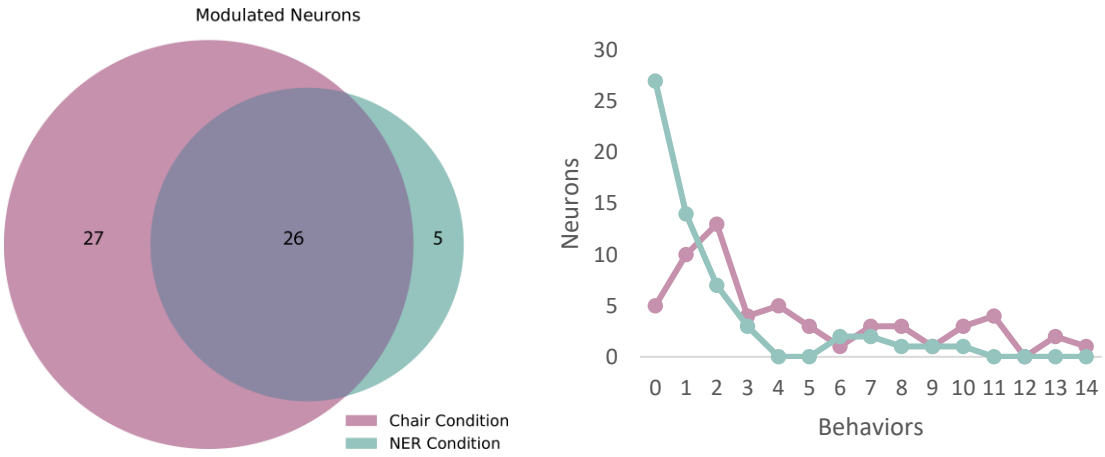

Figure 24. On the left, Venn diagram showing the condition/s associated with the modulation of single-units. In particular, in the first subject (W), we found a total of 27 responsive neurons to at least one scored behavior, where 11 are significantly firing in both conditions (41%), 14 are responsive only in the chair-condition (52%) and 2 only in the NER condition (7%). In the second subject (R), we found a total of 31 responsive neurons, where 15 are significantly activated in both conditions (48%), 13 are responsive only in the chair-condition (42%) and 3 only in the NER condition (10%). On the right, a frequency plot representing how many behaviors are modulated by how many neurons, independently from the condition.

In the freely-moving condition, we classified more behaviors that could correlate with the neural activity, some of them comparable with the chair condition (i.e. grasp, active food to the mouth…), whilst other are strictly related to an unconstrained condition (i.e. scratch, step…). Figure 25 shows a neuron responding selectively when the monkey was yawning, which is testable only in the NER condition, and Figure 26 an example neuron responding reliably during quadrupedal walking.

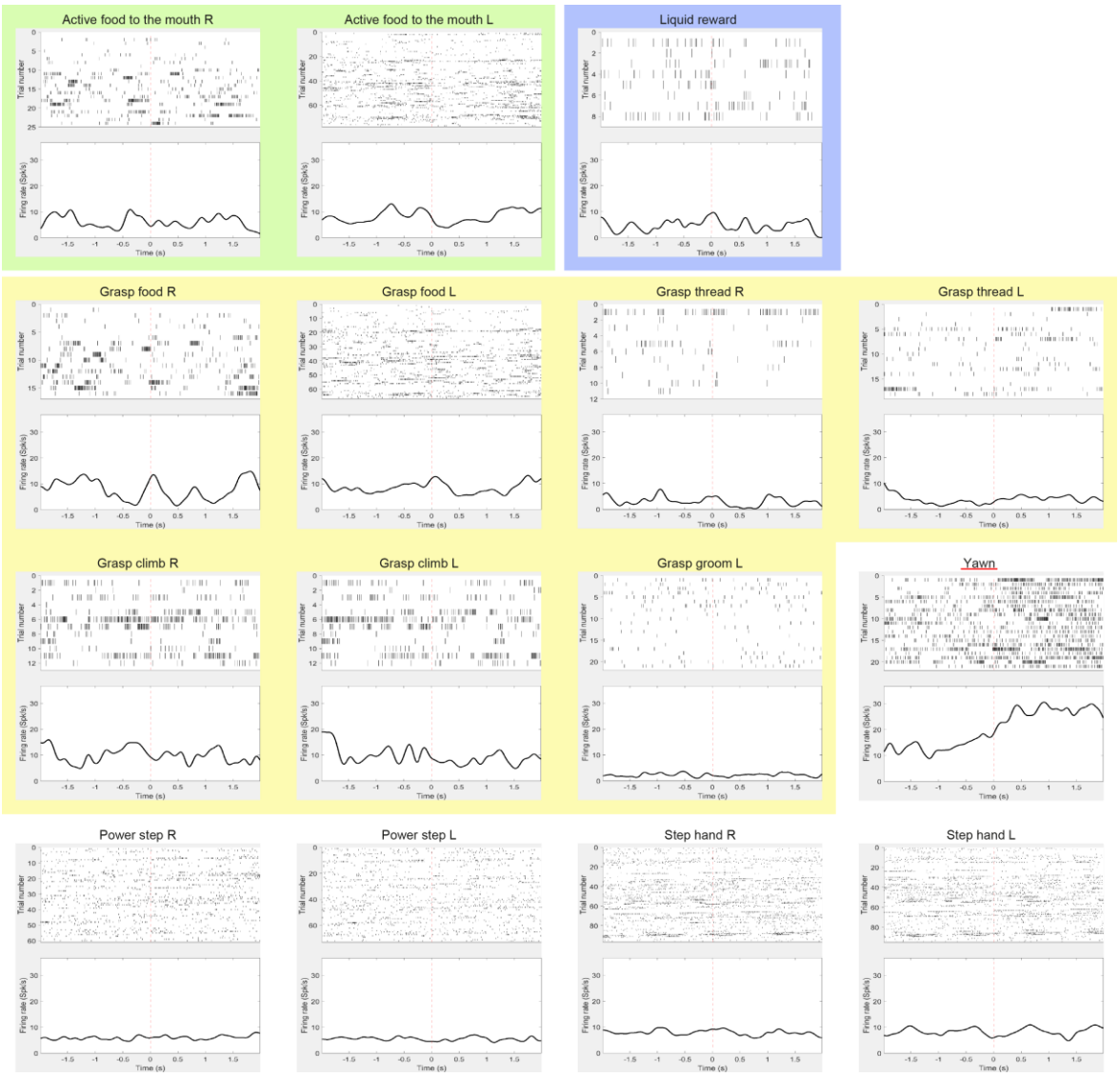

Figure 25. Single-unit activity during the NER condition, where we can observe a significant response for the "Yawn", a behavioral event found only while the monkey was free to move around the enclosure.

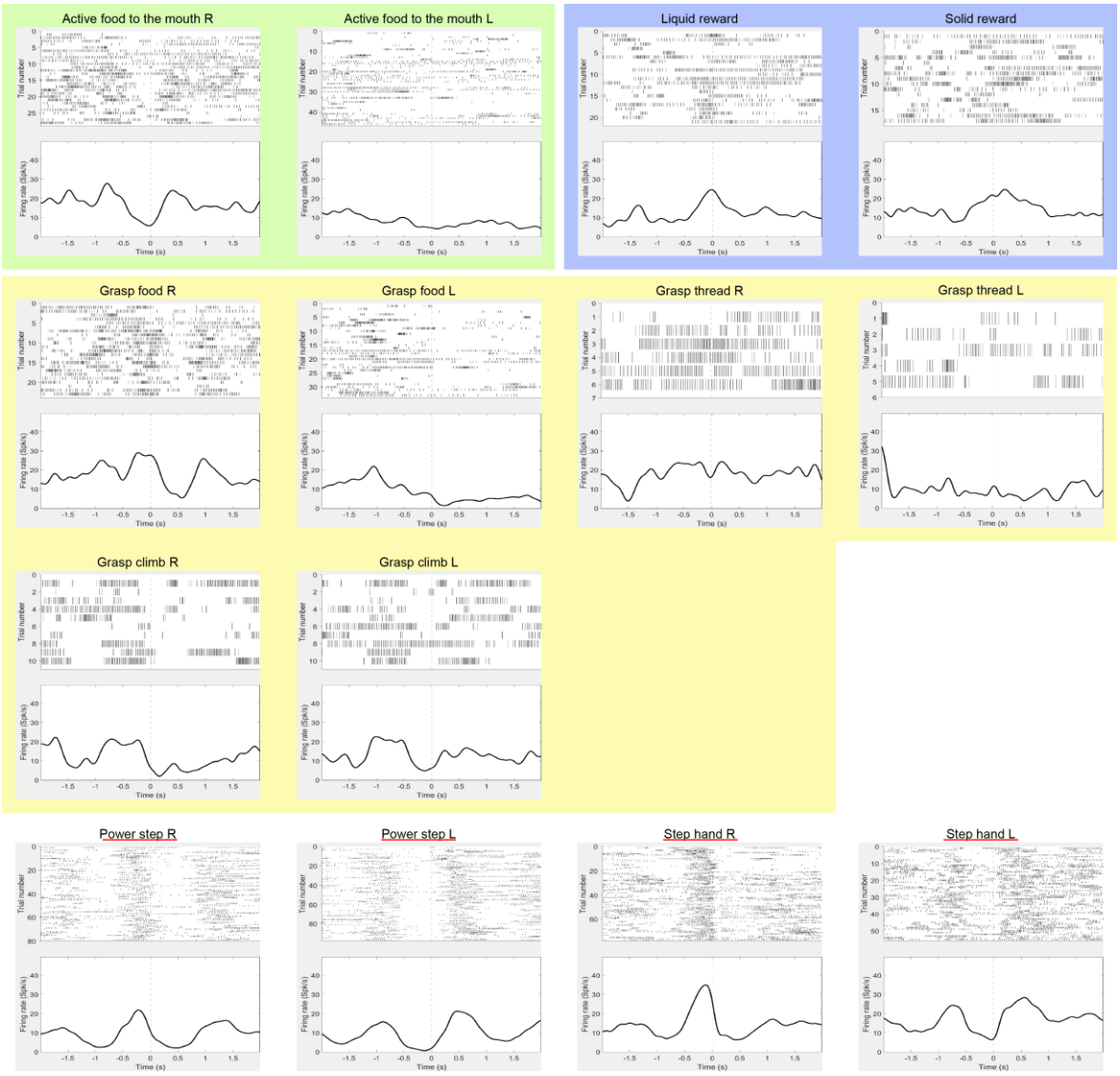

Figure 26. Single-unit activity during the NER condition, where we can observe a significant response for the quadrupedal walking, a behavioral event observable only during the unconstrained movement.

Figure 27 shows the overlap between neuronal tuning between the two conditions (chair and NER) for different behaviors. It is clear that actions involving a robust mouth component (liquid reward, active food to the mouth) are generally those showing the greater number of neurons encoding these behaviors in both contexts. In contrast, distal manual actions, especially when aimed at grasping non-food items, are those showing more distinct neuronal substrates in the two conditions.

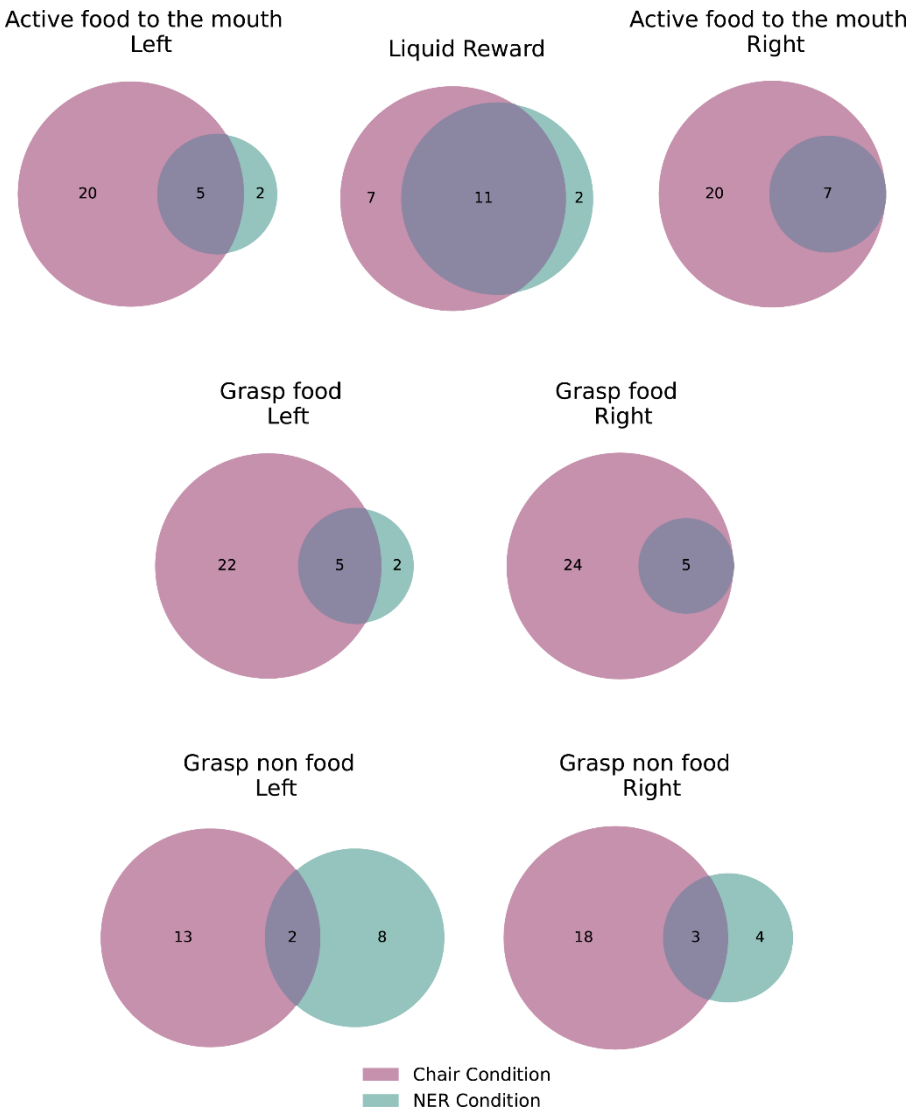

Figure 27. Venn's diagrams representing all the responsive neurons for both monkeys in the two conditions. The first three plots show actions involving the hand-mouth movements, such as "Active food to the mouth" with left and right hand and mouth-related behavior, as "Liquid reward". The other four plots represent behavioral events related to the hand motor actions, such as "Grasp food" with left and right hand, and "Grasp non food" that implies grasping objects (carabiner, rope, etc.).

4.2 Firing features of single neurons: comparison between conditions

The lack of matching between many neurons' functional properties characterized in the primate chair when assessed in the NER, may be due to the greater behavioral variability in the latter context, as also suggested by the greater number and frequency of occurrences of active behavior in the NER relative to the chair. To better scrutinize this issue, we approached the same data with a different type of analysis, where we characterized the single units found in both subjects and, with a behavior-blind approach, we described how the neurons responded during the session in terms of patter of activity. We defined the "bursts" of each neuron based on a set of fixed parameters (see Methods), independent of the condition ("Chair condition" and "NER condition")

and then looked for the possible match between each neuron's individual bursts and the associated behaviors.

First, we assessed single neurons' firing features and compared them in the two contexts (Figure 28). We found a positive correlation in both monkeys between the average firing rate during the Chair condition and during the NER condition (Figure 28A, *linear correlation*, W: r=0.98, p=4.46e^{-41}; R: r=0.92, p=1.81e^{-24}), and between the peak firing rate during the Chair and during the NER condition (Figure 28B, *linear correlation*, W: r=0.93, p=2.15e^{-27}; R: r=0.90, p=1.28e^{-22}). The median burst duration in the chair condition was positively correlated with that in the NER condition (Figure 28C, *linear correlation*: W: r=0.57, p=2.12 e^{-6}; R: r=0.49, p=4.89 e^{-5}). We also found a positive correlation between the percentage of bursts matched with behavioral events in the two different conditions (Figure 28D, *linear regression*: W: r=0.52, p=2.24 e^{-5}; R: r=0.26, p=0.04). Note that the NER condition shows more bursts synchronized with behaviors, but this may be also a byproduct of the greater number of categories and, as a consequence, of the scored events in this context.

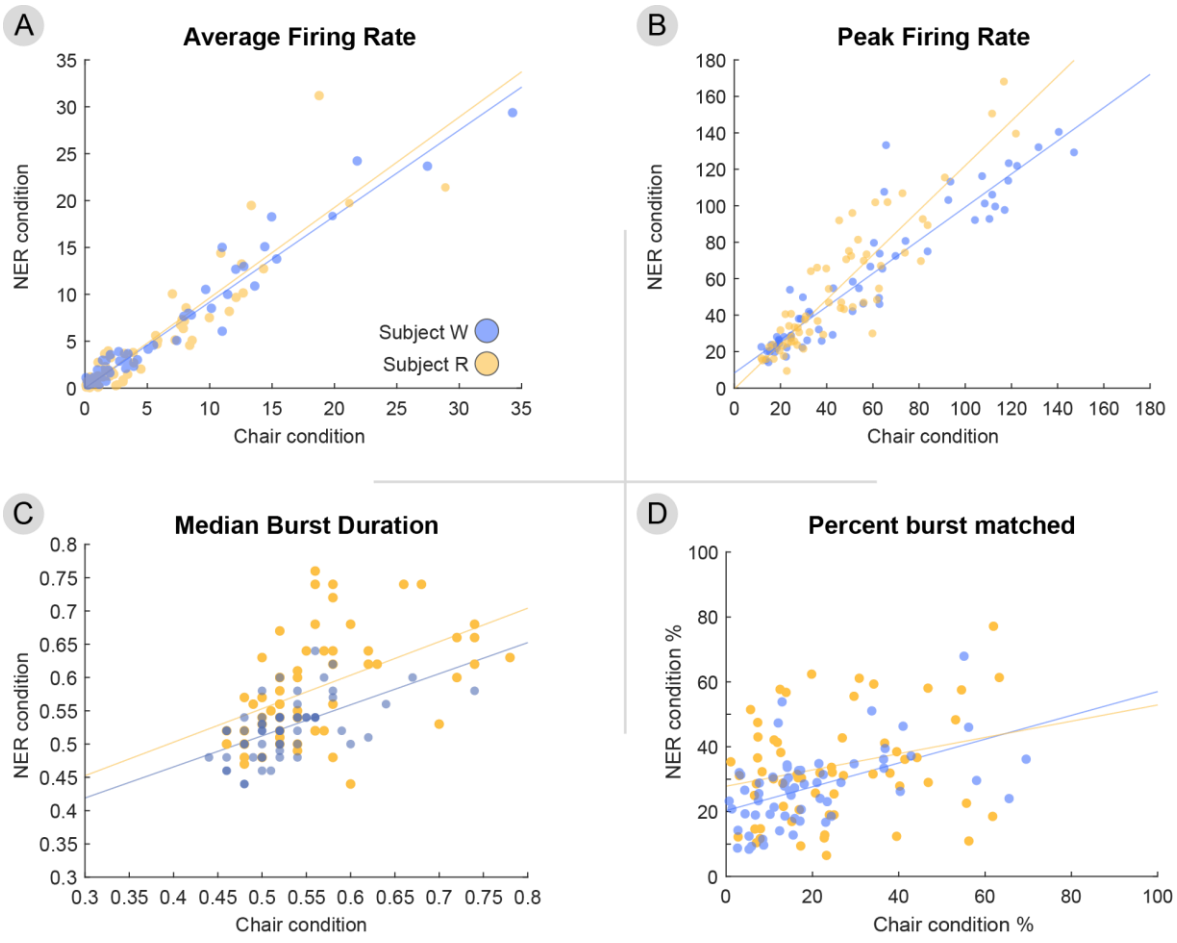

Figure 28. Linear correlations of the: A) average firing rate, B) peak firing rate, C) median burst duration and, D) percentage of burst matched with a behavior between the chair and NER conditions for both monkeys. Data for subject W are shown in blue; subject R is shown in yellow.

For each neuron, we analyzed which percentage of bursts are matched to a behavioral event in each condition (e.g. Figure 29). Subsequently, we looked at what percentage of occurrences of that behavior the single unit was bursting and we plotted the neuron firing rate synchronized to the beginning of the bursts within a fixed time window [-0.5 +1.5 s] relative to the behavioral event (shown with colored markers) closer to the burst onset.

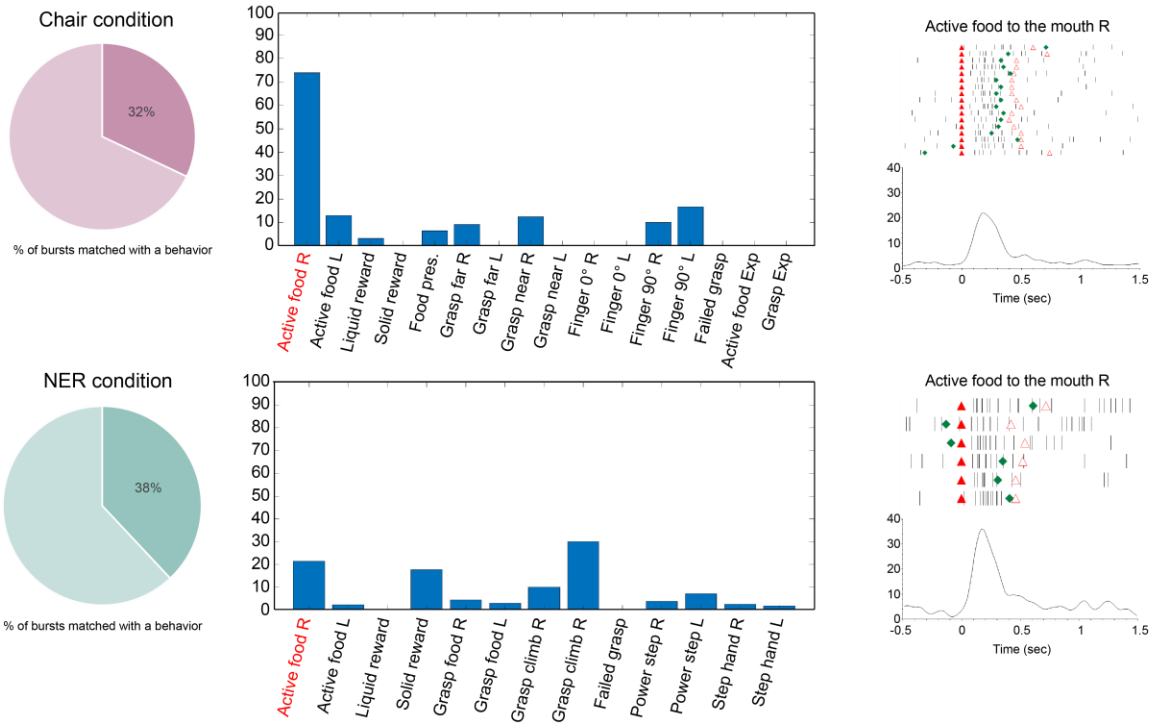

Figure 29. An example of a neuron from subject R that has 32% of its bursts in the Chair condition matched with a behavioral event and 38% in the NER condition. During the Chair condition, this neuron generates bursts in 71% of the trials of "Active food to the mouth R" (n=17), while during the NER condition this happens during 20% of the trials of the same event (n=6). In the raster plot, the red triangle corresponds to the onset of the burst, the white triangle to the offset, and the green symbol to the behavioral event of interest ("Active food to the mouth R").

To directly investigate the similarity between neuronal responses in the two conditions, we correlated the percentage of occurrences matched with a burst in the Chair condition with the NER condition for both monkeys together. As shown in Figure 30, we found single-units with a greater percentage of "Active food to the mouth R" occurrences matched with a burst during the Chair condition than during the NER condition (*linear correlation*, r=0.57, $p=1.22e^{-11}$).

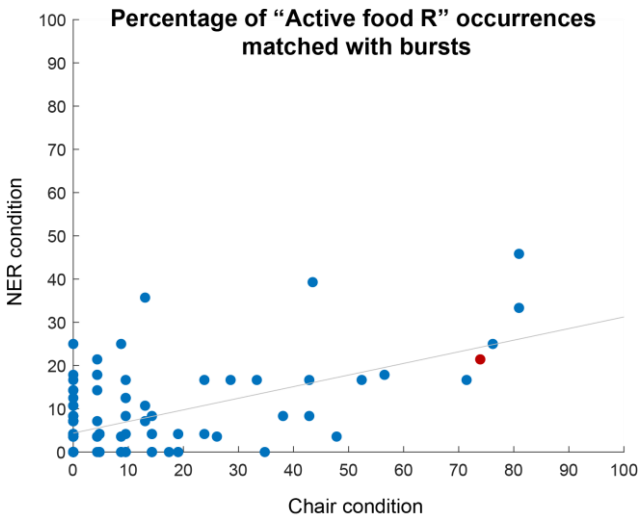

Figure 30. Linear correlation between the percentages of "Active food to the mouth R" occurrences matched with a burst during chair and NER conditions for both monkeys. In red, the example from Figure 29.

Next, to correlate each behavior with every other behaviors in both conditions, we built a similarity matrix for the neurons recorded in both subjects together, where we looked at the responses of all the recorded neurons across all behaviors and conditions (see Methods). The results (Figure 31) show that mouth and hand motor actions are maximally similar in the Chair condition and, to a lesser degree, in the NER condition as well, where we also observed a clustering of behavioral events related to manual actions aimed at different goals, such as climbing or quadrupedal walking ("Power step" and "Step hand"). Looking at the correlation between the two conditions, our findings show a positive correlation between the "Active food" and "Liquid reward" in restraint and freely moving conditions, as well as between the behavioral events related to the subject grasping of food items.

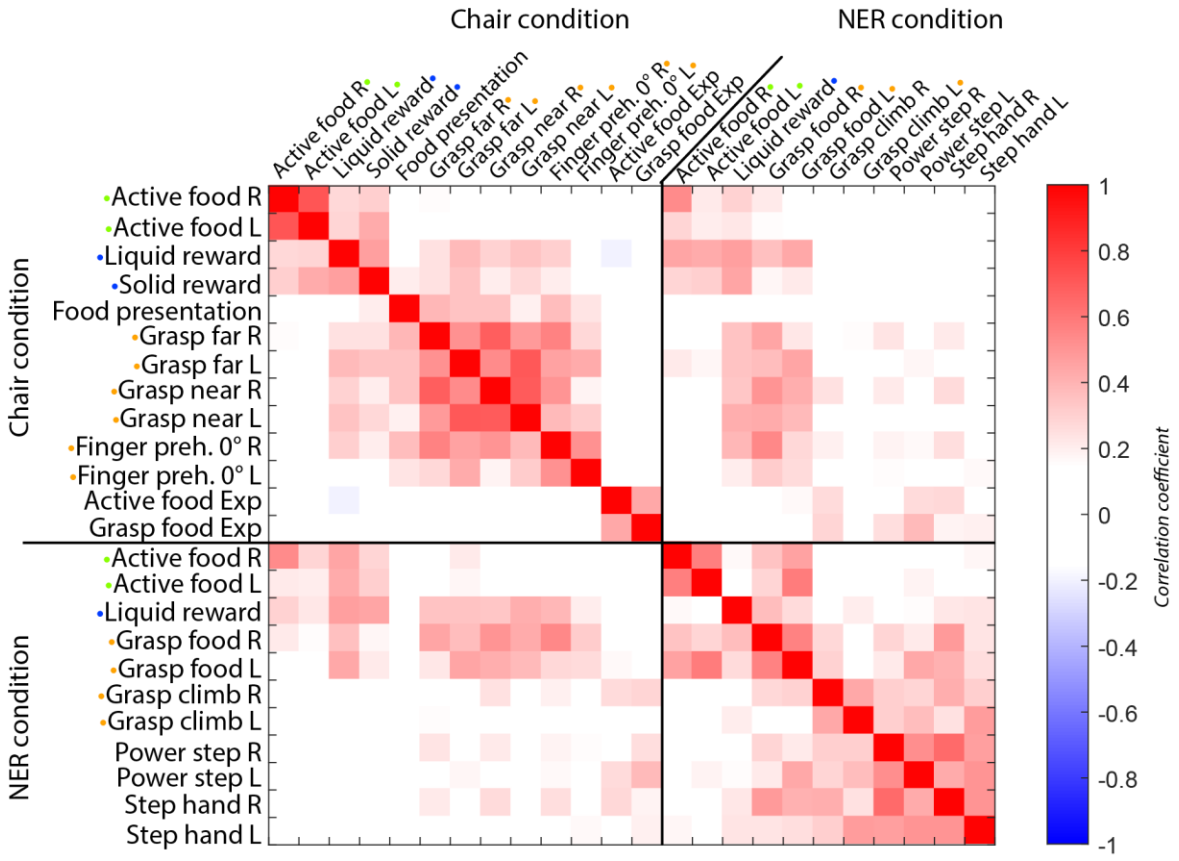

Figure 31. Pairwise correlation between burst-behavior scores of the two conditions. For each neuron and for each behavior, we used the percentages of occurrences of a particular behavior matched with a burst across all neurons to calculate pairwise correlations between different behaviors, both intra- and inter-conditions. Only scored behaviors shared by both subject are taken into account. Comparable behaviors are reported with a colored dot, in particular: hand-mouth actions in green; mouth actions in blue and manual actions in yellow.

5. Discussion

In this study, we developed a two-step approach to compare the traditional, head-restrained neurophysiological approach to the investigation of the cortical motor system with an unconstrained, neuroethological approach in rhesus macaques. During the first step of each experimental session, we focused on a trial-based neurophysiological protocol, while during the second step we aimed at analyzing how the neural activity maintains or changes its relationship with the monkey's behavior during a more ethologically relevant situation.

This study is part of a larger effort of the neuroscientific community (Genzel and Yartsev, 2019; Berger et al., 2020; Nourizonoz et al., 2020), which aims to develop new paradigms allowing a more ecologically valid understanding of the brain-behavior relationship. These new paradigms, allowing to extend freely moving recordings typically performed in rodents and other small animals (McNaughton et al., 1994; Buzsáki et al., 2003; Whitlock et al., 2012) to non-human primates, are making possible to tackle new and more complex questions previously impossible to be addressed with conventional methodologies (Datta et al., 2019; Testard et al., 2021).

In the present work, we recorded neural activity in two macaques within a newly developed protocol and setup allowing us to compare neural activity from the two explored conditions, the chair and the NER. First of all, the results showed a remarkable stability of the single-unit signals throughout the whole session. Our unsupervised spike-sorting approach based on the merge of the raw data collected in the chair and in the NER with the same data logger device, could ensure the comparability of the neuronal data at the single cell level in the two contexts, beyond any possible technical issue. Indeed, we found highly significant and positive correlation of mean firing rate, peak firing rate and median burst duration of the same cells recorded in the two conditions.

Our main analyses demonstrated the stability of the functional properties of a set of neurons, particularly those encoding mouth-related behaviors. In fact, 95% of single-units responsive for mouth behaviors during the NER condition modulated their discharge for similar movements during the Chair condition as well. Behavioral events involving the "mouth", typically include a defined pattern of movements which are repetitive and only to a lesser extent dependent on, e.g. variable axial and/or distal component or visual input changing with the monkey's gaze position. Therefore, it is not surprising that single-units encoding for mouth behaviors appear to be the most robustly tuned across the two contexts.

In contrast to mouth-related neurons, single-units responding to manual actions were much less frequently observed to maintain their functional properties across conditions. Only a 22% of neurons modulating for hand motor actions during the NER condition exhibited a similar coding

during the Chair condition. One possibility for this discrepancy is that behaviors involving distal effectors are part of complex dynamics where different postural variables crucially contribute to the final movement, and strongly depend on context-dependent postural and synergistic controls that are strongly reduced or even eliminated in the constrained condition typical of the head-fixed condition in the primate chair setting. The contribution of a kinematic analysis could help to untangle this issue. Noteworthy, a recent study in freely-moving rats showed that posterior parietal cortex and frontal motor cortex are tuned to posture of the head, neck and back, and with synchronous recordings from both regions they could decode ongoing behavior (Mimica et al., 2018). In this latter study, they analyzed kinematic parameters by means of retroreflective markers tracking technology; we implemented in our setup a comparable tracking method for the head, as shown in "Peripersonal space testing" (Paragraph 3.6.3), mostly used for another study within the framework of the project in which this experiment has been carried out. However, to obtain an accurate estimation of the monkey's posture, we need to devise a way to measure in 3D the head/body-axis angle, which can be predictive of axial muscle involvement and postural changes in a variety of apparently mostly distal forelimb actions. To achieve this goal, future analyses will integrate the use of markerless pose estimation, based on transfer learning with deep neural networks that achieves results with minimal training data (Nath et al., 2019; Bala et al., 2020; Labuguen et al., 2021). Using a combination of retroreflective markers and markerless tracking, we could extract the position and posture of the subject during the NER condition, thereby finding a way to directly investigate the possible involvement of postural component in the encoding of complex behaviors in an unconstrained situation.

Besides the axial component, discrepancies of hand neurons between conditions could be also explained by the different visuo-motor coding, which might be influenced by gaze direction. To better investigate the oculomotor contribution, future studies could integrate new eye-tracking techniques already used for chickens (Schwarz et al., 2013) and rodents (Payne and Raymond, 2017), which exploit magnetic sensing providing a continuous trace of the eye movements while the animal is free to behave naturally.

Our findings showed, in general, stronger and more frequent modulations of single neuron activity during the primate-chair than the NER condition, with 46% of responsive single-units for different behaviors in the chair lacking a correspondent modulation for any behavior during the NER condition. Thus, the obvious question is whether and to what extent we can rely on the neuronal properties characterized in head-fixed and highly constrained condition if a sizeable fraction of them is not predictive of single neuron coding when moving to a more ecologically relevant context.

What about the ethological and ecological validity of the results during the first condition? Establishing possible equivalence between the two contexts is of paramount importance if, for example, a translational aim of the research on the motor system is to apply the knowledge gathered in the lab to repair injuries or devise neuroprosthetic solutions to spinal cord lesions that should ideally function in the highly unconstrained settings of the real life. Our results allow to partially answer to this question: indeed, 45% of neurons respond in both conditions, with similar modulations in both chair and freely-moving situation, despite a relevant number of differences and specificities in the neuronal coding properties in the two contexts. In fact, thanks to the similarity matrix we showed positive correlations between conditions for mouth behaviors and for behavioral events related to the subject grasping food items. Nevertheless, it is not yet clear how much of the neural activity is preserved between the two conditions and how well it overlaps.

Undoubtedly, the freely moving setting is challenging because of the high number of uncontrollable variables, which add noise to the neural signal although it is still clear the relationship of the investigated area with specific manual and orofacial behaviors. To better estimate the correspondence between conditions, further analyses should include other types of neural signals, simultaneously recorded with the individual neurons spiking activity, that is, multi-unit activity and local field potentials. The latter have recently been showed to describe awake and rest states in freely moving macaques (Milton et al., 2020), and may provide broader but also more robust information on the ongoing behavior. Moreover, in recent years we are gaining powerful tools of analysis, including machine learning-based methods that can be applied on large dataset, which are or critical importance to elucidate and understand the neural basis of behavior organization and evolution over time (Datta et al., 2019; Keemink and Machens, 2019). As a future promising direction, focusing on different machine-learning methods will be a priority: we can train and test a decoder to recognize different behaviors across contexts based on neural signal readout, and this approach may lead to identify more general rules underlying the relationship between a larger variety of premotor neural signals and unconstrained behavior. These new approaches will also help to promote novel methodologies to be exploited in the neuroprosthetic field, which often lack the fundamental understanding of the brain-behavior relationship in a natural context, which is critical for ecological validity and clinical relevance of every result.

This novel approach shows a firm step into the neuroethology field, where the NHP studies are still lacking. Different unresolved neurophysiological questions would benefit from this validated setup for freely-moving macaques. Our project will extend towards three main topics: space coding, distal affordances and social interaction. Indeed, the setup and approach validated in this study will allow to investigate how the premotor cortex contribute to encode space in motor terms while the

monkey can actively move in the environment and thus, vary its spatial relationship to different objects or even other subjects – humans or conspecifics. In addition, the same setup and approach can make it possible to go beyond recent findings by Berger and colleagues (2020) and test whether and to what extent different premotor areas can signal what the monkey is going to do when it moves in the environment. In particular, when the subject moves based on visual and acoustic cues in order to perform a previously instructed reach and grasp movement on a specific object that has to be reached with a long sequence of locomotor actions (Orban et al. 2021). Finally, an interesting research branch that can emerge from this project involves the coding of social interactions. As the design of the NeuroEthoRoom allows the possibility to split the space in two, and to use different types of barriers (opaque, reflective, or transparent), it makes possible to manipulate the available social information and interactive possibilities, even by recording from two different subjects and neural data loggers simultaneously (Zhang and Yartsev, 2019).

In conclusion, the methodology and setup presented here, based on the results so far obtained, indicate the possibility to record continuously from a non-human primate in traditional settings and unconstrained contexts, to analyze comparatively the functional properties of neurons recorded in both conditions, and supporting the need of new approaches to generalize the brain mechanisms identified in the laboratory to ecologically and ethologically relevant conditions. Our findings will lead the way to new projects and new ways to study the neural underpinnings of natural behavior in non-human primates.

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