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MONKEY VENTRAL PREMOTOR NEURONS DURING CONSTRAINED AND FREELY MOVING CONDITIONS: FIRING FEATURES

NEURONI DELLA CORTECCIA PREMOTORIA VENTRALE DELLA SCIMMIA IN CONDIZIONI DI RESTRIZIONE E DI LIBERTA' DI MOVIMENTO: PROPRIETA' DI SCARICA

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ABSTRACT – English

Neuroscientific investigations in non-human primates paved the way for a direct investigation of the relationship between brain and behaviours in primates. However, due to highly-controlled traditional settings limitations, the findings obtained so far may be poorly generalizable to the unconstrained settings of the real life, thus hindering the possibility to develop effective brain machine-interfaces to restore brain functions.

In this study, we conceived a novel approach to the investigation of the functioning of primates' ventral premotor cortex in controlling motor behaviours in naturalistic contexts. We implemented a two-step approach, recording monkey's neural activity first in a head-restrained laboratory setting (Chair, CHR), and then during a condition in which the animal was left free to move in a large cage (the NeuroEthoRoom, NER), allowing us to compare the findings obtained with classical neurophysiological settings to those obtained on the same neurons recorded during spontaneous naturalistic behaviours. To this aim we identified time epochs with the highest spiking activity (bursts) and looked for their possible match with behaviours in both conditions (CHR and NER).

In the CHR and NER conditions we found bursts related to mouth and upper limb behaviours during reach, grasp and bring to mouth actions directed to food or objects. However, we found that neurons burst activity in relation to the above mentioned behaviours in the NER condition is lower; this finding along with the high percentage of bursts that appears not to be matched with identified manual and orofacial behaviours, suggests a broader and still unexplored involvement of the ventral premotor cortex in the motor control of naturalistic behaviour. Our findings demonstrate that a two-step approach can open up new avenues to an ecologically valid understanding of the brain-behaviour relationship.

ABSTRACT – Italiano

Studi neurofisiologici sui primati non umani hanno aperto la strada allo studio della relazione tra cervello e comportamento nei primati. Tuttavia, a causa delle limitazioni metodologiche dei setting sperimentali tradizionali altamente controllati, i risultati ottenuti fin'ora sono poco generalizzabili ai contesti di vita quotidiana inficiando la possibilità di sviluppare efficaci *brain-machine interfaces* per riparare le funzioni cerebrali.

In questo studio, abbiamo sviluppato un nuovo approccio per indagare il coinvolgimento della corteccia premotoria ventrale dei primati nel controllo del comportamento in contesti naturalistici. Abbiamo sviluppato un approccio a due fasi, registrando l'attività neurale delle scimmie in una condizione a testa fissa di laboratorio e mentre si muovono liberamente in un vasto recinto (la NeuroEthoRoom, NER), per poter confrontare l'attività degli stessi neuroni nei tradizionali setting sperimentali e durante comportamenti spontanei e naturalistici. A questo scopo abbiamo identificato le epoche temporali con maggiore attività di spike e cercato una loro possibile associazione con i comportamenti studiati nei due contesti (CHR and NER).

In entrambe le condizioni abbiamo trovato risposte (burst) in relazione a comportamenti diretti a cibo e oggetti che coinvolgono la bocca e gli arti superiori. Tuttavia, l'attività di burst dei neuroni in relazione a tali comportamenti risulta inferiore nella condizione NER; ciò, insieme all'alta percentuale di burst che non hanno un match con i comportamenti manuali e orofacciali identificati, suggerisce un ampio ed inesplorato coinvolgimento della corteccia premotoria ventrale nel controllo dei comportamenti naturalistici. I risultati dimostrano come questo approccio in due fasi possa aprire la strada a una comprensione ecologicamente valida della relazione tra cervello-comportamento.

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1. INTRODUCTION

Animal research has been fundamental to answer many questions on human biology that couldn't have been investigated with non-invasive techniques in human subjects (Roelfsema et al., 2014). Neuroscientific investigations, in particular, for years aimed at explaining the relationship between brain activity and behaviour, attempting to identify the processes, the mechanisms and the structures involved; since non-human primates are phylogenetically close to humans, their brain has some important homologies to the human brain in terms of structure and functional organization; therefore, they represent the preferable animal model to study complex sensorimotor and cognitive functions that are uniquely present in primates (Buffalo et al., 2019).

Classical neurophysiological studies on the cortical underpinnings of voluntary behaviour in non-human primates have deeply investigated the functioning of the motor cortex. While initially the dominant idea of the motor system conceived it as a passive apparatus controlled by higher order brain areas to control behaviours, nowadays, the frontal motor and premotor cortices are known to have, along with prefrontal and parietal area's with which they share mutual connections, an active role in selecting, planning and controlling the execution of complex adaptive behaviours finely tuned with the context in which they are performed (Kandel et al., 2015).

So far, neurophysiological studies on non-human primates typically suffered from fundamental technical limitations due to tethered recording systems, which in turn implied to impose subjects highly constrained conditions, and thereby allowing to investigate only simple, highly trained and thus often stereotyped upper limb, eye or mouth movements unrepresentative of natural behaviours (Jackson et al., 2007). Nowadays, thanks to advances in technology, it is possible to overcome traditional limitations in the recording approaches by

leveraging wireless recording system and studying neuronal activity in freely behaving animals. This paradigm shift is making possible to address a wider set of behaviours in more ecologically relevant settings, providing the opportunity to understand the neural basis of naturalistic behaviours.

1.1 Cortical control of motor behaviours: classical neurophysiological studies

According to Brodmann's partitions (Brodmann, 1909), the motor cortex is composed by two main areas: the Brodmann's Area 4 (BA4) and the Brodmann's Area 6 (BA6) that respectively correspond to the primary motor cortex (F1) and premotor cortex and that extend from the central sulcus to the arcuate sulcus. In the latest decades, anatomical and functional studies allowed to discover a more complex organisation of the frontal motor cortex, revealing that it is composed by a mosaic of cytoarchitectonically different areas (Rizzolatti et al., 1998). The supplementary and pre-supplementary motor areas (respectively F3 and F6) represent the most mesial portion of the premotor cortex (Tanji et al., 1996); moving laterally, the dorsal premotor cortex (PMd) occupies the portion on the cortical surface dorsal to the spur, subdivided in a rostral (F7) and a caudal (F2) halves bordering with areas F6 and F3, medially; the most lateral part is represented by the ventral premotor cortex (PMv), located on the cortical surface lateral to the spur of the arcuate sulcus and composed by a rostral (F5) and a caudal (F4) part (Figure 1).

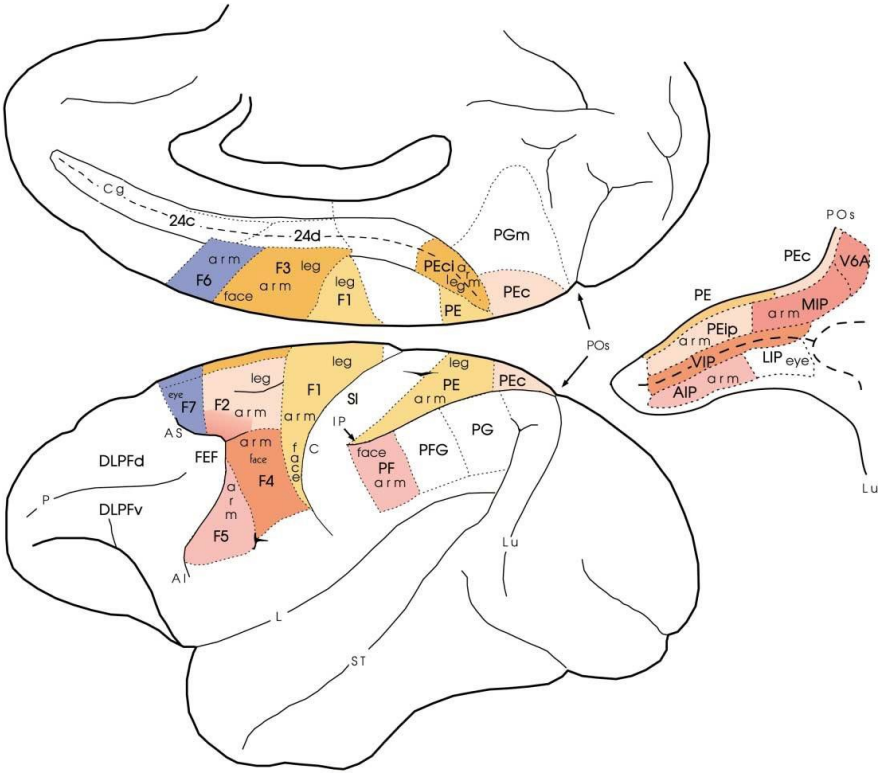

Figure 1. Mesial and Lateral Views of the monkey brain showing the parcellation of the motor cortex, posterior parietal and cingulate cortices. *Frontal motor areas are indicated with the letter F according to Rizzolatti et al. (1998). Parietal areas are defined according to Pandya and Seltzer (1982); those buried within the intraparietal sulcus, shown in an unfolded view of the sulcus in the right part of the figure, are defined according to Rizzolatti et al. (1998). Parieto-dependent motor areas and the parietal areas circuits are represented by illustrating related areas with the same colour. Prefronto-dependent areas are depicted in blue. AI, inferior arcuate sulcus; AS, superior arcuate sulcus; C, central sulcus; Cg, cingulate sulcus; DLPFd, dorsolateral prefrontal cortex, dorsal; DLPFv, dorsolateral prefrontal cortex, ventral; FEF, frontal eye field; L, lateral fissure; Lu, lunate sulcus; P, principal sulcus; POs, parieto-occipital sulcus; ST, superior temporal sulcus (Rizzolatti and Luppino, 2001).*

For years, following Woolsey and colleagues' stimulation experiments (1952), among students dominated the idea that a single motor map existed in the motor cortex in particular distal movements where evoked from area 4, whereas area 6 mostly concerned proximal and

axial movements. However, Rizzolatti and colleagues (1981a, 1981b), in experiments carried out on monkeys, demonstrated that BA6 neurons fire during active mouth movements and can also be activated during tactile stimulation of distal parts of the body (hands and mouth). Additionally, Gentilucci and colleagues (1988) microstimulation experiments demonstrated the presence of distal movements near the central and arcuate sulcus and distal and proximal movements both in F4 and in the rostral portion of F1. These findings, along with Kurata and Tanji (1986) experiment that recorded distal neurons near the arcuate sulcus and proximal neurons in both BA6 and BA4, demonstrate the existence of at least two functionally independent motor maps in the primates' motor cortex, suggesting that the cytoarchitectonic differences between BA4 and BA6 are more likely due to differences in function and not to somatotopic representations as Woolsey and colleagues (1952) suggested.

The organisation of the frontal motor cortex has been analysed in detail by Graziano and co-workers (2002, 2005): by means of intracortical microstimulation (ICMS) with train of pulses of an order of duration (>500ms) approximating the duration of the monkey's behaviours, they highlighted a map complex, multi-joint actions in different zones of the motor cortex. Indeed, long-train ICMS caused the monkey to perform ethologically relevant actions commonly present in its normal repertoire, such as closing the hand in a grip while bringing the hand to the mouth and opening the mouth, extending the hand as for preparing to grasp an object, displaying arm, head, or face movement pattern typical of defensive movement and moving all four limbs as if leaping or climbing (Figure 2).

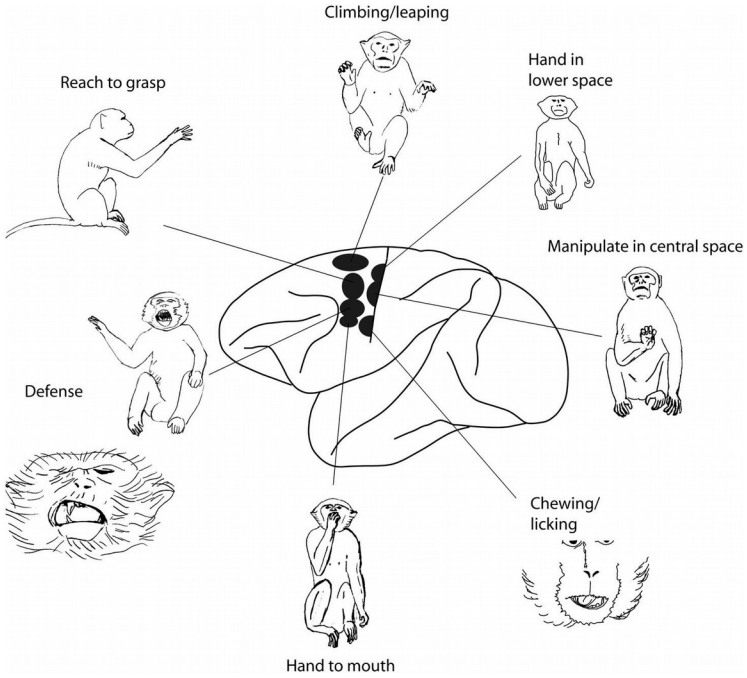

Figure 2. ***Action map in the motor cortex***. *Actions evoked by electrical microstimulation on the behavioural relevant timescale of 0.5 seconds (Graziano and Aflalo, 2007).*

These complex motor outputs suggest that the motor areas are not only involved in motor control but also in functions traditionally considered proper of higher order associative cortical areas; indeed motor areas are involved in sensory motor transformations to perform goal directed actions, action recognition and decisional processes for action initiation (Rizzolatti and Luppino, 2001). These higher order motor-based functions depend on the organization of different connectional patterns (Figure 1): the posterior motor areas (parieto-dependent motor areas), from F1 to F5, receive sensory information thanks to their connections with the parietal lobe, and use them to generate motor actions, for peripersonal space representation and for action recognition. These processes occur in several parallel circuits, each involved in specific sensori-motor transformations (Rizzolatti et al., 1997, 1998); in particular, F1, F3 and part of F2 exploit somatosensory information, whereas F4, F5 and the rostro-ventral part of F2 use visual information (Luppino and Rizzolatti, 2001). Since

the anterior motor areas (prefronto-dependent motor areas), F6 and F7, are mainly linked to the prefrontal cortex (Gerbella et al., 2013; Caminiti et al., 2017), they receive higher order signals, such as contextual and motivational information, but relatively poor sensory information from the parietal cortex, being therefore more likely involved in specifying when and in which circumstances the activity generated in the parieto-dependent areas must be turned into an overt action (Luppino and Rizzolatti, 2000).

The ventral premotor cortex receives inputs from striate and extrastriate (Markowitsh et al., 1987), parietal (Borra et al., 2008) and prefrontal (Lu et al., 1994) cortices, and sends information to motor areas (Luppino et al., 1993) and to the spinal cord (Keizer and Kuypers, 1989); thanks to this connectivity PMv is involved in the execution of sensory motor tasks transforming sensory information into actions execution. The ventral premotor cortex neurons contribute to the decision making process, by leveraging multimodal stimuli including somatosensory (Romo et al., 2004) visual (Pardo-Vasquez et al., 2008) and auditory (Lemus et al., 2009) signals used to perform discrimination task, where neurons become active both in response to sensory stimulation and during the entire decision process.

1.1.1 Ventral premotor cortex: functional properties of area F5

Area F5 is located in the rostral part of PMv and contains hand and mouth partially overlapping representations, as revealed by both extracellular recording and intracortical micristimulation experiments (Maranesi et al., 2012; Rizzolatti et al., 1988). This area exhibits some interesting features that differentiate it from the primary motor cortex; indeed, several studies discovered purely motor neurons that code the action goal regardless of the effector used to carry out the action (Bonini et al., 2011; Rizzolatti et al., 1998), or the single sequence

of muscle activations required to achieve the same action goal depending on the context (Umiltà et al., 2008). In particular, these neurons are known to discharge for “grasping” (i.e., taking possession of an object), tearing, breaking and holding actions (Rizzolatti et al., 1988). Moreover, within each of these categories there are some neurons that code specific types of hand shaping, such as, for example, precision grip, whole hand prehension or finger prehension (Bonini et al., 2012).

In addition to purely motor neurons, F5 hosts neurons with visuomotor response properties, the so-called “canonical neurons”, and “mirror neurons”. Canonical neurons respond to the presentation of three-dimensional objects as well as during the preparation and the execution of reaching-grasping actions directed to them (Murata et al., 1997), suggesting that these neurons can transform the intrinsic physical features of an object (size, shape, weight) into the appropriate motor plan required to interact with it. This is made possible thanks to F5 mutual connection with AIP (*Anterior Intraparietal Area*), located into the intraparietal sulcus, with which it creates a circuit to perform specific sensorimotor transformations necessary to generate “potential motor actions” (Rizzolatti et al., 2002).
Mirror neurons, instead, fire during the execution of an action and during the observation of an action performed by another subject, suggesting they may play a role in action recognition (Gallese et al., 1996).

It is important to underline that the above-mentioned neurons (purely motor, canonical and mirror) can’t be considered as segregated categories of neurons functionally distinct that act separately, but the adjectives “motor”, “canonical”, and “mirror”, are more likely to describe different functional properties that the same neuron can have; for instance more recent studies have demonstrated that canonical and mirror neurons are not anatomically segregated in different sectors of areas F5, as previously thought (canonical in F5p and mirror

in F5c), and “canonical” and “mirror” properties can often apply even to the same single neuron (Bonini et al., 2014).

1.1.2 Ventral premotor cortex: functional properties of area F4

The premotor area F4 is located in the caudal part of inferior BA6, just rostral to BA4 (area F1). It contains a representation of head, trunk, arm and mouth movements (Gentilucci et al., 1988). Proximal, axials and arm movements tend to be represented dorsally, whereas mouth movements are represented mostly in its ventral part.

Motor neurons in this area are known to discharge during neck, upper trunk, arm, mouth (biting, chewing or sucking) and face goal-directed movements, especially when combined; indeed, many neurons in this area fire during bringing the hand to the mouth while opening the mouth, or during arm reaching and trunk and neck orienting movements (Fogassi et al., 1996a).

Somatosensory, visual and bimodal neurons have also been identified in F4. Somatosensory neurons discharge during tactile stimulation of the face, neck, arms or hands (Rizzolatti et al., 1981); visual neurons are triggered by visual stimuli moved close to the subject, whereas bimodal neurons are triggered by both tactile and visual stimuli (Fogassi et al., 1996b). Different from classical visual neurons, F4 neurons do not respond to common visual stimulation: they are activated by tridimensional stimuli moved close to the subject’s body part that equally trigger the neuron when touched; thus, neurons’ visual receptive fields are “anchored” to their tactile receptive fields, regardless of relative gaze position (Fogassi et al., 1996a), suggesting that F4 neurons operate in body-centered coordinates, encoding the location of stimuli in relation to body parts (Graziano et al., 1994). Furthermore, some F4 neurons encode also auditory stimuli in terms of their location with respect to the monkey

(Graziano et al., 1999), suggesting that a multimodal representation of the nearby space is created in area F4. Moreover, a small part of neurons in area F4 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). Thanks to its mutual connection with VIP (*Ventral Intrparietal Area*), an area located into the intraparietal sulcus that contains neurons with similar sensorimotor properties (Colby et al., 1993; Duhamel et al., 1991; Schlack et al., 2005), F4 takes part in a circuit that transforms extrinsic characteristics of an object, such as its position in space, in the correct motor plan to reach it (Matelli and Luppino, 2001; Rizzolatti and Matelli, 2003). Finally, both VIP and F4, if stimulated, generate defensive-like movements (Cooke and Graziano, 2004; Graziano et al., 2005), suggesting that they might be involved in planning defensive actions in the peripersonal space (Cléry et al., 2015): VIP may play a role in the generation of a multisensory head-centered representation of the nearby space (Duhamel et al., 1998), whereas F4 may be more involved in generating defensive and avoidance actions.

1.2 Wireless recording system

1.2.1. Why implementing wireless technology?

Traditional neuroscience studies using non-human primates have allowed to achieve great knowledges on neuronal activity, cognitive functions and on a vast number of processes and mechanisms of brain functioning (Roelfsema and Treue, 2014). Non-human primates, indeed, can be trained to carry out relatively complex sensory discriminations and motor tasks; this, along with sophisticated recording techniques to study brain activity on awake subjects, allowed researchers to identify the functional roles and properties of single nerve cells, networks and cortical and subcortical areas subserving perceptual, motor and cognitive

functions (Moore and Armstrong, 2003; Roelfsema, 2006). However traditional neural recording systems, composed by movable microelectrodes connected with cable to amplifiers and recording equipment, requires recording cells' activity in constrained conditions (Lemon, 1984); indeed, typical experiments on non-human primates engage monkeys in physical restraint and head-fixed settings by using the so-called primate chairs. These experimental conditions allow to control for many parameters such as, for example, head position, gaze direction, body and arm posture increasing the results' internal validity, but at the same time they limit studies to artificially constrained behaviours, reducing their ecological validity (Berger et al., 2020): monkeys cannot explore the environment, neither freely interact with other subjects, they can mostly perform behavioural tasks characterized by relatively limited, often stereotyped and repeated movements, in a restricted workplace, that mainly involve upper limbs and that are unrepresentative of natural behaviours (Jackson et. al, 2007). Therefore, although classical neurophysiology experiments provided insights into the underlying mechanisms of brain functioning it is not clear to what extent these results can be generalised to unconstrained natural behaviours, making necessary to implement freely behaving experimental conditions especially while investigating the mechanisms underlying motor functions: several studies, indeed, have reported that body boundaries are able to affect the encoding of sensory information (Caggiano et al., 2009, Rizzolatti et al., 1981b) necessary to generate potential motor actions.

Freely moving experiments by means of tethered recording systems has been successfully employed in small species such as rats (O'Keefe, 1971) and small primate species, for instance squirrel monkeys (Ludvig et al., 2004) or marmosets (Courellis et al., 2019; Nummela et al., 2017), however they cannot be implemented with larger non-human primates, which could easily remove and damage cables and devices. To overcome tethered

system limitations wireless recording systems have been implemented; thus, even if in unconstrained conditions it is more difficult to quantify animals' behaviours and confounding factors are more difficult to control, freely moving paradigms together with wireless recording systems allow to overcome traditional limits of non-human primate studies and enable researchers to investigate natural behaviours, ecologically relevant questions, and to improve reliability and ecological validity of the data.

Successful wireless technology has already been implemented on various animal species such as rats (Grieves et al., 2020), bats (Omer et al., 2018; Yartsev and Ulanovsky 2013), non-human primates (Berger et al., 2020; Roy and Wang 2012) and insects (Harrison et al., 2011). Their capability to address ecologically relevant questions has been fully demonstrated; for instance, they have led to *social-place cells* discovery in Egyptian fruit bats (cells that represent the position of conspecific in allocentric coordinates) clearing the way to better understand complex behavioural processes like social interaction (Omer et al., 2018). Moreover, it has also been demonstrated the advantage of using wireless recording techniques, rather than traditional approaches, in studying natural behaviours; for instance, thanks to a wireless multi-channel single-unit recording techniques, it was possible to record marmoset's neuronal activity during social vocalization, a specie-specific behaviour that couldn't have been studied in traditional experiments because in constrained conditions marmosets show an inhibition in vocal behaviour (Roy and Wang, 2012).

Finally, wireless technology could represent a turning point in the translational research helping to develop more sophisticated brain machine-interfaces (BMI) based on intracortical extracellular recording. Indeed, intracortical signals can be decoded to control external devices and partially restore motor functions in spinal cord lesioned patients (Aflalo et al., 2015; Bouton et al., 2016; Collinger et al., 2013; Gilja et al., 2015; Hochberg et al.,

2012; Wodlinger et al., 2015); in this research field studying neuronal activity in freely moving subjects can highlight brain's involvement in whole-body behaviours and help to restore lost function with a prothesis while the patients is performing other movements in parallel (Berger at al., 2020). In light of these findings it is clear that wireless system represents a useful technology able to improve basic and translational research.

1.2.2 Wireless neurophysiological studies on the motor system

So far, few studies on freely moving animals with wireless neural recording systems have been implemented in non-human primates' motor system. In 2007, Jackson and co-workers, using both tethered and wireless recording systems, contrasted for the first time the activity obtained during constrained and unconstrained paradigms in order to determine whether results obtained under constrained conditions generalize to freely moving conditions. In particular they recorded neurons from *Macaca nemestrina*'s primary motor cortex in three experimental conditions: monkeys performing a torque-tracking task on a primate chair, monkeys freely behaving and natural sleep. In each of awake conditions they found correlations between neural activations and motor activity recorded by means of electromyogram (EMG); the results obtained by the use of the two different types of methodologies partially overlap, however the utilization of wireless recording system in unrestrained conditions is important to extend the data obtained under classical restrained conditions.

Another study that took advantage of wireless technology in investigating motor control has been conducted by Berger and colleagues (2020). They implemented a wireless recording system in a freely moving experimental environment, the Reach Cage, in order to investigate movement planning and goal directed movements in unrestrained rhesus

macaques. They trained fully unrestrained monkeys to perform spatially and temporally well-structured memory-guided reaching actions into the Reach Cage equipped with a visuo-haptic interaction system. Thanks to this experimental setup they were able to study motor goal encoding even beyond the immediate reachable space and during ongoing walking movements (not only in the immediate reachable space while the monkey stood still in a primate chair as previous studies did). Moreover, by using a markerless video-based motion capture software, that allowed to control for head, shoulder, elbow and wrist trajectories, they could control many confounding factors likewise in restrained monkey experiments. Thanks to this methodology they have 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. These studies provide an example of wireless recording systems' potential to deepen and improve knowledges so far obtained with classical methods.

2. AIM OF THE STUDY

Classical neurophysiological studies on cortical control of behaviours carried out on non-human primates achieved a great amount of discoveries on motor cortex functioning. However, the limitations due to traditional experimental setups allowed to investigate simple action, often stereotyped and difficult to generalize to naturalistic behaviour. This could represent an obstacle to truly understand primate's cerebral functioning underlying their vast behavioural repertoire and the possibility to develop effective interventions to repair brain damage.

Therefore, in this study, new wireless recording system, synchronized with a multi-camera system, was implemented in non-human primates (rhesus macaques) to study whole-body movements and complex behaviour. First, monkey's neuronal activity was recorded during simple but reproducible motor behaviours of interest in a traditional experimental setup where monkeys, by means of a primate chair, were constrained in head-fixed conditions. Second, the same neuronal activity was recorded while the monkeys were freely behaving and exploring the environment into an enclosure enriched with various stimuli aimed to elicit natural behaviours, in principle comparable to those the monkeys were trained to perform in the restrained condition.

This experimental setup was developed in order to study non-human primates' ventral premotor cortex in a more ecologically relevant manner, to contrast the single-units' activity in both constrained and freely moving behaving conditions and to analyse whether data obtained in the first condition can be generalized to a more naturalistic one.

3. MATERIAL AND METHODS

3.1 Subjects and surgery

The study involved 2 males *Macaca mulatta* (Mk1, 8 years, 13 kg; Mk2, 10 years, 13.5 kg). Before recordings, the monkeys were trained by means of positive reinforcement to perform the actions described below.

Once the training was completed, both monkeys underwent surgeries in deep anaesthesia and aseptic conditions to implant on their skull a head-holder (head-post) and the intracortical chronic electrodes. For both surgeries animals were prepared for the anaesthesia with atropine administration (0.03 mg/kg) 15 minutes prior to the induction of anaesthesia. Next, anaesthesia 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).

All experimental protocols complied with the European (Directive 2010/63/EU) and national (D.lgs 26/2014) laws on the protection of animals used for scientific purposes, they 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).

3.2 Apparatus and experimental paradigm

The monkeys underwent a training to be recorded in both constrained condition (chair condition), typical of the classical neurophysiological experiments, and unconstrained condition (freely moving condition). To this purpose, they were trained to spontaneously enter and sit in a primate chair from their home cage. Then, they were both habituated to two

different experimental environments: the first one consisted of a primate chair where the monkey sat and interacted with the experimenter during a variety of motor actions, whereas the second one, the “NeuroEthoRoom” (NER), consisted of an enclosure where the monkey could freely undertake a variety of spontaneous activities. Finally, the monkeys returned into the primate chair to be brought back to their home cage.

In every session each monkey entered the laboratory, was head-fixed, and performed various motor actions in the chair (CHR) condition for about 30 minutes; next, the head was released and the monkey started the freely-moving (NER) condition in the NeuroEthoRoom, for about 30 minutes.

3.2.1 Chair condition

Throughout the experiment the monkey sat in a primate chair placed at the centre of a transparent plexiglass enclosure, the NeuroEthoRoom (Figure 3), equipped with a System of 8 colour cameras aimed to record macaques’ behaviours throughout the session (for further details see paragraph 3.3.1).

Figure 3. The NeuroEthoRoom*. The picture illustrates a view from the outside of the large plexiglass enclosure where the sessions are recorded.*

In the CHR condition the monkey's head was fixed and the animal could reach and grasp target objects or food items to bring them to the mouth by means of an opening in the front side of the chair.

The CHR experimental session included different type of motor actions that mainly engage monkey's mouth, hands and arms. In particular the monkey had been trained to stand still while food was presented far or near the monkey itself (*Food presentation*) and later to grasp it (*Grasp food*) with the right and the left hand and bring it to the mouth (*Active food to the mouth*); next the monkey had to stand still while received food (*Solid reward*) and juice (*Liquid reward*) given directly to the mouth by the experimenter; finally it did grasping actions with the right and the left hand on different type of objects allowing different wrist rotation (*Finger prehension 0°*, when the carabiner is presented horizzontally, *Finger prehension 90°, when the rope is presented vertically*). Each behaviour, for each forelimb, was repeated for at least 7 trials (for further details see table 1 in paragraph 3.3.2).

The motor actions have been chosen in order to be compared to those spontaneous actions the monkey does in the NER where it can freely move.

3.2.2 Freely-moving condition

In the NER condition, the monkey could move freely in the environment and perform spontaneous actions using the objects and the enrichment stimuli provided in the NeouroEthoRoom prior to the initiation of the session.

The NER, is a transparent plexiglass structure (Width: 208 cm, Height: 205 cm, Depth: 181 cm) equipped with two large doors on the front side wall that allow the experimenter to enter the NER and prepare the environment before each session. The front

side wall is also equipped with two smaller vertical sliding doors, which allow the monkey to move autonomously from the chair to the NER and begin the session.

The NER was enriched before the initiation of the session with different items (Figure 4): a wooden structure where to climb and sit on, climbing holds attached on the NER walls and a rope to climb in order to reach the upper level of the cage; the wooden structure and the NER have breaches to dispense liquid rewards and food from the outside by means of syringes, toothpicks or hooks lowered from openings on the NER roof. During the session the monkey could pick up food hidden in the wall openings, directly given by the experimenter, or lowered from the roof, forage on the floor, climbing and displaying specie-specific behaviours.

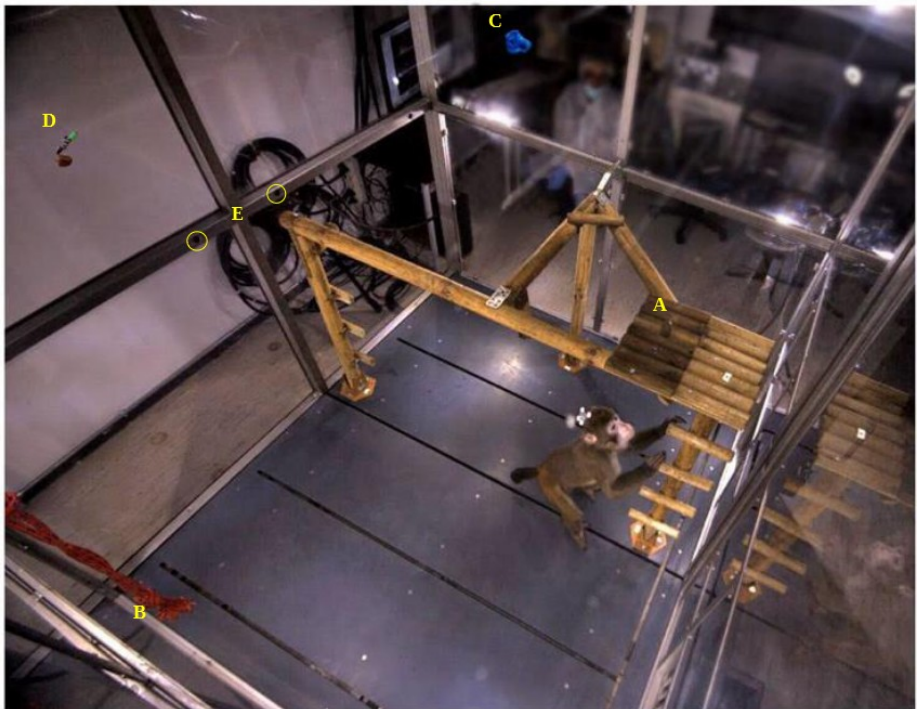

Figure 4. Freely-moving condition setup. *The picture illustrates a view from the inside of the NER where the monkey interacts with some of the enrichment stimuli: the wooden structure (A), the rope (B) and the climbing holds (C) the monkey uses to reach the highest level of the NER; hooks (D) and openings (E) to dispense food and liquid reward.*

3.3 Behavioural analysis

3.3.1 Behavioural recording

Monkey's behaviours were recorded in both conditions with the same system (SIMI, Munich, Germany) composed by eight high-resolution cameras mounted on movable arms attached to the four corners of the NER, at two different heights (mid and top of the enclosure). Dual Gigabit Ethernet Machine vision cameras (mvBlueCOUGAR-XD, Matrix Vision) with a resolution of 1936×1214 set to 50 frame-per-second acquisition rate were used. The cameras were equipped with a global shutter with sensor size 1/2" format (5.86 μm pixel), a manual C-Mount Lenses with 5 mm focal length (CCTV Lens, KowaOptical Products Co., Ldt) and LEDs ring lights. Each camera had two RJ-45 Gigabit Ethernet connectors with screw-locking and two Industry standard 12-pin locking connectors to provide transmission of images and signals to the computer, and a synchronization box connected to both cameras and computer to synchronize frames acquisition. They fed their signal to a computer with a dedicated software, *Simi Motion Capture*, necessary for the video recording of the experimental sessions.

3.3.2 Ethogram definition

Prior to the neural recordings, in order to construct the ethogram, several video recordings of different experimental sessions were observed, considering that the cerebral area of interest, (the ventral premotor cortex) is known to be involved in planning and controlling hand, mouth, head, trunk and arm movements, in reaching and grasping actions and that their neurons have motor, canonical and mirror properties (see Introduction).

Then, we categorized the behaviours of interest performed in both experimental conditions, CHR and NER, in instantaneous events (*point events*) and events with a certain

duration (*state events*) differentiating actions performed with the left and right forelimb (Table 1). Most behaviours can occur in both conditions, although of course they are much more variable and combined with different postural and eye-hand coordination in NER relative to CHR condition. In addition, in the NER the monkey can freely move and perform more actions than in the CHR condition.

EXPERIMENTAL CONDITION	BEHAVIOUR	TYPE OF EVENT	OPERATIONAL DESCRIPTION
CHR e NER	*Active food to the mouth Right*	Point event	Monkey actively places food into the mouth with the right hand. Start when the right hand reaches the mouth. If bimanual add a comment
CHR e NER	*Active food to the mouth Left*	Point event	Monkey actively places food into the mouth with the right hand. Start when the left hand reaches the mouth. If bimanual add a comment
CHR e NER	*Liquid reward*	Point event	Monkey passively receives liquid reward directly into the mouth by means of a syringe. Start when the mouth touches the syringe
CHR e NER	*Solid reward*	Point event	Monkey passively receives solid food (fruit pieces) directly into the mouth by means of a toothpick. Start when the food touches the mouth
CHR e NER	*Grasp food Right*	Point event	Monkey grasps food pieces with the right hand. Start when the hand touches food. Grasp in a hole (NER): start when the finger enter the hole. If bimanual add a comment
CHR e NER	*Grasp food Left*	Point event	Monkey grasps food pieces with the left hand. Start when the hand touches food. Grasp in a hole (NER): start when the finger enter the hole. If bimanual add a comment

CHR e NER	*Failed grasp*	Point event	Monkey tries to grasp food pieces with the left/right hand, but fails. Start when the hand touches food. Failed grasp in a hole (NER): starts when the finger enter the hole
CHR e NER	*Undefined*	Point event	Monkey performs movements not better explained in the ethogram
CHR	*Finger prehension 0° Right*	Point event	Monkey grasps a carabiner with the right hand. Start when the hand closes around the carabiner
CHR	*Finger prehension 0° Left*	Point event	Monkey grasps a carabiner with the left hand. Start when the hand closes around the carabiner
CHR	*Finger prehension 90° Right*	Point event	Monkey grasps a rope with the right hand. Start when the hand touches the rope
CHR	*Finger prehension 90° Left*	Point event	Monkey grasps a rope with the left hand. Start when the hand touches the rope
CHR	*Food presentation*	Point event	Monkey stand still and food is presented close or far from it
NER	*Grasp food with mouth*	Point event	Monkey eats food with the mouth (it doesn't gasp it with hands). Start when the mouth touches the food
NER	*Grasp solid reward Right*	Point event	Monkey grasps food given by the experimenter with the right hand. Start when the right hand touches the food
NER	*Grasp solid reward Left*	Point event	Monkey grasps food given by the experimenter with the right hand. Start when the right hand touches the food
NER	*Grasp for climbing Right*	Point event	Monkey grasps the climbing holds or the wooden structure (not the rope) with the right hand for climbing. Start when hand touches the object. If bimanual add a comment

NER	*Grasp for climbing Left*	Point event	Monkey grasps the footholds or the wooden structure (not the rope) with the left hand for climbing. Start when hand touches the object. If bimanual add a comment
NER	*Grasp thread Right*	Point event	Monkey grasps a nylon thread with the right hand. Start when hand touches the nylon thread. If bimanual add a comment
NER	*Grasp thread Left*	Point event	Monkey grasps a nylon thread with the left hand. Start when hand touches the nylon thread. If bimanual add a comment
NER	*Grasp rope Right*	Point event	Monkey grasps a rope with the right hand. Start when hand touches the rope. If bimanual add a comment
NER	*Grasp rope Left*	Point event	Monkey grasps a rope with the left hand. Start when hand touches the rope. If bimanual add a comment
NER	*Autogrooming*	State event	Monkey does autogrooming. Start at the first touch. Stop the moment the monkey stops touching itself
NER	*Grasp for grooming Right*	Point event	Monkey grasps itself with the right hand for grooming. Start when the fingers are closed
NER	*Grasp for grooming Left*	Point event	Monkey grasps itself with the left hand for grooming. Start when the fingers are closed
NER	*Walk*	State event	Monkey moves from one location to another (not climbing). Context independent. Start when first limb touches the floor; minimum two steps with the hands. Stop when last limb touches the floor and the monkey doesn’t move for at least 2 seconds

NER	*Power step Right*	Point event	Monkey grasps with the right hand the wooden structure or the cage for walking. Only those power steps included within the walk behaviour, those not included are considered as *undefined*. Start when the right hand touches the surface for grasping it
NER	*Power step Left*	Point event	Monkey grasps with the left hand the wooden structure or the cage for walking. Only those power steps included within the walk behaviour, those not included are considered as *undefined*. Start when the left hand touches the surface for grasping it
NER	*Step hand Right*	Point event	Monkey steps (hand flat) with the right hand on the floor/wooden structure/cage for walking. Only those steps included within the walk behaviour, those not included are considered as *undefined*. Start when the right hand touches the surface
NER	*Step hand Left*	Point event	Monkey steps (hand flat) with the left hand on the floor/wooden structure/cage for walking. Only those steps included in the walk behaviour, all steps not included in the walk are considered as *undefined*. Start when the left hand touches the surface
NER	*Rest*	State event	Monkey stands still: in this moment monkey isn't walking. Start when the rear-end touches the ground for at least 2 seconds. Stop when the rear-end gets up
NER	*Scratch*	Point event	Monkey scratches itself. Start at the first touch of the monkey’s body
NER	*Yawn*	Point event	Monkey yawns. Start when mouth starts opening
NER	*Threat*	Point event	Monkey threatens. Start when mouth starts moving

Table 1. ***Ethogram.*** *The table reports the behaviours of interest specifying their operational description, the type of event (point or state event) and in which experimental condition they can be observed: in both chair and*

freely moving condition (CHR e NER), only in the chair condition (CHR), only in the freely moving condition (NER).

3.3.3 Data analysis

The videos of each experimental session were analysed by means of dedicated software (Behavioural Observation Research Interactive Software, BORIS - Friard and Gamba, 2016), allowing to score the ethograms' behaviours for the entire duration of the recording by playing simultaneously and synchronously the 8 cameras' video recordings.

To be more accurate in behaviours' logging we used the frame-by-frame mode which allows one to slow down the video recordings in order to capture the exact moment in which an action happened with a 20 milliseconds resolution. To increase accuracy and the trustworthiness of behavioural scoring, the video recordings were observed many times by independent observers, and then the inter-rater reliability using the Cohen's kappa statistic was calculated. Finally, we generated an output for each session, containing all the behaviours with the exact time of their occurrence.

3.4 Neural recording

Neural recordings were performed using 32-channel Floating Microelectrode Array (FMA), with alternated electrodes of 4 and 2.5 mm in length implanted so as to cover all the cortical convexity extending between the inferior arcuate sulcus, just ventral to the cross with the superior arcuate, and the central sulcus. Ventral premotor cortex, in its rostral (F5) and caudal (F4) halves, was covered in both monkeys' left hemisphere (Figure 5). Each FMA was connected through an OMNETICS connector to the recording system, a 128 channel neural

data logger (https://deuterontech.com/) synchronized via a radio signal to the rest of the recording devices along the whole session.

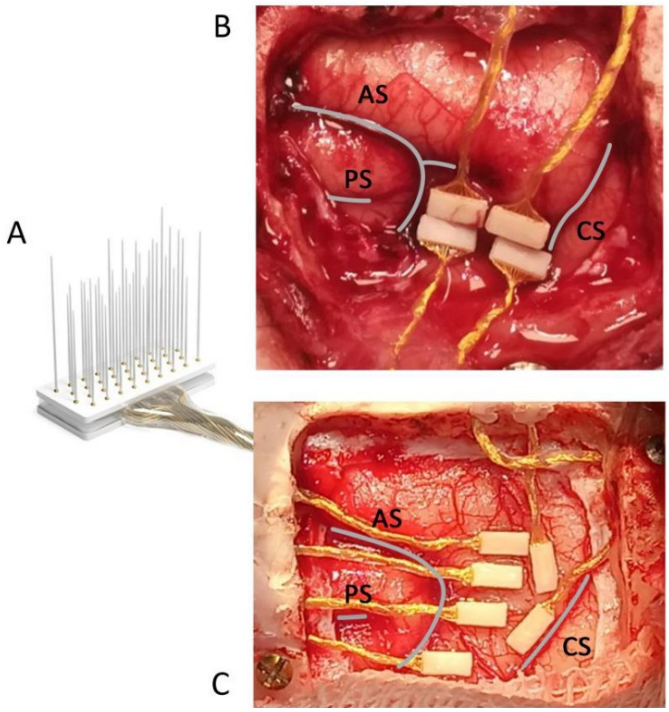

Figure 5. Floating microelectrode arrays (FMAs) implanted in macaques, Mk1 and Mk2*. A) Schematic representation of a FMA with 36-channels; B) Image of microelectrode arrays placement in Mk1 and, C) Mk2. Anatomical landmark descriptions: CS - central sulcus; AS - arcuate sulcus and PS - principal sulcus.*

The original signal was grounded and referenced using low impedance dedicated electrodes in each FMA, and recorded with a band-pass filter set on the range 2 - 7000 Hz at a conversion rate of 32000 Hz for each channel. The system can thus sample single and multi-unit activity together with most of the Local Field Potential frequency bands. Neural signals were amplified, digitized and stored in a MicroSD memory card (64 GB), so as to prevent any possible transmission error. The device was powered by a small external battery connected via a short cable (Figure 6B). Once the logger device was linked to the electrode arrays into the chamber (Figure 6A), all the components were sealed within a cover screwed on top of the chamber (Figure 6C). In addition, the logger had a magnetic on/off switch, so that it could be

turned on and off also when the device was sealed into the protective chamber on the head of the animal, with no need to physically interact with the monkey or remove any component. All formal signal analysis were performed off-line.

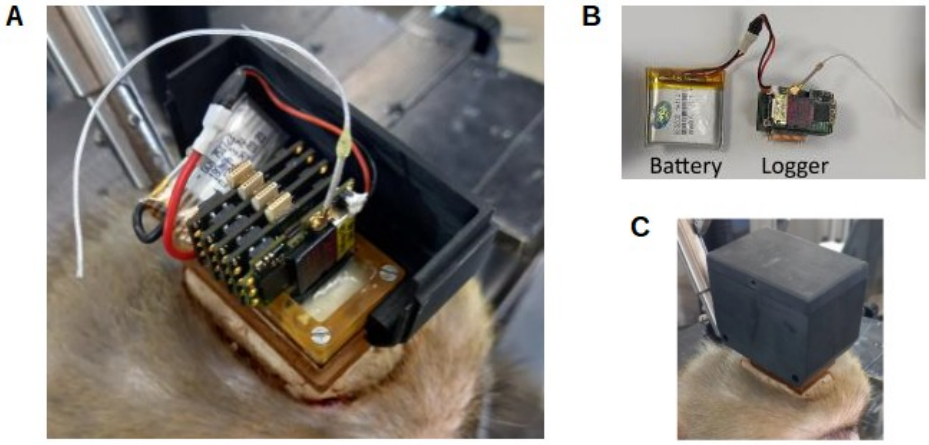

Figure 6. The recording chamber divided in its components. *A) the recording chamber open; B) the battery connected with the recording system (RatLog-128 from Deuteron technologies); C) the cover sewed around recording components.*

3.5 Data Analysis

To compare the neural activity among the CHR and NER condition, we firstly analysed the experimental sessions focusing, in each condition, on the relationship between scored behaviours and the neuronal activity recorded in the experimental session. We considered behaviours that occurred at least seven times during the experimental session focusing on *point events.*

Finally, we correlated the results obtained in the two experimental conditions to compare single units' response to similar behaviours performed in different contexts (primate chair and NER) under the null hypothesis that if a neuron tested in the CHR condition responds in relation to a behaviour, it should do so in the NER condition in relation to the same or a similar behaviour.

3.5.1 Single units extraction

All formal signal analyses were performed off-line with fully automated software (MountainSort, Chung et al., 2017), using -3.0 standard deviations of the signal-to-noise ratio of each channel as threshold for detecting units. Importantly, we used the same data logger for the acquisition of the signal in both CHR and NER condition of each session, and the spike sorting procedure was therefore performed at the same time on the whole dataset of the two conditions in a merged file, in order to eliminate any possible drift or variation in the isolation criteria.

Units were distinguished into single and multi-units using the noise overlap, a parameter that can vary between 0 and 1, with units with value below 0.1 considered as a single and all the waveforms belonging to cluster with high noise overlap values forming the multi-unit signal. Single unit isolation was further verified using standard criteria: by visual inspection of the Inter Spike Interval distribution and the waveform shape. Possible large amplitude artefacts were removed by visual inspection and all the remaining waveforms that could not be classified as single units formed the multi-unit activity.

3.5.2 Burst analysis

We studied the relationship between neural activity and behaviours starting from the single unit activity with the following assumption: if a neuron generates a pattern of spiking activity (burst) in relation to a given behaviour in the CHR condition then it should do so when a similar behaviour is performed in the NER.

Operationally, we considered as a burst every interval in which the firing rate of a single neuron exceeds the 95th percentile of its firing rate distribution, computed separately in the CHR and NER conditions because some neurons could exhibit different average firing

rate between conditions. First, for each neuron, we calculated the smoothed firing rate for a given condition by binning the spiking activity of each neuron in 20ms time bins, smoothing with a 100ms Gaussian kernel. Then, we defined the start and stop times of each burst as the first and last time bin in which the firing rate distribution exceeded the 95th percentile for at least 300ms consecutively.

For each neuron we matched the identified bursts with behaviours, looking for possible behaviours in the interval ranging from 500ms before the start of the burst to 500ms after the end of the burst. If multiple behaviours fell within the burst, we chose the one closest to the burst mean point. Conversely, if a behaviour fell within more than one burst-related interval, it was associated only to the burst that has its mean point closest to it. Busts without matched behaviours were defined “empty”.

We analysed neurons’ firing features comparing them between conditions (CHR and NER); in particular, we computed the average firing rate, the peak firing rate, the maximum position of Inter-spike Interval (ISI), the coefficient of variance of ISI, the bust index (computed according to Constantinidis et al., 2002) and the median bursts duration of each neuron in every condition and calculated the correlation of this parameters in the two different contexts.

Then, for each neuron and for each behaviour, we calculated the percentage of trials that have a burst matching that particular behaviour, relative to its total occurrences. Finally, we used these percentages across all neurons to calculate the pairwise correlations between different behaviours, both intra- and inter-conditions, obtaining a matrix of correlation coefficients (r). This matrix was plotted with a threshold level corresponding to the r expected by a significance level $\alpha=0.05$ and the current sample size (N).

4. RESULTS

The entire experimental session lasted about one hour per monkey: each monkey was tested for about 30-40 minutes in the constrained condition (CHR condition) and for about 30-40 minutes in the freely moving condition (NER condition). During the sessions we recorded neuronal activity from 128 electrodes in each monkey; in particular, in Mk1 we recorded from all the four implanted arrays (Figure 7A), whereas in Mk2 we recorded from arrays C, D, E and F (Figure 7B), isolating a total of 98 single units (Mk1: n = 60; Mk2: n = 38).

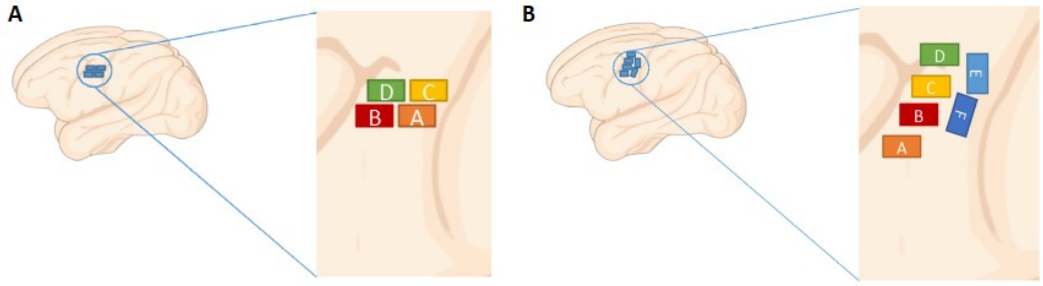

Figure 7. Chronic arrays implantation. *The picture schematically illustrates the insertion sites of chronic arrays in the ventral premotor cortex: A) left hemisphere of Mk1, B) left hemisphere of Mk2.*

4.1 Firing feature of single neurons: comparison between conditions

After extracting single units (see Materials and Methods) we examined neurons' firing features and compared them in the CHR and NER conditions (Figure 8). In both monkeys, we found a positive correlation between the average firing rate (Figure 8A, Mk1: r= 0.98, $p=4.47e^{-41}$; Mk2: r=0.96, $p=4.15e^{-21}$), and the peak firing rate (Figure 8B, Mk1: r=0.93, $p=2.15e^{-27}$; Mk2: r=0.89, $p=9.41e^{-14}$) of the two conditions. We also found a positive correlation, in both monkeys between the two conditions for the maximum position of the Inter-spike Interval (ISI; Figure 8C, Mk1: r=0.71, $p=1.57e^{-10}$; Mk2: r=0.56, $p=2.52e^{-04}$), for

the coefficient of variance of ISI (Figure 8D, Mk1: r=0.90, p=9.1e^{-23}; Mk2: r= 0.82, p=2.13e^{-10}) and for the burst index (Figure 8E, Mk1: r=0.90, p=1.28e^{-22}; Mk2: r=0.60, p=6.73e^{-05}). From burst analysis it emerged that the median bursts duration in the CHR condition was positively correlated with that in the NER condition in both monkeys (Figure 8F, Mk1: r= 0.57, p=2.13e^{-06}; Mk2 r=0.36, p=0.03); in particular, in Mk1 the median burst duration ranges between 0.44–0.74 seconds in the CHR condition and between 0.44-0.64 seconds in NER condition, whereas in Mk2 it ranges between 0.44-0.74 seconds in the CHR condition and between 0.48-0.70 seconds in NER condition. Furthermore, the explored firing features generally did not significantly differ between the two conditions (except for the coefficient of variance of ISI in Mk1, the peak firing rate and the median burst duration in Mk2), suggesting that the isolation of individual neurons remained stable across conditions.

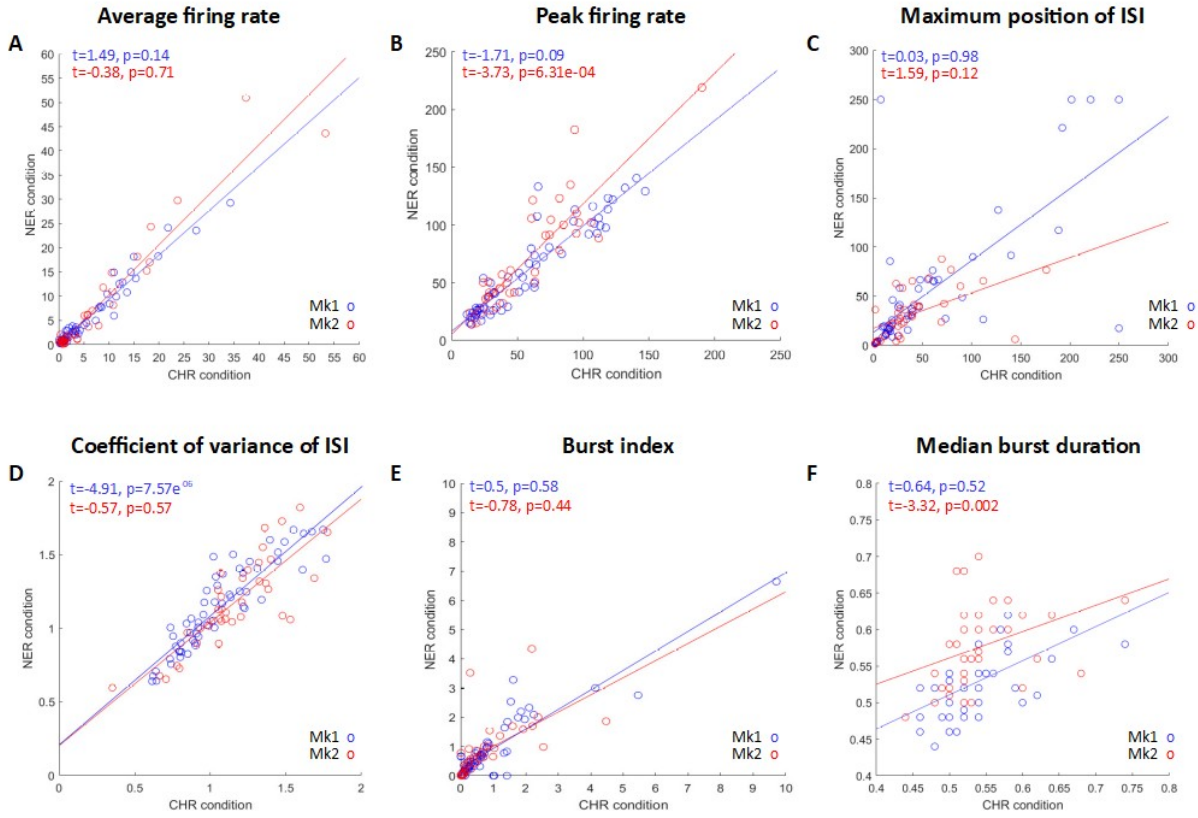

Figure 8. Firing features. *Linear correlations of the: A) average firing rate, B) peak firing rate, c) maximum position of ISI, D) coefficient of variance of ISI, E) Burst index and F) median bursts duration. Data of Mk1 are shown in blue; data of Mk2 are shown in red.*

4.2 Burst and behaviours synchronization: comparison between conditions

We analysed one session per monkey and scored the behavioural events as previously described (see Methods). Figure 9 illustrates the distribution of behavioural events along the sessions' timeline in the CHR and NER conditions and the point events (behaviours that are instantaneous) that occurred at least 7 times in the sessions of both monkeys.

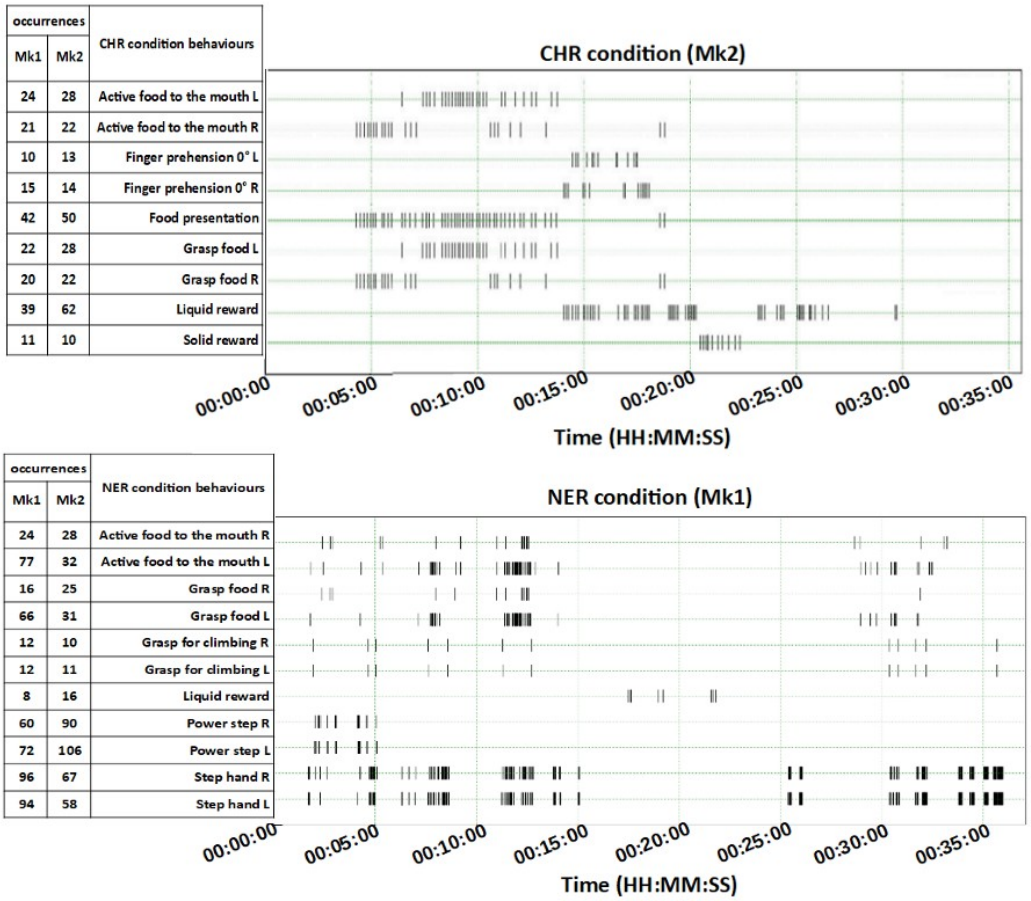

Figure 9. Behavioural scoring. *The plots illustrate an example of the timeline of behavioural events during the CHR condition for Mk2 and the NER condition for Mk1 and the relative number of behavioural events classified for both monkeys in the CHR (above) and in the NER (below) conditions.*

For each neuron, we analysed the synchronization of neurons' responses with the behaviours of interest during the session. To this purpose, we selected behavioural events that occurred at least 7 times in the session of both monkeys (see Figure 9) and matched them with bursts (see Methods). Thus, we obtained *matched* and *empty* bursts: the former has a behavioural event within 500ms before the start and 500ms after the end of the burst, whereas the latter are not associated with any behaviour within these time limits. We found a smaller number of matched than empty bursts in both conditions; in particular, the median of matched bursts is 14.01% in the CHR condition and 24.77% in the NER condition (Figure 10).

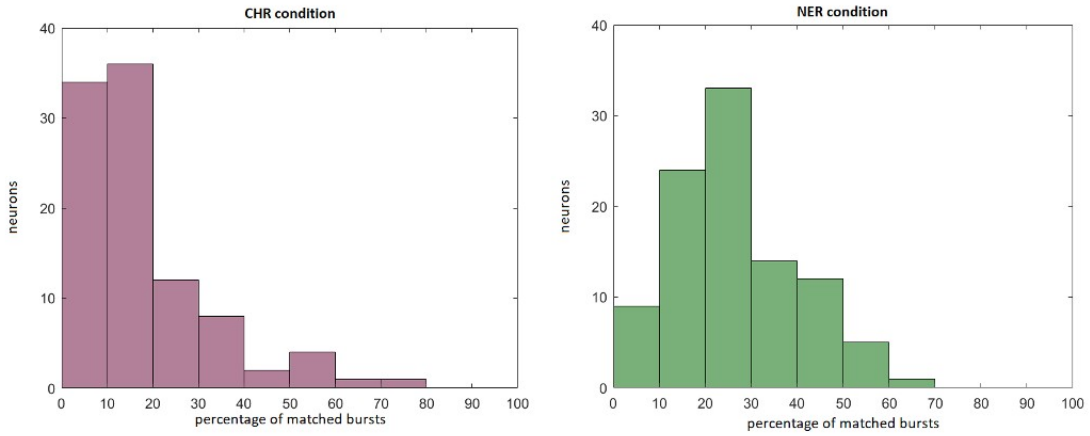

Figure 10. Matched bursts. *The two histograms illustrate the distribution of the percentage of bursts matched with a behaviour, across neurons, in the CHR (on the left) and in the NER (on the right) conditions for both monkeys.*

Furthermore, we analysed matched bursts of each single unit (e.g., Figure 11, 12 and 13); specifically, we looked at what percentage of occurrences of any given scored behaviours each single unit generated a burst; we focused especially on behaviours comparable between the two conditions. We plotted the neuron firing rate synchronized, first, to the occurrence of each behaviour in relation to which the neuron generated a burst, within a fixed time window [-1 +1 s] around the behaviours of interest; second, we aligned the same trial to the beginning

of each burst within a fixed time window [-0.5 +1.5 s] relative to the behavioural events closer to the burst onset (indicated with a coloured marker).

Unit 38a, recorded from Mk1 (Figure 11), seems to generate a burst during mouth and upper limb behaviours in both conditions; however, in the CHR condition it shows a preference for mouth behaviours, whereas in the NER condition it mainly responds to hand behaviours. Indeed, concerning mouth behaviours, in the CHR conditions the neuron generates bursts when the food touches the monkey's mouth while it is actively placing it with the right or the left hand into the mouth ("Active food to the mouth R", "Active food to the mouth L"), but also when it passively receives juice ("Liquid reward") given by the experimenter; when recorded in the NER condition, its responses to mouth behaviours appears to be weaker and less generalized to every mouth behaviour, bursting especially during "liquid reward" delivery. Moreover, concerning upper limb behaviours, a higher percentage of bursts are elicited in the NER condition when the monkey touches the wooden structure and then grasps it with the ipsilateral hand (left hand) in order to climb, whereas in the CHR condition the same neuron generates fewer bursts in relation to the moment when the monkey grasps food, closing the right hand fingers around it ("Grasp food R").


U38a (Mk1)
CHR condition
NER condition
59%
25%
active food to the mouth R
active food to the mouth L
liquid reward
grasp food R
grasp food L
grasp no food R
grasp no food L
solid reward
food presentation
Power step R
Power step L
Step hand R
Step hand L
Grasp no food R/L
CHR = Finger prehension 0° R/L
NER = Grasp for climbing R/L
Active food to the mouth R – CHR condition
Active food to the mouth R – NER condition
Active food to the mouth L – CHR condition
Active food to the mouth L – NER condition
Liquid reward – CHR condition
Liquid reward – NER condition
Grasp food R – CHR condition
Grasp food R – NER condition
Time (sec)

Figure 11. Single unit*. An example of a neuron from subject Mk1 that has 59% of its bursts in the CHR condition and 25% in the NER condition matched with a behavioural event. During the CHR condition, this neuron generates bursts in 45% of the trials of "Active food to the mouth R" (n=21), in the 75% of the trials of "Active food to the mouth L" (n=24), in the 41% of the trials of "Liquid reward" (n=39), in the 50% of trials of "grasp food R" (n=20), in the 5% of trials of "Grasp food L" (n=22), in the 13% of "Finger prehension 0°R" (n=15) and it doesn't generate any burst in relation to "Finger prehension 0° L" (n=10). During the NER condition it generates bursts in the 8% of the trials of "Active food to the mouth R" (n=24), in the 3% of the trials of "Active food to the mouth L" (n=77), in the 50% of the trials of "Liquid reward" (n=8), in the 13% of trials of "Grasp food R" (n=16), in the 3% of trials of "Grasp food L" (n=66), in the 8% of trials of "Grasp for climbing R" (n=12), and in the in the 75% of the trials of "Grasp for climbing L" (n=12). In each red box, the raster plot on the left represents the neuron firing rate aligned to the behaviour of interest (depicted with the*

purple symbol for the CHR condition and with the green symbol for the NER condition) named above the box. In the raster plot on the right, the red triangle corresponds to the onset of the burst, the upside down triangle to the offset, and the purple or green symbol to the behavioural event of interest in the CHR and in the NER condition respectively.

Unit 30b recorded from Mk2 (Figure 12) in the CHR condition shows a preference for a specific hand behaviour. In particular, this neuron generates a burst prior to the moment the monkey grasps with its right hand an object with all fingers with the hand in pronation ("Finger prehension 0° R"). This response in relation to reaching/hand-shaping action is generalized in the NER condition to hand behaviours performed towards objects and food morsels; indeed, the neuron generates bursts prior to the moment when the monkey touches the food in order to grasp it ("Grasp food R") and when it grasps the wooden structure in order to climb ("Grasp for climbing R") with the right hand.

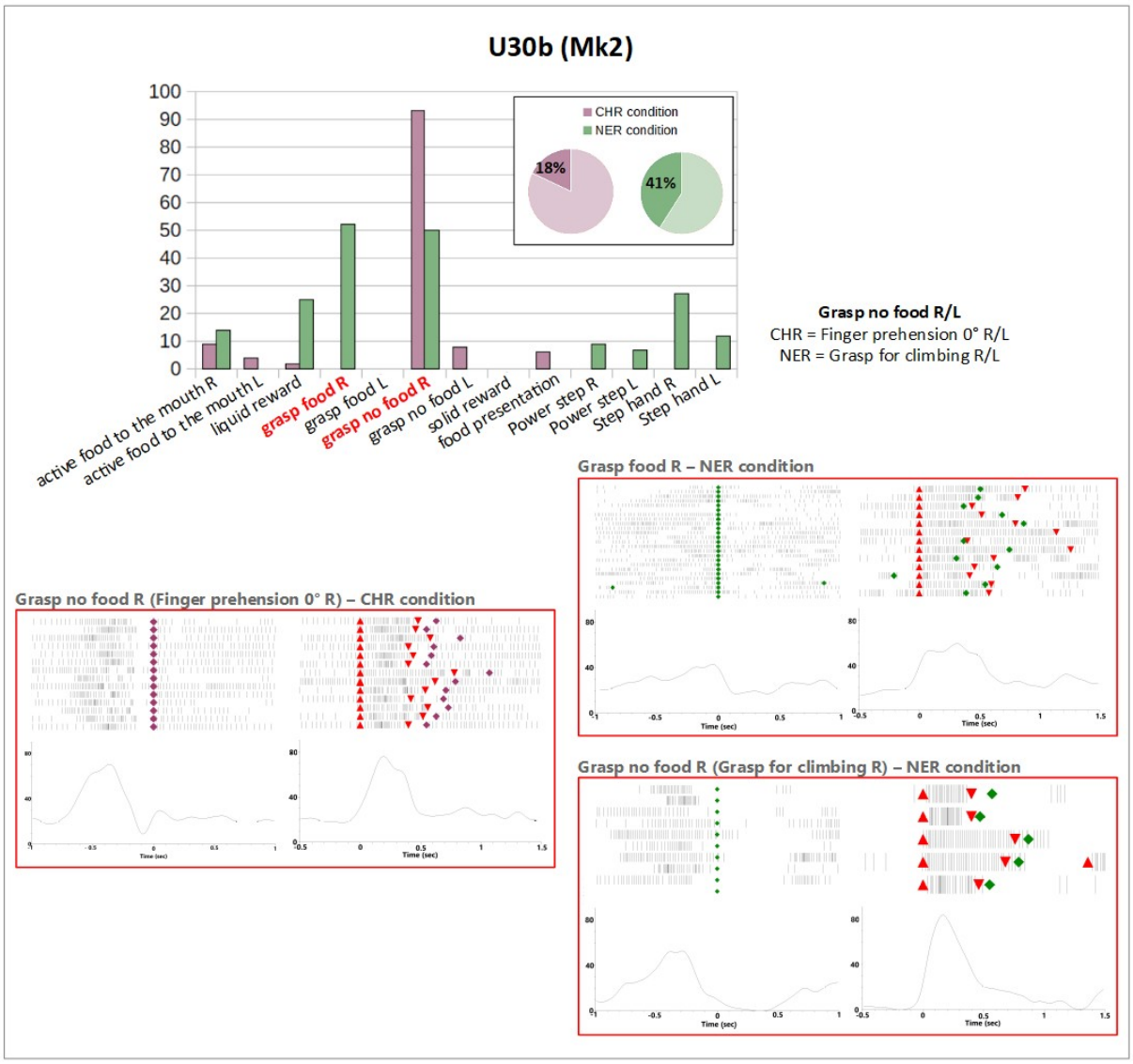

Figure 12. Single unit*. An example of a neuron from subject Mk2 that has 18% of its bursts in the CHR condition and 41% in the NER condition matched with a behavioural event. During the CHR condition, this neuron generates bursts in 93% of the trials of "Finger prehension 0° R" (n=14). During the NER condition it generates bursts in the 52% of the trials of "Grasp food R" (n=25) and in the 50% of trials of "Grasp for climbing R" (n=10). In each red box, the raster plot on the left represents the neuron firing rate aligned to the behaviour of interest (depicted with the purple symbol for the CHR condition and with the green symbol for the NER condition) named above the box. In the raster plot on the right, the red triangle corresponds to the onset of the burst, the upside down red triangle to the offset, and the purple or green symbol to the behavioural event of interest in the CHR and in the NER condition respectively.*

Unit 107a recorded from Mk2 (Figure 13) is an example of a neuron becoming active only in the NER condition, where it responds during locomotion actions. In particular, it

generates bursts prior to the moment in which the monkey touches the wooden structure with its right hand in order to grasp it for climbing ("Grasp for climbing R"), and when the monkey touches the wooden structure with the left hand and then lays it in order to walk ("Power step L"). In the CHR condition the neuron doesn't seem to be strongly elicited by any behaviour generating very few bursts only prior to the moment when the left and right hand fingers close around an object ("Finger prehension 0° R/L").

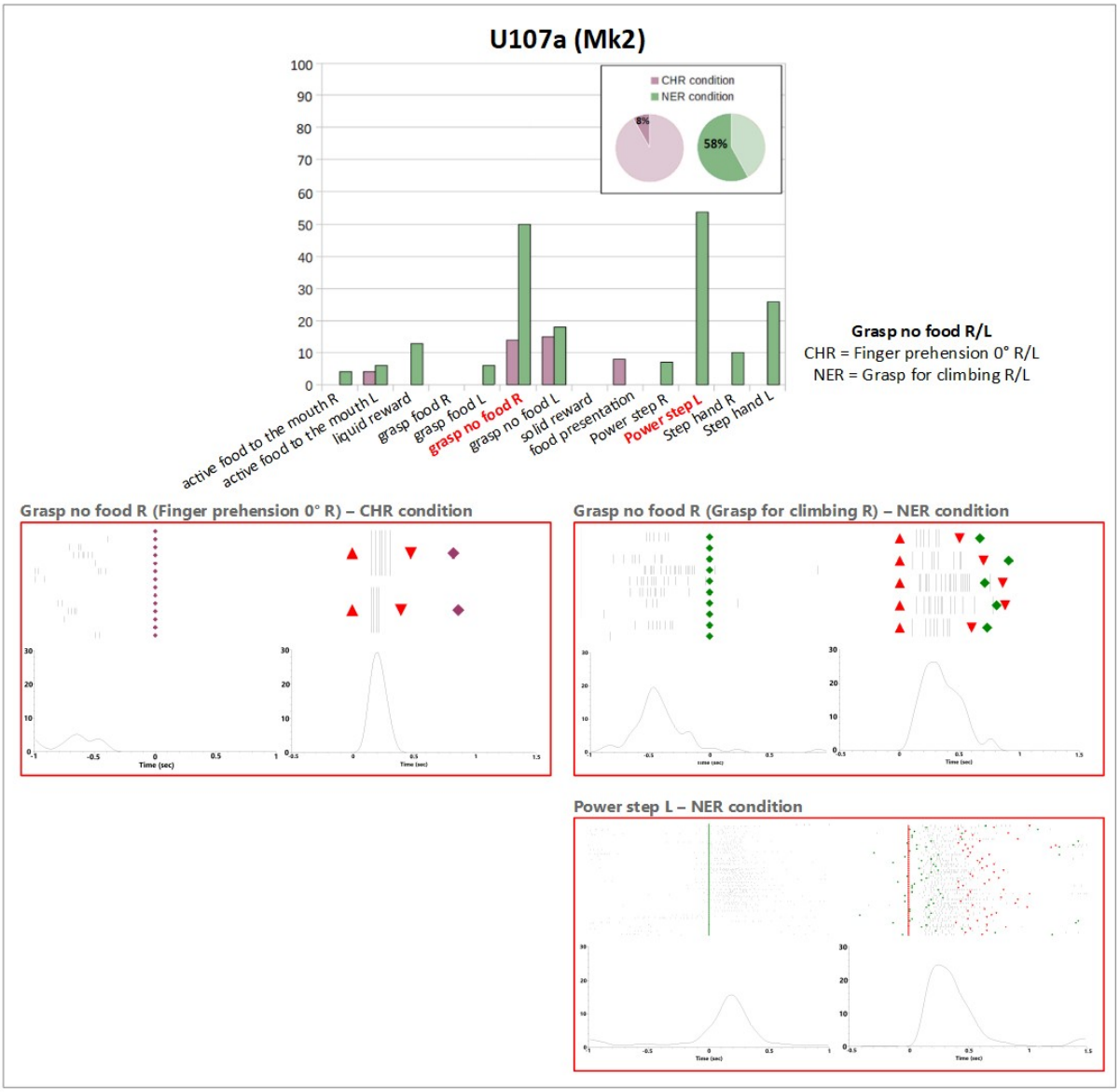

Figure 13. Single unit*. An example of a neuron from subject Mk2 that has 8% of its bursts in the CHR condition and 58% in the NER condition matched with a behavioural event. During the CHR condition, this neuron generates bursts in 14% of the trials of "Finger prehension 0° R" (n=14), and in 15% of the trials of "Finger*

prehension 0° L" (n=13). During the NER condition it generates bursts in the 50% of the trials of "Grasp for climbing R" (n=10) and in the 53% of trials of "Power step L" (n=106). In each red box, the raster plot on the left represents the neuron firing rate aligned to the behaviour of interest (depicted with the purple symbol for the CHR condition and with the green symbol for the NER condition) named above the box. In the raster plot on the right, the red triangle corresponds to the onset of the burst, the upside down red triangle to the offset, and the purple or green symbol to the behavioural event of interest in the CHR and in the NER condition respectively.

To obtain a global picture of the burst-behaviours synchronization across the entire population of recorded neurons, we calculated the average percentage of matched bursts across neurons (n=98), in both conditions (Figure 14). We found that the burst-behaviour association is similar between CHR and NER conditions, for both mouth and hand actions. In addition, behaviours specific of NER conditions ("Power step" and "Step hand") elicit lower bur comparable percentage of matched bursts.

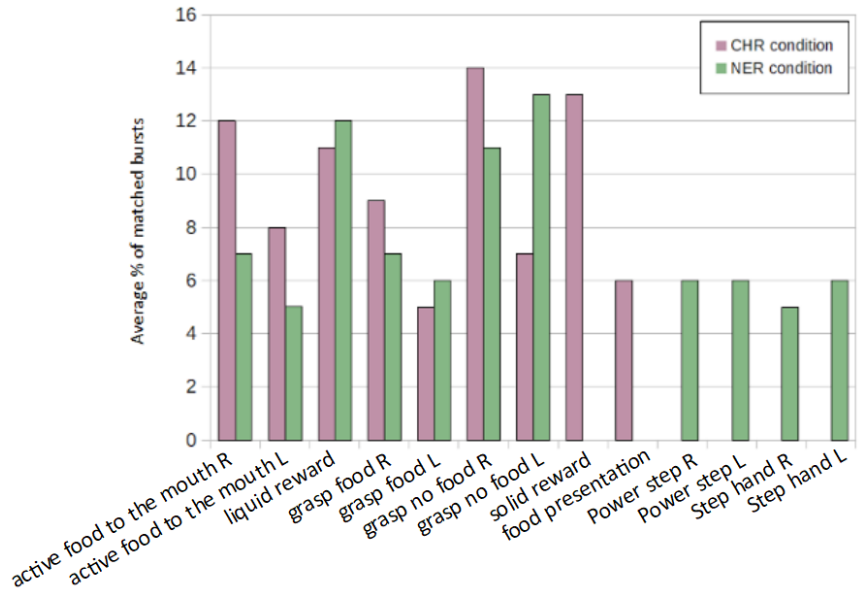

***Figure 14*. Burst-behaviours synchronization.** *The picture illustrate at what percentage of occurrences of the behaviours neurons of both monkeys (n=98) burst, in both conditions. Axis Y shows the percentage of bursts matched for the behaviour of interest (axis x) across neurons. "Grasp no food R/L" behaviour corresponds to "Finger prehension 0° R/L" in the CHR condition and to "Grasp for climbing R/L" in the NER condition.*

We then asked whether and to what extent the percentage of matched burst for a given behaviour in the CHR condition generalizes to the same behaviour in the NER condition. Thus, we computed the correlation between the percentages of matched bursts across neurons for each pair of behaviours (e.g., “Liquid reward” of CHR and NER, Figure 15A), obtaining the matrix below (Figure 15B).

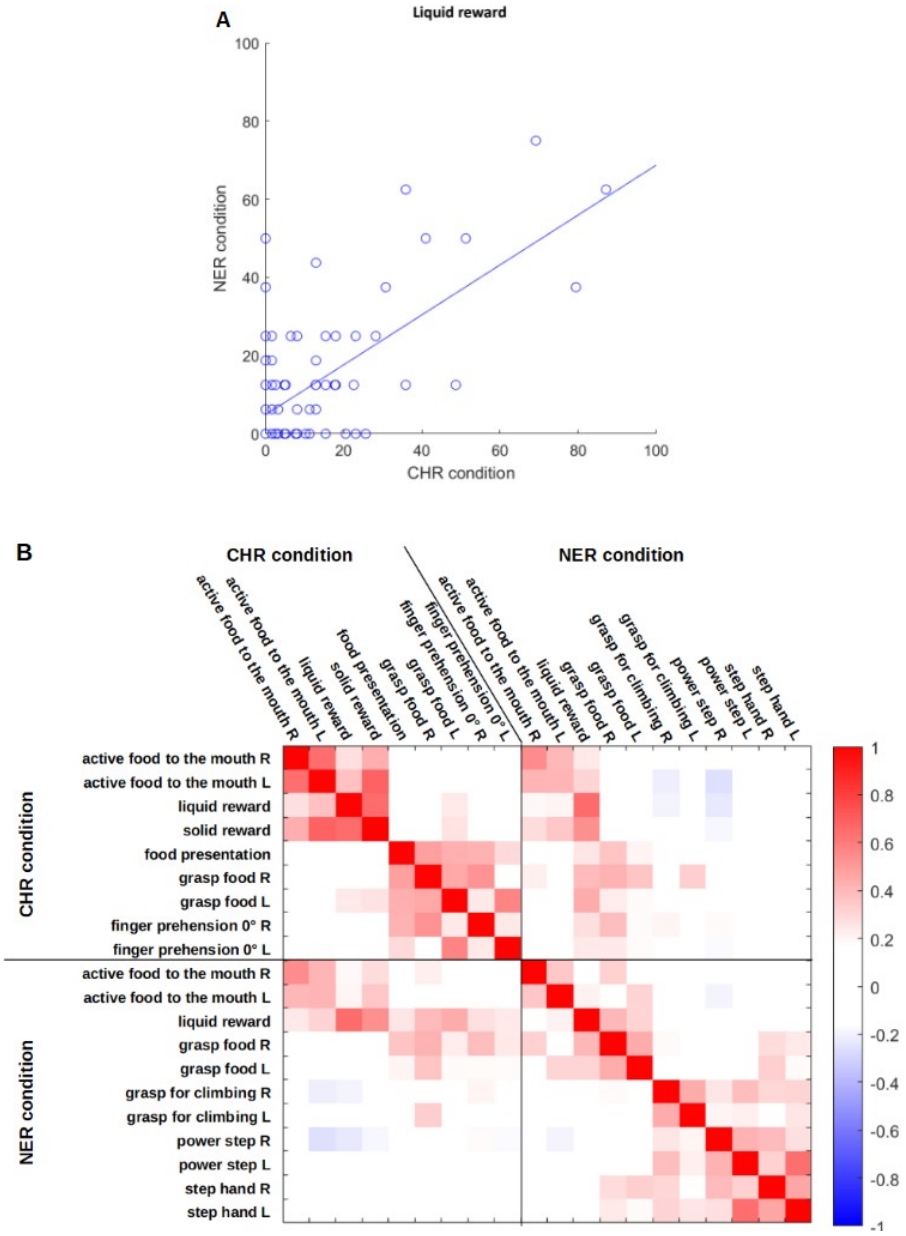

Figure 15. Pairwise correlation between burst-behaviour scores of the two conditions. *Panel A) shows an example of pairwise correlation inter-condition: the picture illustrates the linear correlation between the*

percentage of "Liquid reward" occurrences matched with a burst during CHR and NER conditions for both monkeys ($r=0.65$, $p=4.36e^{-13}$). B) for each neuron and for each behaviour, we used the percentages of occurrences of a particular behaviour matched with a burst across all neurons to calculate pairwise correlations, intra- and inter-conditions, between different behaviours. Significant positive correlations are depicted in red, significant negative correlation in blue (significance threshold: $r=+ - 0.17$, $\alpha=.05$).

From pairwise correlations intra-condition it emerged that in the CHR condition there is a significant and positive correlation among mouth-related behaviours as well as between upper limb actions. Instead, in the NER condition, we found that mouth and volitionally controlled behaviours directed to food (such as "Active food to the mouth", "Liquid reward" and "Grasp food") are significantly, mostly positively, correlated; in addition, whole-body behaviours such as locomotion are significantly correlated as well ("Grasp for climbing", "Power step" and "Step hand"). Moreover, the correlations between behaviours in the NER condition, even if significant, appears to be generally weaker than those in the CHR condition, likely because of higher variability among behaviours in the NER.

Finally, from pairwise correlation inter-condition it emerged a significant correlation between mouth behaviours performed in the two different contexts, especially for the "Liquid reward" behaviour and grasping food actions; however, considering behaviours specific of the NER condition that mainly involve the upper limbs in locomotion actions ("Grasp for climbing", "Power step", "Step hand"), we did not find significant correlations between them and upper limbs behaviours performed in the CHR condition.

5. DISCUSSION

In the last decade neuroscientific investigation aimed at understanding the relationship between brain activity and behaviour in a more ecologically relevant manner, trying to answer complex questions about the capacity of brain activity to generate adaptive behaviours. By means of animal model and wireless recording systems it has been possible to investigate brain activity in freely moving animals, recently even non-human primates (e.g. Berger et al., 2020; Jackson et al., 2007), paving the way to the development of more effective intervention to repair brain damage.

In this study, we conceived a novel approach to the investigation of the functioning of primates' ventral premotor cortex in controlling motor behaviours in naturalistic contexts. By implementing a two-step approach, we could compare the findings obtained with classical neurophysiological settings to those obtained on the same neurons recorded during spontaneous naturalistic behaviours.

One of the main challenges of naturalistic behaviours is that they are highly variable and poorly repeatable, thus violating one of the fundamental scopes of head-restrained, highly stereotyped laboratory tasks. Nonetheless, naturalistic behaviours must share with similar behaviours tested in the primate chair some hallmark features, supposed to underlie a possibly shared neural coding. This shared coding would be the base to generalize neurophysiological findings in the laboratory to explain the brain-behaviour relationship in the wild. Given the difficulties of identifying temporal epochs where neuronal discharge can be tested, as commonly done in laboratory tasks (e.g., Bonini et al., 2014a, 2014b), here we tested a novel approach based on the identification of trains of spikes with reproducible features (bursts) and looked for their possible match with behaviours across contexts (CHR and NER). Thus, instead of focusing on well-defined time epochs and looking how much the neurons fire in

these epochs across contexts, we opted for a more useful, or at least alternative approach, focusing on when the neurons fire and in relation to which observed behaviours (if any) across contexts.

First, we sorted merged neural data from both conditions and checked if the firing features of single neurons could demonstrate their stable isolation across the two contexts within a session. We found that the different firing features were positively correlated and non-significantly different between the two conditions, confirming that we were recording the same single units across conditions. Importantly, these findings allowed us to exclude that any difference in single neuron response properties in relation to the tested behaviours in the two contexts could be due to changes in the neuron isolation quality.

In both the CHR and NER conditions we found responses (burst) related to mouth and upper limb behaviours during reach, grasp and bring to mouth actions directed to food or objects. Our results are consistent with the chronic arrays implantation sites, although in Mk2 we recorded from a slightly more medial part of the premotor cortex, likely encompassing the lateral part of F2 (the caudal halves of dorsal premotor cortex). This area, indeed, is known to have functional properties similar to the ventral premotor cortex, with neurons involved in planning and controlling arm reaching, wrist and finger movements, but not the mouth/face (Raos et al., 2002). Interestingly, the above-mentioned behaviours with distal effectors when tested in the NER generally elicit a lower number of bursts. Considering that in the freely moving condition the final movement results from the contribution of many more variables that are strongly reduced or even eliminated in a head-fixed condition, we hypothesize that a lower percentage of responses in the NER condition could be due to other variables encoded by the premotor cortex activity when the animal is actively behaving.

Behaviours in the CHR and in the NER conditions can differ widely: movements performed in constrained and highly-controlled traditional paradigms, as those of the CHR condition, are often simple, stereotyped when a specific task with several repeated trials is used, and mainly involve the upper limbs (Jackson et al., 2007); in contrast, in a freely moving context they tend to be spontaneous and performed in a more complex, dynamic and synergistic manner, likely exploiting cortico-subcortical motor synergies involving the whole body, including head/gaze (Mushiake et al., 1997) and axial components (Maranesi et al., 2012; Mimica et al., 2018). These considerations could also explain why the pairwise correlations within the NER condition tend to be weaker than those in the CHR condition: the higher neural variability associated with the complex and continuously changing conditions of free movement could reflect the high variability that characterized spontaneous whole-body movements, which may be dealt or accounted for by a variable intervention of subcortical systems. Moreover, studying neurons functional properties starting from their spiking activity (burst) and matching it with behaviours afterwards, we found a high percentage of bursts that hadn't a match with our ethogram's behaviours in both conditions (although it remains clear the relationship of single units with specific manual and orofacial behaviours). This could be affected by limitations due to observation-based methods, but could also represent a further confirm on how much more complex could be the involvement of the premotor cortex in motor control of behaviours. This results, indeed, could be consistent with a mixed-selectivity mechanism, in which action-relevant information is distributed across a neural population to support flexible behaviours (Lehmann and Scherberger, 2013; Takahashi et al., 2017).

Whether and to what extent the variability in the observed behaviour can account for the mismatch between CHR and NER conditions may be investigated by means of kinematic analysis. Video-based markerless tracking of motion have already been successfully

implemented to measure and control tridimensional head, shoulder, elbow, and wrist trajectories (Berger et al., 2020) in non-human primates performing reaching tasks in mildly constrained conditions; similarly, retro-reflective marker tracking technologies can be used, at a certain extent even in monkeys, to control for postural variables during ongoing behaviours, as recently done in rats in order to understand how these factor affect neural activity and may be decoded during motor planning and whole body navigation (Mimica et al., 2018).

Since ventral premotor cortex neurons are known to have visuo-motor properties (Murata et. al 1997; Gallese et al., 1996) and can also be influenced by gaze position (Fuji et al., 1998; Lehmann & Scherberger, 2013), measuring this variable could constitute an added value to explain part of the variance of neuronal discharge in freely-moving context. Nonetheless, it is important to note that in the CHR condition monkeys were not required to maintain fixation, and it is well established that during grasping actions free gazing monkeys tend to exhibit a highly reproducible, stereotyped behaviour (Maranesi et al., 2013), likewise humans (Flanagan & Johansonn, 2003), which is necessary to provide visual information needed for predictive motor control of the hand. Monitoring the gaze will be undoubtedly important also to better discriminate purely motor and visuo-motor responses, as well as to investigate purely visual properties and their relevance in naturalistic and social contexts. Eye-tracking methods have been recently brought to a more ethologically-relevant applicability to investigate oculomotor behaviour in chickens (Schwarz et al., 2013), rodents (Payne and Raymond, 2017) and non-human primates (Milton et al., 2020) while the animals were free to behave naturally.

Further studies could implement wireless technologies along with kinematic analysis and eye-tracker methodologies in order to deepen knowledge on how the variables mentioned above may affect neural activity and play a role in a multidimensional control of motor

behaviour. Moreover, including other types of neural signals, simultaneously recorded with the individual neurons' spiking activity (multi-unit activity and local field potentials), may allow to better estimate the correspondence of neural activity with behaviour. Local field potentials, for instance, have recently been showed to describe awake and rest states in freely moving macaques (Milton et al., 2020). Furthermore, to better elucidate and understand the neural basis of behaviour organization and evolution over time, the neural activity that hadn't found a match with behaviours could be analysed in further studies by means of machine learning-based methods (Datta et al., 2019; Keemink and Machens, 2019) that allow to train and test a decoder to recognize different behaviours across contexts based on neural signal readout; this approach may lead to identify more general, hidden rules underlying the relationship between a larger variety of premotor neural signals and behaviour despite a relevant source of noise which may ultimately be found to carry relevant information on finer granularity of the studied behaviours. Clarifying the complexity of whole-body naturalistic behaviours and their neural bases is vital to develop more sophisticated brain machine-interfaces to restore lost function in spinal cord lesioned patients while performing multiple movements in the highly unconstrained settings of the real life.

Our new paradigm is part of the larger effort of the neuroscientific community (Genzel and Yartsev, 2019; Berger et al., 2020; Nourizonoz et al., 2020), to allow a more ecologically valid understanding of the brain-behaviour relationship and resolve neurophysiological questions that couldn't be explored with traditional methodologies. By improving the NeuroEthoRoom setup we could explore in an ecologically relevant manner not only motor planning and control, but also, for example, space coding in motor terms while the animal is freely behaving, and social interaction. Concerning the latter, indeed, the NER set up can allow monkeys to freely interact in a more naturalistic context while recording simultaneously

and continuously their neural activity, adding more accurate gaze and movement monitoring to gain tools for a deeper investigation of a larger variety of socio-cognitive processes (Hopper et al., 2020).

In conclusion, the results so far obtained with this novel approach reveal the possibility to record continuously from a non-human primate in traditional settings and unconstrained contexts allowing to compare neurons' functional properties in both conditions, and thus to refine a methodology for generalizing brain functioning from highly-controlled to ecologically relevant contexts, paving the way to understand the neural underpinnings of natural behaviour in non-human primates.

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