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NEURONAL REPRESENTATIONS OF EXECUTED AND WITHHELD ACTIONS IN THE MACAQUE PRE-SUPPLEMENTARY MOTOR AREA F6

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ABSTRACT

To plan an action and performing or withholding it requires an agent to consider contextual information such as the presence of another individual, the type of object or physical barrier preventing the subject to act. Studies in the last thirty years have assigned to the pre-supplementary motor area F6 a role in the encoding of high-order aspects of motor planning, but its relative contribution in coding executed or withheld actions of self and others is unknown. We trained monkeys to perform a Go/No-go visuomotor task and to observe an experimenter doing the same task. We identified five different populations of neurons responding to specific contextual information during task execution: some showed facilitate (Hexc cells) or suppressed (Hsup cells) response during Go trials, others were facilitated (HLexc) or suppressed (HLsup) during both Go and NoGo trials, and another set was modulated only during Nogo trials with a suppressed response (Lsup). Our findings suggest that these different sets of neurons may provide signals orchestrating releasing and withholding mechanisms underlying self and others' goal directed actions.

Evolutionary pre-face

The execution of a motor behaviour is not limited to the role of primary motor cortex and of the muscles under its control. The intention and the goal of the agent are themselves a fundamental part of the action itself. All of these aspects are bound to the sensory input available during the preparation and execution of an action. Indeed, modern theory of action planning and motor control maintain a circularity in perception and action, supported by the results of several studies on the parietal, premotor and prefrontal cortex, which form a network of anatomically connected areas underlying sensory-motor transformation.

In addition, the action context, including elements such as space, obstacles, rules or even other individual, have to be taken into account to plan the final motor output. The possibility to integrate these aspects also help us to learn new rules when existing-conditions change. For example, when we are driving a car the switch from green to red light is a sign that we must respect and stop the car. This simple example shows how the entire set of variable that characterized a context can change a motor program in its opposite.

Thanks to its anatomical location and functional proprieties, the rostromesial pre-supplementary motor cortex – also known as area F6 in the macaque or pre-SMA in human – appears to be a key region connecting high level motor and socio-cognitive functions to action execution.

1.1 General view on the premotor cortex

The premotor cortex was identified just at the beginning of the 20th century, when Brodmann, preformed a cytoarchitectonic parcellation of the brain in different cortical areas based on their morphological features. Brodmann thought that anatomically similar areas should have similar function. Starting from this consideration, area 4 and 6 were called "agranular cortex" because of the absence of the inner fourth layer (the granular one). This is both in the human and in the monkey brain (Fig.1)
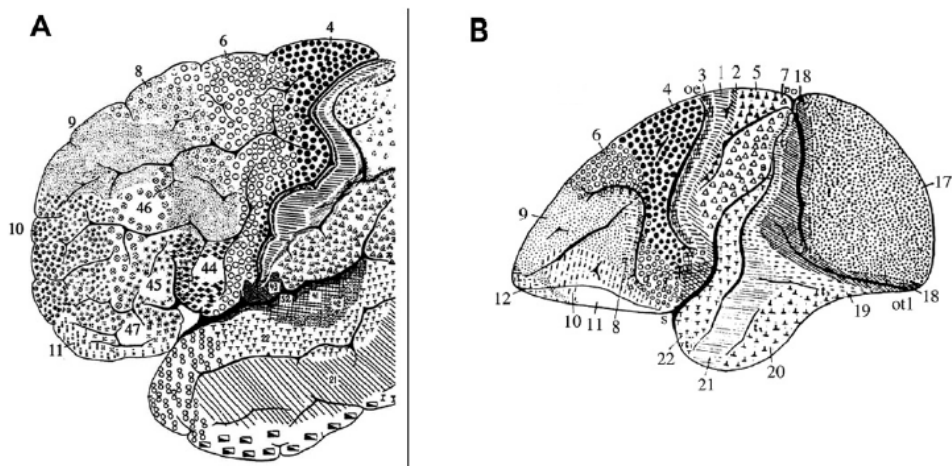
Figure 1 A: Frontal cortex of the human brain as parcellated in the cytoarchitectonic maps of Brodmann (Brodmann 1909); B: Cytoarchitectonic map of the monkey cerebral cortex (Brodmann 1905)

From Woolsey works we know that area 6 of the macaque cortex represents, like in human a secondary motor area and the mesial part of it has been named "supplementary" (Woolsey et al., 1952).

Matelli and co-workers divided area 6 of the macaque in different areas: F2, F3, F4, F5, F6 and F7 (Fig.2). All these areas together represent the premotor cortex. Areas F2 and F7 form the dorsal premotor cortex (PMd), areas F4 and F5 are the ventral premotor cortex (PMv), whereas areas F3 and F6 constitute the mesial premotor cortex (PMm) (Matelli and Luppino 1996). All these areas have been studied both with electrophysiology recording and with microstimulation (Rizzolatti and Luppino, 2001). Thanks to these two different methodologies a multiplicity of functional features have been demonstrated.
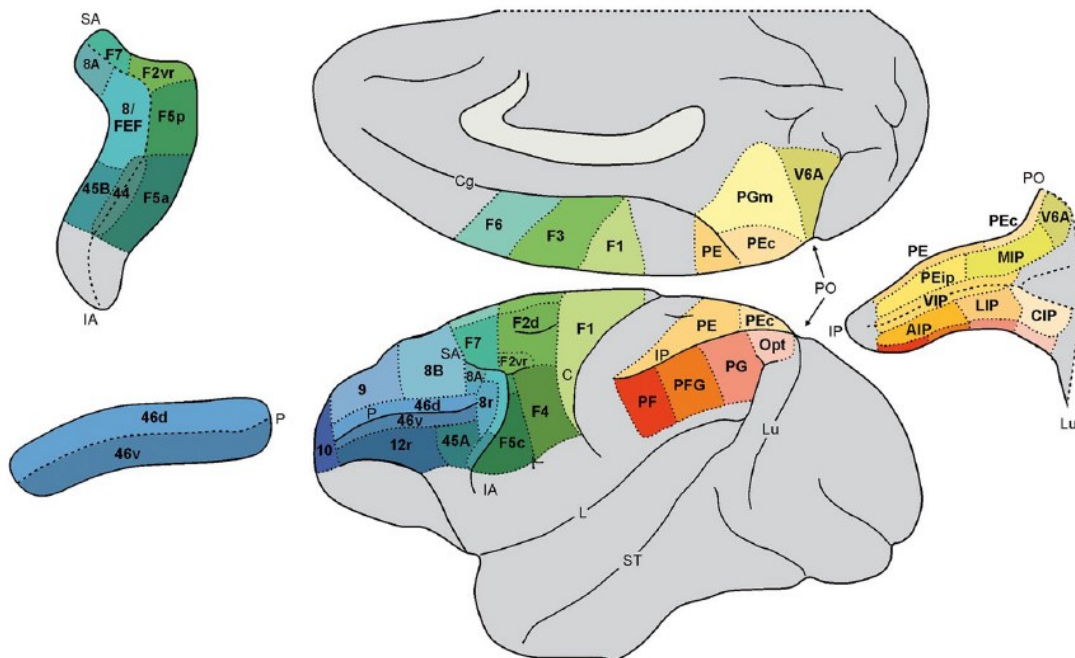
Figure 2 *Lateral and mesial views of the macaque brain showing parcellation of the frontal and posterior parietal cortex. The prefrontal cortex is subdivided according to Carmichael and Price (1994), except for its caudo-ventral part (Gerbella et al. 2007). Agranular frontal areas are classified according to Matelli et al. (1991) and Belmalih et al. (2009). The parietal areas are named according to Rozzi et al. (2006). The areas located within the arcuate and the principal sulci are shown in an unfolded view of the sulci in the left part of the figure, and the areas located within the intraparietal sulcus in the right part of the figure. Dashed lines indicate the architectonic borders. C central sulcus, Cg cingulate sulcus, IA inferior arcuate, L lateral fissure, Lu lunate sulcus, P principal sulcus, PO parietal-occipital sulcus, SA superior arcuate, ST superior temporal sulcus (Gerbella et al. 2017).*

Considering the connections of the different premotor areas, usually reciprocal with most of the target areas, it is possible to categorized two groups of premotor areas. In

particular, parietal areas project almost exclusively to caudal premotor regions, called parieto-dependent motor areas (i.e. F2, F3, F4 and F5). Area F6 and F7, the most anterior are connected with rostral prefrontal cortical regions and are called prefronto-dependent motor areas (Fig.3) (Rizzolatti, Luppino 2001).
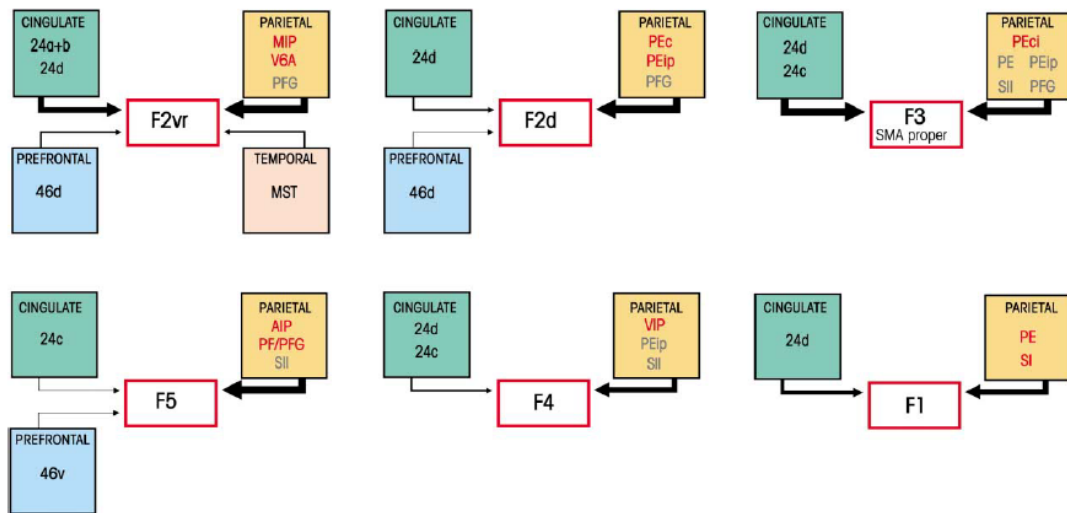
Figure 3: Schematic Diagram Showing the Extrinsic Afferent Connections of the Parieto-Dependent Motor Areas. The thickness of the connecting arrows reflects the strength of the connections. Areas indicated in red represent the parietal areas that are the sources of predominant inputs for the relative motor area. Parietal areas that are the sources of minor projections are indicated in grey. (Luppino; Rizzolatti, 2001)

Intrinsic connections with regions belonging to the premotor cortex maintain these distinctive features: prefronto-dependent areas – F6 and F7 - do not communicate directly with the primary motor cortex (F1) but only with other motor areas with which they are closely connected (Luppino et al., 1993). In contrast, the parieto-dependent areas are directly connected with F1 in a roughly somatotopic manner (Fig.4) (Muakkassa and Strick, 1979; Matelli et al., 1986; Luppino et al., 1993)
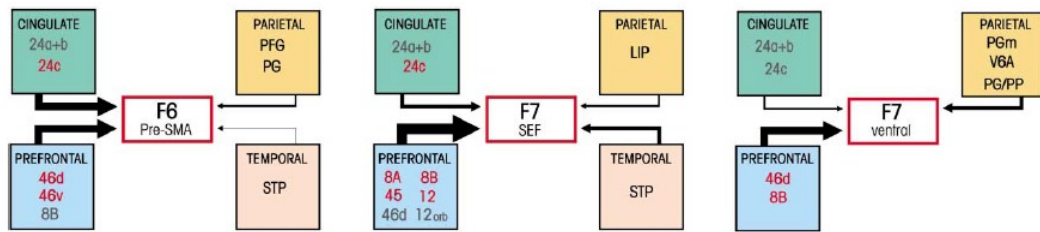
Figure 4: Schematic Diagram Showing the Extrinsic Afferent Connections of the Prefronto-Dependent Motor Areas. The thickness of the connecting arrows reflects the strength of connections. Areas indicated in red represent the frontal and cingulate areas sources of major inputs for the relative motor area. Frontal and cingulate areas sources of minor projections are indicated in grey. (Luppino; Rizzolatti, 2001)

In the last three decades F6 has been studied a lot. The great interest on this area is explained because it is not simply involved in the mere analysis of sensory stimuli, but thanks to its connection with the prefrontal and cingulate cortex, it seems that it play an important role in high-order function i.e. working memory, temporal planning of action and motivation. Nowadays it is widely accepted that rostral pre-supplementary motor area F6 plays a role in high level control of complex movements. It is also well known that it receives inputs from both prefrontal and cingulate cortices and from caudal premotor areas. Thanks to these connections, F6 plays a role in setting behavioural goals, organizing higher order action sequences based on learned spatial or non-spatial information and motivational state (Geyer et al., 2012).

1.2 Anatomical organization of area F6

Premotor cortex is divided into several areas: adjacent to the primary motor area F1 there are area F4 (laterally), F2(dorsally) and F3 in the mesial wall. More rostrally there are the ventral premotor area F5 there are the ventral premotor area F5, the dorsal premotor area F7 and the mesial pre-supplementary motor area F6, which lies in the mesial wall of the hemispheres just rostral to area F3 (Fig.3).

1.2.1 Cytoarchitectonic organization

Area F6 is located in the Brodmann's area 6 in the posterior part of the frontal lobe in particular in the mesial agranular frontal cortex. This portion of cortex has been named agranular because of the lack of the layer IV (the granular one). Considering the cytoarchitectonic features of this cortex it comes out that, generally, area F6 is rich in cells, but its density is lower than that of F3.

The first layer is thick (205 .t 9 Fm), whereas the second layer is poorly developed. The III layer of F6 is larger if compared with layer II. In layer III there are little pyramidal cells, whereas in the deeper layer V, there are bigger pyramidal cells, which make it darkly stained (Matelli et al., 1991; Belmalih et al., 2007). The limit between area F6 and the prefrontal cortex is defined by the recurrence of the granular layer.

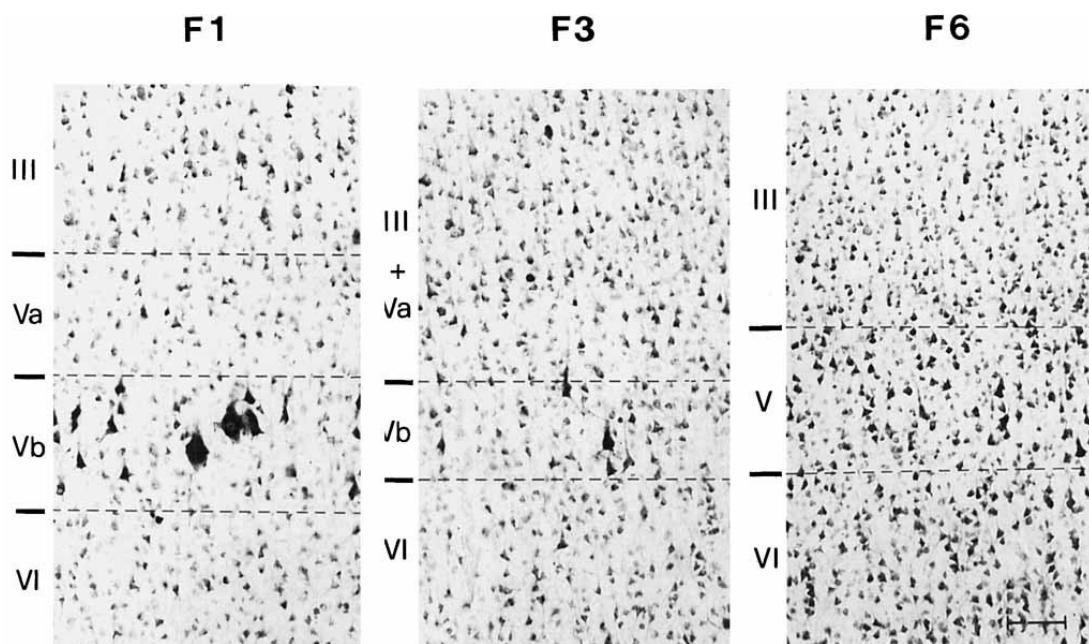*Figure 5 High-power photomicrographs of F1, F3, and F6. The figure allows a direct comparison of lower part of layer III and layer V in the various areas. (Matelli et al. 1991)*

Furthermore F6 has a dark, very evident layer V. This layer is well demarcated from the less dense layers III. In addition it is also important to note that, differently from F1 and F3, the rostral mesial cortex does not have a layer Vb, this layer is

important because it is where we can find giant pyramidal cells in directly connection with spinal cord (Fig.6).
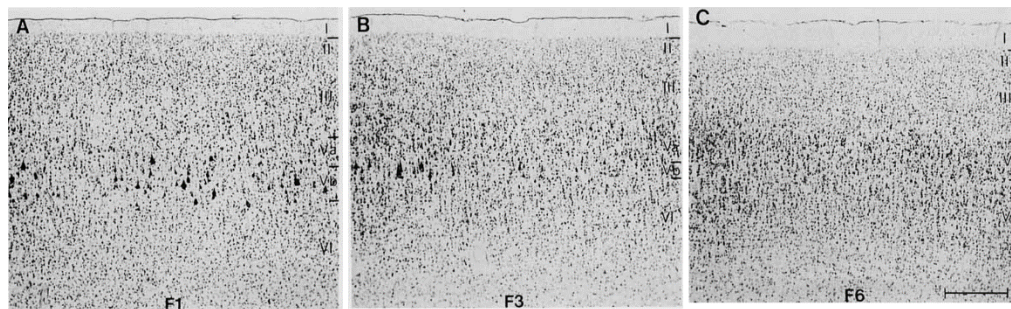

Figure 6: Photomicrographs of F1 (A), F3 (B), and F6 (C) (Matelli et al., 1991)

Going in depth we find an irregular border with the layer VI that appears not so dark as the layer V.

This cytoarchitectonic features allow to make a subdivision into a mesial sector (F6) and a dorsal one (F7). As a matter of fact, the two rostral sectors are different for the layer V - that is darker in F6 than in F7 - and in layer VI – that it is a single laminae in F6 and differently two in F7 (Matelli et al., 1991).

In conclusion, it is important to underline that this cytoarchitectonic differences between mesial and dorsal areas reflect a different anatomo-functional organization, and this is true for both F3-F2 and F6-F7. In fact rostral premotor areas can be involved in the generation of motor behaviour only indirectly.

1.2.2 Connections of F6

Corticocoritcal connections

Generally, all the premotor areas, share both cortical and subcortical connections.

Using a neuronal tracer some authors identified the various connections that F6 establishes with different cortical areas. Luppino and co-workers found that F6 has intrinsic connections especially with F7 and F5 and with F3, F2 and F4, but not with F1. Injections of retrograde tracer showed that most of input reaching F6 come from

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the prerolandic areas i.e. the anterior premotor (F5, F7), cingulate (agranular cingulate cortex 24c) and prefrontal regions (see fig. 7). Concerning the connections between the two mesial cortical areas (F3 and F6) we know that efferences from pre-SMA to SMA are richer than those from F3 to F6 (Matelli et al. 1986; Luppino et al. 2003; Gerbella et al 2011). In a recent study, Albertini and co-workers (Albertini et al. 2020) injected an antero-retrogade tracer along the rostro-caudal cortex of F6 with the purpose to mark the different anatomical connectivities at different antero-posterior positions (Fig.7). The labelling of neurons gave the possibility to appreciate the anatomo-functional organization and the connectivity of F6 with other cortical regions. The histograms show the percentages of marked cells that appear in other cortical areas after the injection of tracer. Grouping these marked areas on the basis of similar functional proprieties, it came out that the majority of F6 connections are with the mesial motor cortex (MCC), the supplementary motor area (SMA), the dorsal premotor cortex (PMd) and the ventral premotor cortex (PMv). A great percentage of marked cells in these areas are mostly related to the caudal portion of F6.
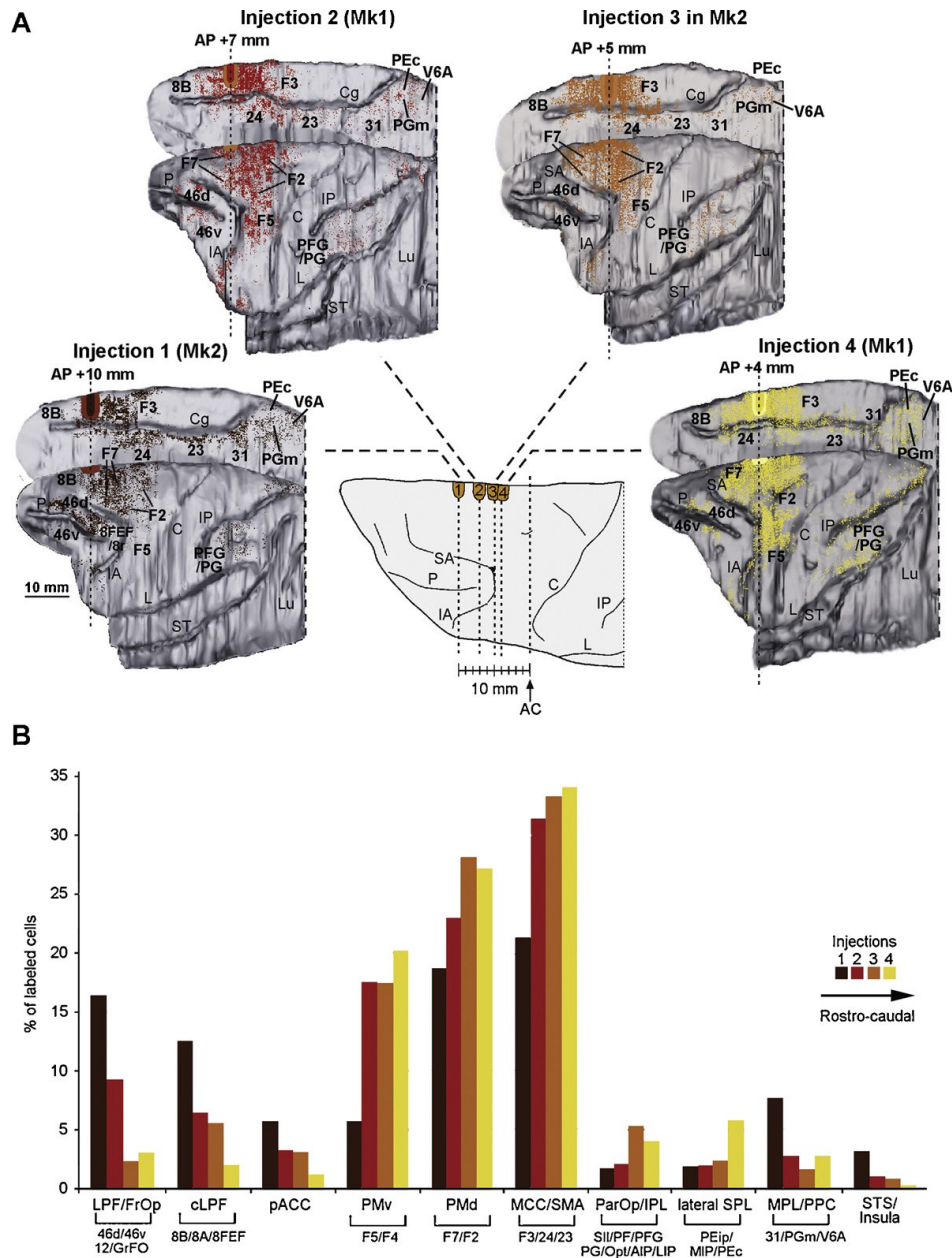
Figure 7: Anatomical connectivity of F6 sectors located at different antero-posterior positions. (A) Three dimensional anatomical reconstructions illustrating the distribution of labelled cells after injections in four different spots of F6 at different antero-posterior position. The labelling is shown in dorsolateral and medial views of the injected hemisphere: each dot corresponds to one labeled neuron. The location of each injection is shown as a filled area. Dashed lines indicate the position of the injection site (0 corresponds to the anterior commissure). To facilitate the comparison, all the lateral views of the brain are shown as a left hemisphere and the mesial views a right hemisphere. The 2D reconstruction in the centre is a composite view of all the injection sites, shown as red circles, mapped on a template hemisphere. (B) Histograms illustrating the percentage of labeled cells in the various cortical regions following each injection (in colour code). The areas (listed under the histogram) are grouped based on anatomo-functional similarity. Abbreviations: ACC, anterior cingulate cortex; Cg, cingulate sulcus; cLPC, caudal lateral prefrontal cortex; FrOp, frontal operculum; IP, intraparietal sulcus; IPL, inferior parietal lobule; LPC, lateral prefrontal cortex; Lu, lunate sulcus; MCC, mesial motor cortex; MPL, medial parietal lobule; ParOp, parietal operculum; Pmd, dorsal premotor cortex; PMv, premotor cortex; PPC; posterior parietal cortex; SMA, supplementary motor area; SPL, superior parietal lobule; ST, superior temporal sulcus (Albertini et al. 2019).

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Beyond the frontal lobe, F6 has very meagre connections with the postrolandic regions. Area F6 is linked with area PG and PFG, and has some connections with the disgranular insula and the superior temporal sulcus (STS) (Luppino et al., 1993).

Interestingly a strong difference between F6 and F3 is that F3 has a direct output to the primary motor cortex, whereas F6 does not have any connection neither to F1 nor to the spinal cord (He et al. 1993; Luppino et al. 1993).

Subcortical connections

F6 has subcortical projection to the striatum, which in turn projects, directly and indirectly to the globus pallidus. These connections create cortico-subcortical circuit (Parthasaraty et al., 1992; Inase et al., 1999). Forming the cortico-basal ganglia loop. The output of the glubus pallidus inhibit the motor nuclei of the thalamus whose modulation affect the selection and initiation of cortical motor plans. A recent work by Albertini and co-workers leveraged antero-retrograde tracers injected in different antero-posterior position of area F6 to show that the region of the putamen caudal to the anterior commissure, related to motor control of the hand and the arm (Alexander and de Long 1985; Nambu 2011), is linked with the caudal area F6, whereas the portion of the putamen rostral to the anterior commissure, which is generally classified as associative (Alexander et al. 1986; Tremblay et al., 2015),was reached by projections from the most anterior part of F6. The rostral part of area F6 is also linked with the caudate nucleus, and provide a functional base for the interpretation of the concomitant rostro-caudal gradients in terms of observed functional properties in same region (see fig. 8), from self-referenced location of target object, rostrally to the encoding of actions of self and others, caudally.
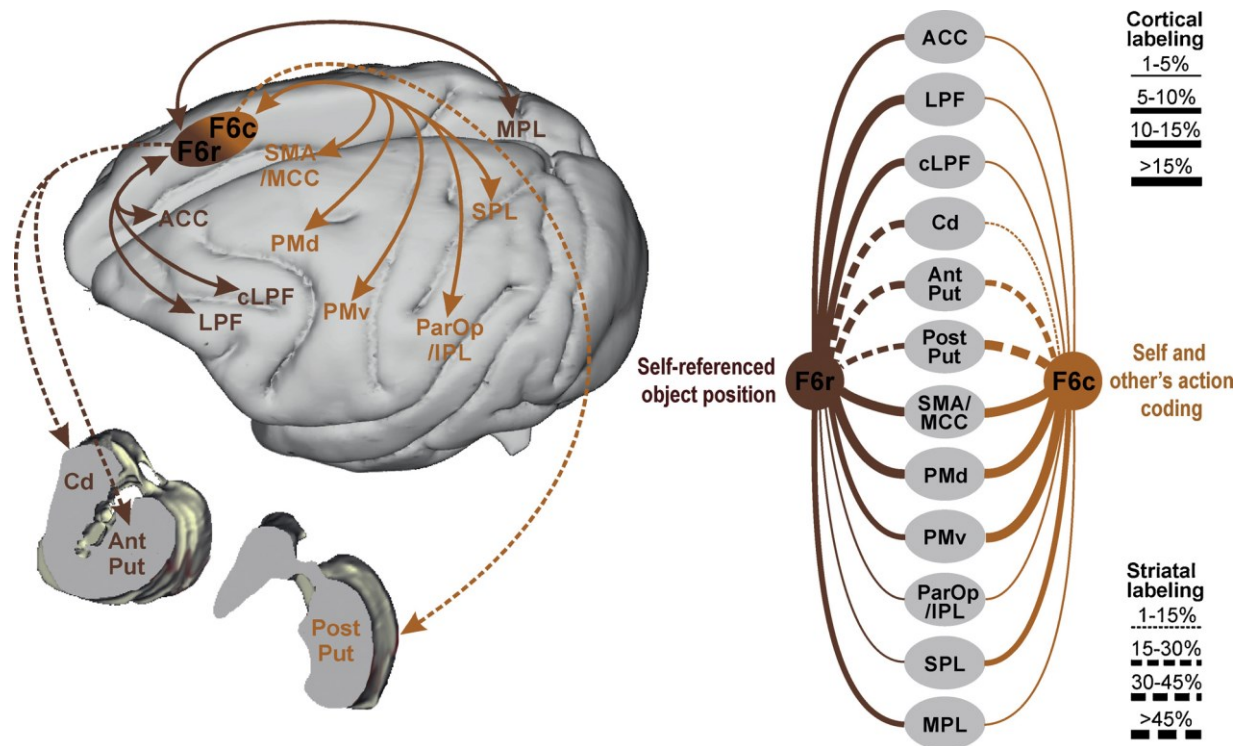
Figure 8: Schematic representation of the connectivity patterns of rostral and caudal part of areaF6. The lines represent the stronger connections observed after the most rostral (injection 1, brown) and caudal (injection 4, orange) injection. The full lines represent the cortical connections, the dashed lines the striatal ones. (Albertini et al. 2019)

1.3 Functional proprieties of area F6

1.3.1 Studies of microstimulation

The rostral mesial cortex, differently from others premotor areas (F2, F3, F4, F5p and F5c) does not have any direct access either to the spinal cord or to F1 (Porter and Lemon, 1993), hence being able to contribute to motor behaviour only indirectly (Schall et al., 1993; Geyer et al., 2012). As a consequence, intracortical microstimulation (ICMS) is purely effective in triggering F6 motor responses. It is necessary to opt for relatively long stimulation train duration (100 ms) with relatively high current intensity. Furthermore, motor responses following ICMS in F6 are facilitated by the application of natural stimuli (reaching for food, pushing away negative stimuli) combined to ICMS pulses (Mitz and Wise, 1987; Luppino et al., 1991). These studies evidenced that the elicited movements are generally slow and

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semi-natural, while in F3 they are faster and triggered by lower and shorter electrical stimuli.

During qualitative testing of neuronal properties the activity recorded in this area was characterized by neurons related to the orienteering of neck and upper face-mouth movements (caudal part of F6 near the border of F3). Some neurons respond also to visual stimuli, but most of them respond to reaching-grasping actions. The activity of these neurons, does not seem to be influenced by the type of grip (Rizzolatti et al., 1990; Tanji et al., 2001). Another aspect of the relation with reaching-grasping action is emphasized by the fact that these neurons does not respond during a non-goal-directed movement. No eye movement has been found. These studies also showed that F6 neurons started their discharge before the beginning of the movement. Furthermore neurons maintained this activity for the whole duration of the action. This change in their activity could start right at the object presentation, before action execution. Considering the activity pattern of reaching-grasping neurons, the authors classified two different and opposite populations during visual presentation and grasping of objects:

1) Neurons with excitatory-excitatory or inhibitory-inhibitory response during both phases of the task;

2) Neurons with excitatory-inhibitory or inhibitory-excitatory activity during object presentation and reaching-grasping, respectively.

The authors claimed that F6 plays a role in the preparation and execution of reaching-grasping actions, when the context offers the appropriate conditions (Rizzolatti et al., 1990).

A different property shown by F6 neurons has been demonstrated by Shima and co-workers (1996). In this experiment the monkeys were trained to perform

different sequences of temporally-ordered movements (correct information about the right order was given – at first – on the basis of visual information, then the monkey had to perform the remaining sequence by memory). The movements that monkeys have to perform were "pull – push – turn" on a manipulandum. After some correct trials the monkey was required to perform a new sequence and to discard the old one. This task allowed the authors to discover a new set of neurons: some cells discharged only during the first part of a trial of any new sequence. Curiously, during the following trials this specific response disappeared. This fact can be explained by the learning of the sequence, thanks to which the monkey could then perform the task on the basis of a mnemonic trace in the subsequent trials. A possible interpretation of these data is that F6 plays a role in the update of a motor plan. In this interpretation any new sequence of movement might requires a new motor strategy. As a matter of fact, when the monkey have to complete the task simply by memory, no updating is needed and neurons do not discharge (Shima et al., 1996).

A complete new paradigm has been tested by Yoshida and co-workers. These authors used a social paradigm to test how F6 respond during social interaction. This choice is explained by the fact that for social interaction the agents need to distinguish between their own and others' action (Decety et al., 2003; Tomlin et al., 2006; Frith et al., 2010). The experimental set was organized to investigate how actions of another agent are encode by F6 neurons. To do this, the monkey actively use the information provided by another monkey's action. Then the observer monkey use this information for guiding its own action. The task was performed by two monkeys sat face to face. In each trial there was always a monkey with the role of actor and the other one with the role of observer. The roles of actor and observer was alternated every two trials. If the entire trial was performed correctly both monkeys received the reward (fig. 9).
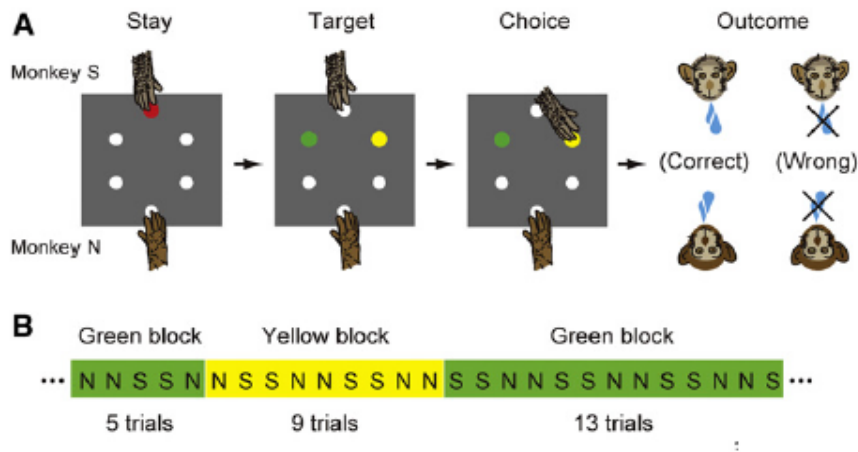
Figure 9: Behavioral task and animals' choice behaviour. (A) Temporal sequence of events in role-reversal task. Shown is an example of a single trial in which monkey S was the actor and monkey N was the observer. The reward outcome differed depending on the colour-reward contingency ("Outcome"). (B) in the task, two monkeys alternated in the role of the actor every two trials, and the colour-reward contingency switched unpredictably every 5-17 trials (blocked design). "Green block" means that the green target was associated with reward. N and S indicate the acting monkey (Yoshida et al., 2011).

The activity of a set of single neurons in area F6, significantly increased during actions performed by the partner: some of them also discharged during action execution, whereas another set specifically encoded partner's action. This latter type of neurons have been named "partner type". This variable was proposed for discarding the possibility that the partner-selective neuronal activity might reflect the animal social hierarchy. Anyway, also with these new variable, the neurons activity was the same, demonstrating the existence of a neural substrate specific for representing others' action (Yoshida et al., 2011).

In the last studies done on F6 (Lanzilotto et al. 2016; Livi et al. 2019; Albertini et al. 2020) it comes out that this area is also involved in the encoding of visually presented objects and types of grip. The authors applied a go-nogo task with three main conditions:

1) Grasping in the light

2) Grasping in the dark

3) No-go condition.

They found purely motor neurons, visuomotor neurons and purely visual neurons. Among purely motor neurons, most of them discharged in different way on the basis of the type of object even in the dark. The different type of discharge reflects a preference for the type of grip. Among the neurons triggered by the vision of the objects (visual and visuomotor) there was a 28% of these cells showing a selectivity for the observed object. Based on a series of test, including visual presentation of object behind a barrier and the comparison of visual selectivities for the different types of objects, the authors proposed that F6 could operate a visuomotor association between the object and the "potential" action that can be done, especially when these actions is a rather unnatural and learned one (such as in the case of ring object, for which the monkey has to be trained to insert the index finger and pull the object, otherwise grasped with a side grip). This results induced to include area F6 in the cortical network for the visuomotor processing of objects.

1.3.2 Studies of single unit in human

Study on human pre-SMA revealed similarity with area F6 of the macaque (Amador N., Fried I., 2004). According with the idea of a phylogenetic continuity between nonhuman primate and the human, these authors thanks to an implantation of intracranial electrodes recorded 61 neurons in the supplementary motor area (SMA proper and pre-SMA) of patients with pharmacologically intractable epilepsy. 26 of these 61 neurons were from the SMA and the remaining part were from the pre-SMA. The tasks they used for testing the functional activity of these areas was composed of an execution part and of a mental imagery. During both the tasks the patients had to perform (or imagine) the apposition of fingers-to-thumb during random sequences. Fingers were numbered by 1 to 4 (respectively: 1 correspond to the index and 4 to the

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little finger). The sequences could be simple or complex. In the simple condition number were repeated i.e. (111, 222, 333), differently in the complex condition number were randomized (231, 312, 132). The right hand (L or R) to use and the right sequence to perform have been presented on a computer monitor. In both tasks a trial began with the onset of the instruction cue (for example, L213, R123). The patient was required to fixate on the instruction cue for 5 seconds.

Authors found that in pre-SMA there is a higher percentage of neurons (31.4% on the total recorded neurons in pre-SMA) than in SMA that have a differentiated response between simple and complex tasks, in addition another 22.9% of neurons in this area displayed a significant difference between neurons response rates for ipsilateral and contralateral movements. Furthermore another interesting aspect is that some neurons that responded selectively to a particular sequences of fingers apposition to thumb. Finally, selective neuron responses to visually prescribed complex or simple sequences as well, selective responses to individual sequences, indicates that the activity of medial frontal neurons encodes information about stimuli. This information is then used for planning the motor response (Hernàndez et al., 2002; Shima et al., 2000). Concerning the trials performed in the imaginary task this study highlights some functional proprieties of the pre-SMA related to mental activity during the imagination of a movement sequence that cannot be accessed in animals. In fact, Amador and co-workers found that a small number of neurons (contrary to any expectation) responded with a higher discharge during the imagery of a movement sequence than during the actual performance of it (Amador N., Fried I., 2004).

3.   *AIMS OF THE STUDY*

F6 has been described as an area involved in a variety of functions in multiple domains. First of all, it has both motor and visual property. Motor response evoked in this area demonstrated it is implicated in the control of forelimb movements. In addition, it exhibits an activity related to the initiation, sequencing and planning (or withholding) of voluntary goal-directed actions. With different task it showed an activity related to the update of a motor plan. Recent studies demonstrated that F6 has a main role in social aspects. As a matter of fact, it has neurons that encode selectively monkey's own action and another agent's action, or both. This work aims to highlight how F6 integrates sensory and motor information for contributing to the representation of executed and withheld action.

4. MATERIALS AND METHODS

Experiments were carried out on one Macaca nemestrina (MK1, male, 9kg) and one Macaca mulatta (MK2, male, 7kg). Before recordings, monkeys were habituated to sit in a primate chair and to interact with the experimenters. They were then trained to perform the visuomotor tasks described below using the hand contralateral to the hemisphere to be recorded. When the training was completed, a head-fixation system was implanted under general anesthesia (ketamine hydrochloride, 5 mg/kg i.m. and medetomidine hydrochloride, 0.1 mg/kg i.m.), followed by postsurgical pain medications. Dexamethasone and prophylactic broad-spectrum antibiotics were administered pre- and postoperatively: Furthermore analgesics were administered intra- and postoperatively. During all surgeries, hydration was maintained with continuous infusion of saline solution. A heating pad was used to maintain the temperature constant. The heart rate, blood pressure, respiratory depth and body temperature were continuously monitored. Upon recovery from anesthesia the animal were returned to their home cages and closely monitored.

All experimental protocols complied with the European law on the humane care and use of laboratory animals (directives 86/609/EEC, 2003/65/CE, and 2010/63/EU), they were authorized by the Italian Ministry of Health (D.M. 294/2012-C and 11/12/2012), and approved by the Veterinarian Animal Care and Use Committee of the University of Parma (Prot. 78/12 17/07/2012).

4.1 Apparatus and Behavioural Paradigm

Both monkeys were trained to perform a visuomotor task by means of the apparatus shown in (Fig.10). The monkey was seated on the primate chair in front of the box.
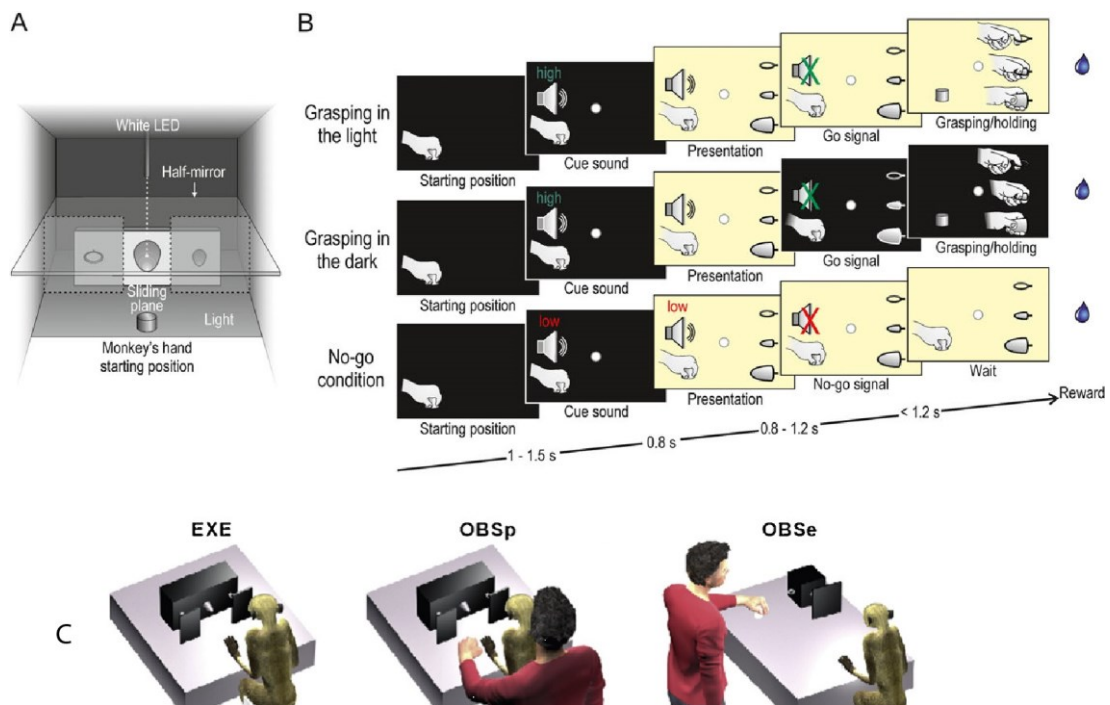
Figure 10 Behavioural paradigm. (A) Behavioural setup for the execution. (B) Temporal sequence of task events. Each trial started when the monkey, with its hand in the starting position, engaged fixation in complete darkness. A high (Go cue) or low (No-go cue) tone was presented and remained on during the subsequent object presentation phase (Obj pres). When the sound stopped (Go/No-go signal), the agent monkey (monkey or experimenter) had to reach, grasp and pull the target (Go trial) or to remain still (No-go trial), maintaining fixation for the entire duration of the trial (Albertini et al. 2020). (C) Behavioural setup for the observation.

The box was divided horizontally into 2 sectors by a half mirror: the upper sector contained a small black tube with a white light-emitting diode (LED) that could project a spot of light on the half-mirror surface; the lower sector contained a sliding plane hosting three different objects. When the LED was turned on (in complete darkness), the half-mirror reflected the spot of light so that it appeared to the monkey as located in the lower sector (fixation point), in the exact position of the centre of mass of the not-yet-visible target object. The object – a ring, a small cone, and a big cone – were chosen because they afforded three different grip types, as follows: hook grip (in which

the index finger enters the ring); side grip (performed by opposing the thumb and the lateral surface of the index finger); whole-hand prehension (achieved by opposing all the fingers to the palm). Objects were presented, one at a time during different experimental trials, through a 7-cm opening located on the monkey's sagittal plane at a reaching distance from tis hand starting position. A stripe of white LEDs located on the lower sector of the box allowed us to illuminate it during specific phases of the tsk. Note that, because of the half-mirror, the fixation point remained visible in the middle of the object even when the lower sector of the box was illuminated.

The task included three basic conditions, as illustrated in Figure 10: grasping in the light, grasping in the dark, and a no-go condition. Each of them started when the monkey held its hand on a fixed starting position, after a variable inter-trial period ranging from 1 to 1.5 s from the end of the previous trial.

1. *Grasping in the light:* the fixation point was presented and the monkey was required to start fixating it within 1.2 s. Fixation onset resulted in the presentation of a cue sound (a pure high tone constituted by a 1200 Hz sine wave), which instructed the monkey to grasp the subsequently presented object (go-cue). After 0.8s, the lower sector of the box was illuminated and one of the objects became visible. Then, after a variable time lag (0.8 – 1.2 s) the sound ceased (go-signal), at which point the monkey had to reach, grasp, and pull the object within 1.2 s. It then had to hold the object steadily for at least 0.8 s. If the task was performed correctly without breaking fixation, the reward was automatically delivered (pressure reward delivery system, Crist Instruments).

2. *Grasping in the dark:* the entire temporal sequence of events in this condition was identical to that of grasping in the light. However when the cue sound (the

same high tone as in grasping in the light) ceased (go – signal), the light inside the box was automatically switched off and the monkey performed the subsequent motor acts in complete darkness. Note that because the fixation point was visible for the entire duration of each trial, it provided a spatial guidance for reaching the object in the absence of visual feedback. In this paradigm, grasping in the light and grasping in the dark trials were identical and unpredictable until the occurrence of the go – signal: thus, action planning was the same in both conditions, and the only difference between them was the presence/absence of visual feedback from the acting hand and the target object.

3. *No – go condition*: the basic sequence of events in this condition was the same as in the other go conditions, but a different cue sound (a pure low tone constituted by a 300 – Hz sine wave) instructed the monkey to remain still and continue fixating the object for 1.2 s in order to receive a drop of juice as a reward. The same sequence of events of the no – go condition as also been employed during a barrier test. In the barrier test, a transparent plastic barrier was interposed between the monkey's hand and the target. The aim of this test was to verify whether object processing by F6 neurons could be different depending on whether the monkey refrained from acting because of physical obstacle (the barrier) or because of an instruction cue (the no – go signal). Hence, we used the go-cue during this test in order to ensure that the monkey refrained from acting because of the presence of the barrier (Bonini et al. 2014). Before formal testing of neuronal activity, the monkey was administered a few trials before starting the acquisition block, in order to ensure it actually understood that the barrier was present.

The task phases were automatically controlled and monitored by LabView-based software, enabling the interruption of the trial if the monkey broke fixation, made an incorrect movement, or did not respect the task temporal constraints described above. In all these cases, no reward was delivered. After correct completion of a trial, the monkey was automatically rewarded with the same amount of juice in all conditions.

The activity of each neuron was recorded in at least 12 trials for each basic condition. In all sessions, we also recorded 12 additional control trials in which the monkey was presented, in complete darkness and with its hand still on the starting position, with the fixation point alone: after a variable time lag (<1 s) from fixation onset, the reward was delivered. These trials were used to verify the possible presence of neuronal responses due to mouth movements/reward delivery, which could otherwise be confounded with hand-related activity, particularly during the holding epoch that precedes the reward delivery. Neurons responding specifically to this condition were not considered as task related in the present study.

4.2 Recording Techniques

Neuronal recording were performed by means of chronically implanted 2D or 3D arrays of linear silicon probes with 8 recording channels per shaft and a variable number of shafts per probe, as follows: one 4-shaft 2D probe in the right hemisphere of MK1; and one 3D probe in the left hemisphere of MK1; two 3D probes in the right hemisphere of MK2 (see Fig. 11). All probes were implanted vertically, approximately 1 mm laterally to the mesial wall.

The signal was amplified and sampled at 40 kHz with a 16 channel Omniplex recording system (Plexon). Different sets of 16 channels were recorded only one time during separate session on different days.
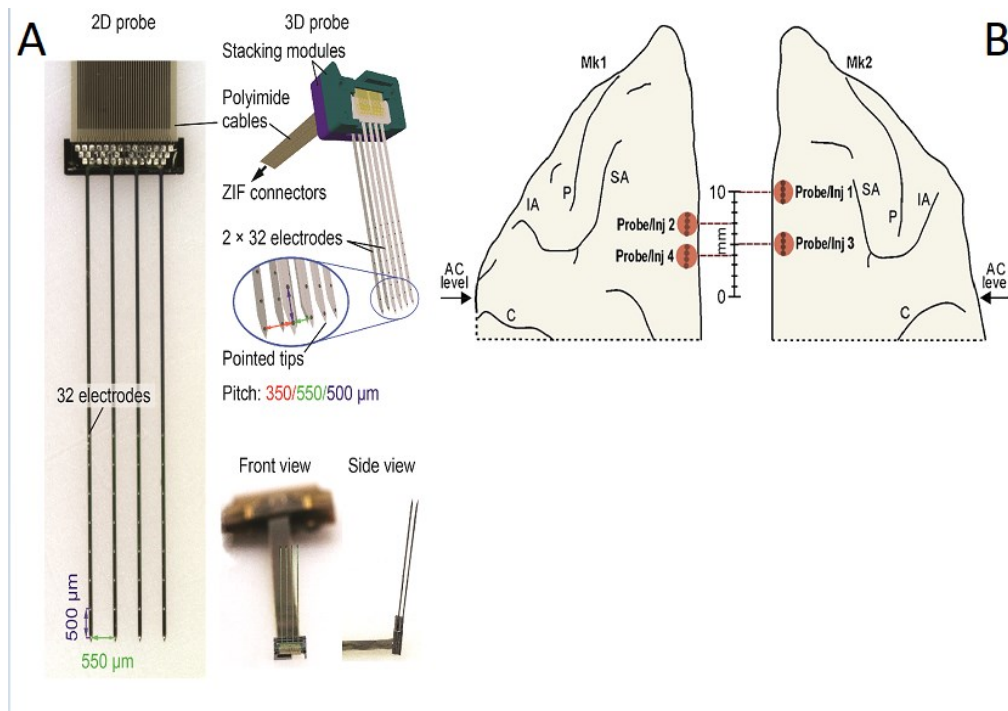
*Figure 11 **(A) 2D and 3D probes. (B) Recorded and injected sites**. The grey dots illustrate the anatomical location of each probe's shafts and the red shaded circles indicate the location of the core of injection sites relative to the implanted probes. (Figure from: Albertini et al. 2019)*

The signal was amplified and sampled at 40 kHz with a 16 channel Omniplex recording system (Plexon). Different sets of 16 channels were recorded only one time during separate session on different days. Online spike sorting was performed on all channels using dedicated software (Plexon), but all final quantitative analysis were performed off-line, as described in the subsequent sections.

4.3 Recording of Behavioural Events and Definition of Epochs of Interest

Distinct contact sensitive devices (Crist Instruments) were used to detect when the monkey (grounded) touched with the hand the metal surface of the starting position or one of the target objects. To signal the onset and tonic phase of object pulling, an additional device was connected to the switch located behind each object. Each of these devices provided a TTL signal, which was used by the LabView-based software to monitor the monkey's performance and to control the generation and presentation of the behavioural paradigm's auditory and visual cue signals.

Eye position was monitored in parallel with neuronal activity with an eye tracking system consisting of a 50-Hz CCD video camera provided with an infrared filter and 2 spots of infrared light. Analog signal related to horizontal and vertical eye positions was fed to a computer equipped with dedicated software, enabling calibration and basic processing of eye position signals. The monkey was required to maintain its gaze of the fixation point (tolerance radius 5°) throughout the task, and the eye position signal was monitored by the same LabView-based software dedicated to the control of the behavioural paradigm. These signal were recorded and stored together with the neural activity and subsequently used to construct the response histogram and the data files for statistical analysis.

Single-neuron activity was analysed in relation to the digital signals related to the main behavioural events, by considering the following epochs of interests:

1. Baseline, 500 ms before object presentation

2. Object presentation, from 0 to 500 ms after switching on the light

3. Pre-movement, from 500 ms before turning off the sound and reaching onset (detachment of the hand from the starting position

4. Movement, from the sound off to 1000 ms after this event.

During baseline, the monkey rested its hand unmovingly on the starting position, was starring at the fixation point, and was already aware of whether the ongoing trial was a go or a no-go trial: these feature enabled us to assess possible variation in neural discharge specifically linked with the subsequent tsk stages within the ongoing behavioural set.

4.4 Data Analysis

4.4.1 Single Unit Analysis

Single unit have been isolated using the ratio of the visuomotor task described above. We made an ANOVA 3 x 2 x 4 (Objects x Condition x Epochs) to define the activity of each single unit, so we classified neurons significantly activated during movement-related epochs relative to baseline. When there was an interaction effect of 2 or more levels we corrected with Bonferroni post-hoc test ($P < 0.05$). We classified all single units as *nonobject-type visuomotor* neurons that showed only a significant effect ($P < 0.05$) in the action execution epoch in the light (Epoch 4) relative to baseline. We classified as *object-type visuomotor* neurons, all single units showing a significant effect ($P < 0.05$) during the object presentation epoch (Epoch 2) relative to baseline. Next, single units were classified on possible modulation of the activity in Epoch 4 as *facilitated* (when the response was stronger than baseline) or *suppressed* (when the response was weaker than baseline, according to Vigneswaren et al. (2013).

4.4.2 Population Analysis

We clustered single unit activity into 5 populations on the basis of their response during the action execution epoch (Epoch 4) in light condition.

1. Neurons facilitated/suppressed only in trials with high sound

2. Neurons facilitated/suppressed both in trials with high and low sound

3. Neurons facilitated/suppressed only in trials with low sound.

Population analysis were carried out taking into account single-neuron responses expressed in terms of normalized mean activity (each neuron relative to the absolute maximum activity value among all conditions and then, for each condition, subtracting the average baseline activity value from each normalized value). Data were converted to raster format into binned format: specifically, we created binned data that contained the firing rate of 200 ms intervals sampled every 20 ms.

To analyse the population activity we performed, on the base of the response to the object preferred, a repeated measures 8 x 4 ANOVA (factors: Condition x Epoch) on the mean activity for each condition (visuo-motor task: go in light, go in dark, no-go, barrier; observation task: go and no-go in peripersonal space, go and no-go in extrapersonal space) within all 5 populations, followed by Fishers's LSD post-hoc test ($P < 0.05$) in the case of significant interaction effects or to identify specific effects of factors with more than 2 levels. Excitatory neurons included in population responding to the low sound were included in the population analysis after inverting their response.

5. RESULTS

We recorded neural activity from 4 rostro-caudal positions along area F6 in both monkeys: two locations in the left hemisphere of monkey 1 (Mk1) and two locations in the right hemisphere of monkey 2 (Mk2) using 32-channel linear multi-electrode silicon probes. The probes were spaced from each other by 4 mm in monkey 1 and by 6 mm in monkey 2 (Fig 11). The entire recorded region corresponds to the functionally defined area F6 (Luppino et al., 1991; Matsuzaka et al., 1992; Nakamura et al., 1999, Albertini et al., 2020)

During all recording sessions (n = 16; 11 in Mk1 and 5 in Mk2), monkeys performed both the VMT and the OT (Fig.10B, C), using the apparatus (Fig.10A) originally devised for previous studies (Bonini et al. 2014; Lanzilotto et al. 2016, 2019).

Neuronal response during VMT and OT

We isolated 225 single units (n = 168 in Mk1 and n = 57 in Mk2) (Fig. 12). The activity of each unit was analysed during the VMT carried out in the light by means of a 3 x 2 x 4 repeated measures ANOVA (factors: Object, Condition, Epoch), followed by Bonferroni post hoc tests ($P<0.05$) in the case of significant interaction effects or to identify specific effects of factors with more than 2 levels.

Of all isolated units, 39 (17%) did not show any task-related response and were therefore classified as unresponsive. Of the remaining, 13 units (6%) were active only during object presentation and hence were classified as visual neuron, 31 (14%) were active both during object presentation and pre-movement, whereas the other 142 were classified as visuomotor.

Based on the response during Go/No-go conditions of the VMT (Fig., we subdivided the 142 visuomotor units into 5 subpopulations (Fig. 12: Hexc, Hsup, HLexc, HLsup, Lsup) that either significantly increased (n = 76; 54%) or decreased (n = 66; 46%) their firing rate relative to baseline in Epoch 4, being therefore classified as facilitated or suppressed, respectively, according to Kraskov et al. (2009). Specifically, we found: neurons facilitated only during go condition (Population Hexc; n = 47; 21%), neurons suppressed during go condition (Population Hsup; n = 19; 8%), neurons facilitated during both conditions (Population HLexc; n = 24; 11%), neurons suppressed during both conditions (Population HLsup; n = 35; 16%) and neurons suppressed only during No-go condition (Population Lsup; n = 17; 5%).
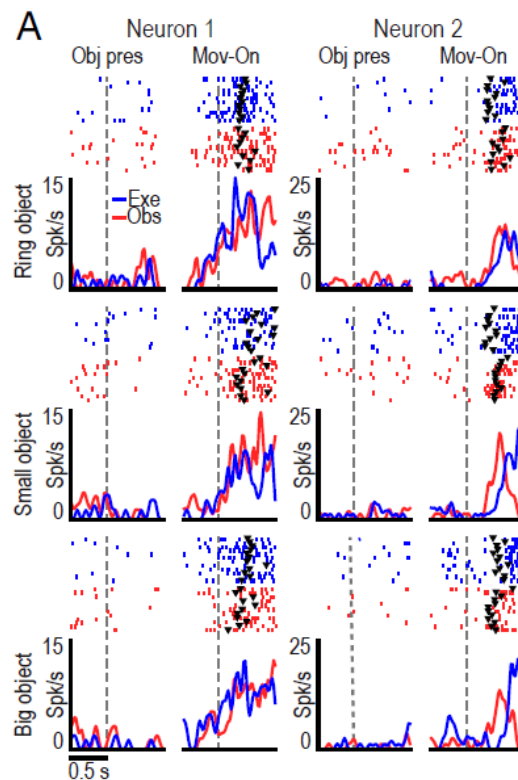

Figure 12: Single-neuron response (raster and line plot) during task execution (Exe, blue) and observation (Obs, red) with the three target objects. Neuron response is aligned to the object presentation (vertical dashed line in the left), and then (after the gap) to the movement onset (i.e. detachment of the hand from the starting position) (Livi et al., 2019).
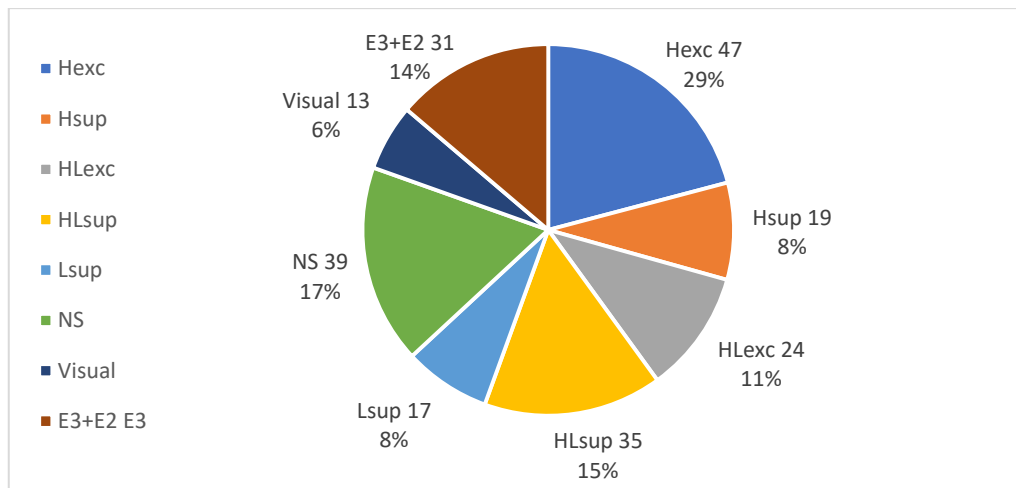
Figure 13 Units and percentage corresponding to each population: Hexc, Hsup, HLexc, HLsup, Lsup, Visual, Unresponsive

Population Hexc

In this population of visuo-motor neurons (Fig. 13), the activity of the cells is related to high sound and, during the VMT, they discharge in the same way ($p > 0.05$) for both Go-light and Go-dark conditions in all comparisons (Go-light: Epoch2: $p < 0.001$ Epoch3: $p < 0.001$ Epoch4: $p < 0.001$; Go-dark: Epoch2: $p < 0.001$ Epoch3: $p < 0.001$ Epoch4: $p < 0.001$). In contrast, in the No-go and Barrier conditions, there was no significant discharge (Fig 13 A).   In the observation task we did not observe any significant activation, moreover during both the Go and No-go trials performed in extrapersonal space, there are not significant activation for all comparisons ($p > 0.05$) (Fig. 13 B).
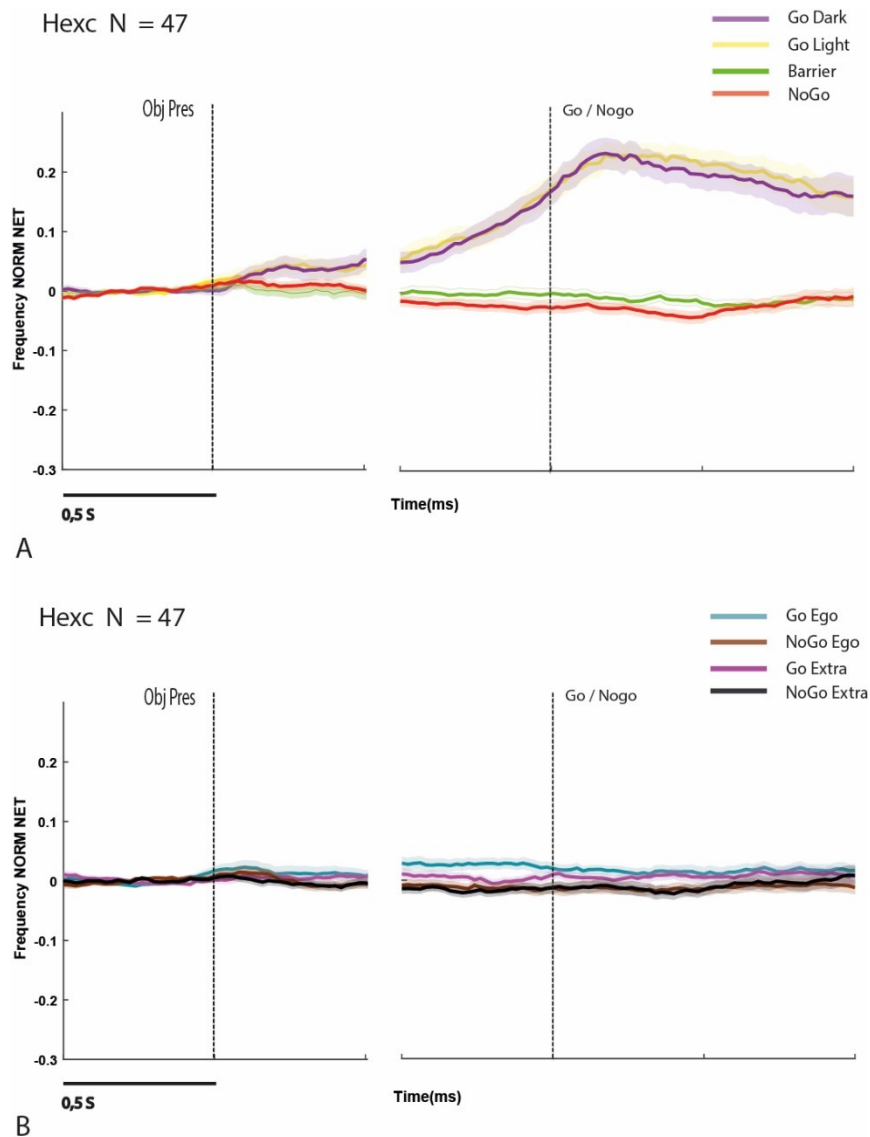
*Figure 13: time course on the net normalized population activity between conditions. **A**: facilitate population during high sound; conditions: Go dark, Go light, Barrier, No-go (VMT). **B**: facilitate population during high sound; conditions: Go ego, No-go ego, Go extra, No-go extra. . The shading around each area represent 1 standard error.*

Population Hsup

This population shows a significant suppression during both the epochs of object presentation and epochs of pre-movement and movement. This response is particularly evident in the condition of go-light and Go–dark, in all the epoch of interests (Epoch 2, 3, 4: F= 2.15, p < 0.05). In the VMT there is a suppression of the activity after the No-go signal (p = 0.04). There is also a suppression for the Barrier condition both

during object presentation and Epoch 4 (Epoch 2: $p = 0.048$; Epoch 4: $p = 0.013$) (Fig. 14A).

During the observation task performed in the peripersonal space there is a suppression of the firing rate in both the epochs of pre-movement and movement during the Go and No-go trials (OTp Go: Epoch 3: $p = 0.003$ and Epoch 4: $p = 0.0008$; OTp No-go: Epoch 3: $p = 0.004$; Epoch 4: $p = 0.001$) (Fig.14B). This population is modulated also during the observation in extrapersonal space: during both the Go and No-go trials, the activity is suppressed in epoch 3 and 4 as compared to baseline (OTe Go: Epoch 3: $p = 0.0005$; Epoch 4: $p = 0.0002$; No-go: Epoch 3: $p = 0.005$; Epoch 4: $P = 0.001$).
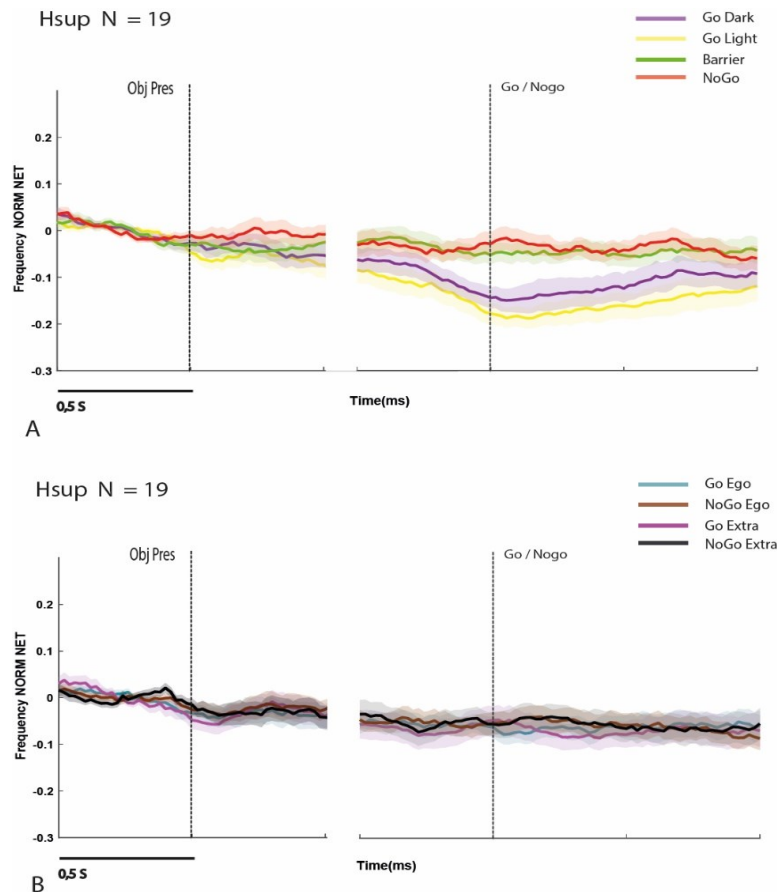
*Figure 14: time course on the net normalized population activity between conditions. **A**: suppressed population during high sound; conditions: Go dark, Go light, Barrier, No-go (VMT). **B**: suppressed population during high sound; conditions: Go ego, No-go ego, Go extra, No-go extra. . The shading around each area represent 1 standard error.*

HLexc

This population seems to have a similar response both for VMT and OT. There is no significant interaction between epochs per conditions ($F= 1.46$; $p = 0.08$). In all the conditions of the VMT there is a significant activity (related to the baseline $p < 0.05$ for all the comparisons) in all the epochs. Starting from object presentation to the end of epoch 4 there is a constant increasing of the activity discharge, also in the barrier condition (Fig. 15A).

In the same way, during the OT performed in peripersonal space there is an increasing activity for actions observed or withheld in Go and No-go trials. Concerning

the action observed or withheld in extrapersonal space, the population activity is different compared with the baseline only during epoch 4 (Go condition: p = 0.006; No-go condition: p = 0.019) (Fig. 15B).

*Figure 15: time course on the net normalized population activity between conditions. **A**: facilitate population during both high and low sound; conditions: Go dark, Go light, Barrier, No-go (VMT). **B**: facilitate population during both high sound and low sound; conditions: Go ego, No-go ego, Go extra, No-go extra. . The shading around each area represent 1 standard error.*

Population HLsup

This population is made of visuo-motor neurons characterized by a suppression of activity at the single neuron level. Generally, there is no significant interaction between

conditions per epochs (F = 1.49431; p = 0.07). The population response, relative to baseline, is significant ($p < 0.05$) in all the epochs of all the conditions except for epoch 2 of Go trial (p = 0.06) in extrapersonal space. In VMT, both in Go and No-go conditions, there is a significant suppression that start with object presentation until the end of the task. The suppression of the Go trials in both epoch 3 and epoch 4 are statistically different from epochs of No-go and Barrier (Go vs No-go: Epoch 3: p = 0.005; Epoch 4: p = 0.001; Go vs Barrier: Epoch 3: p = 0.006 Epoch 4: p = 0.001) (Fig. 16A).

During the observation tasks there is no significant difference between action observed in the peripersonal and extrapersonal space. In the plot it is possible to see a general suppression in all conditions (Go OTp: Epoch 2: p = 0.004; No-go OTp: Epoch 2: p = 0.0003; No-go OTe: Epoch 2: p = 0.016), with no significant difference among epochs ($p < 0.05$) (Fig. 16B).
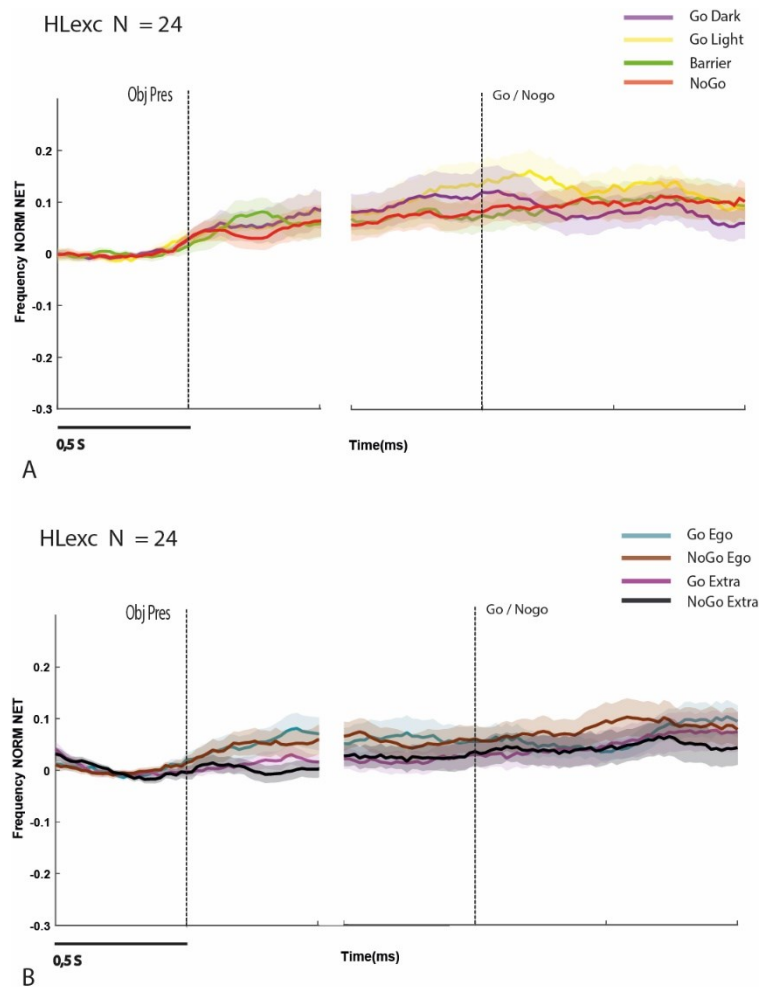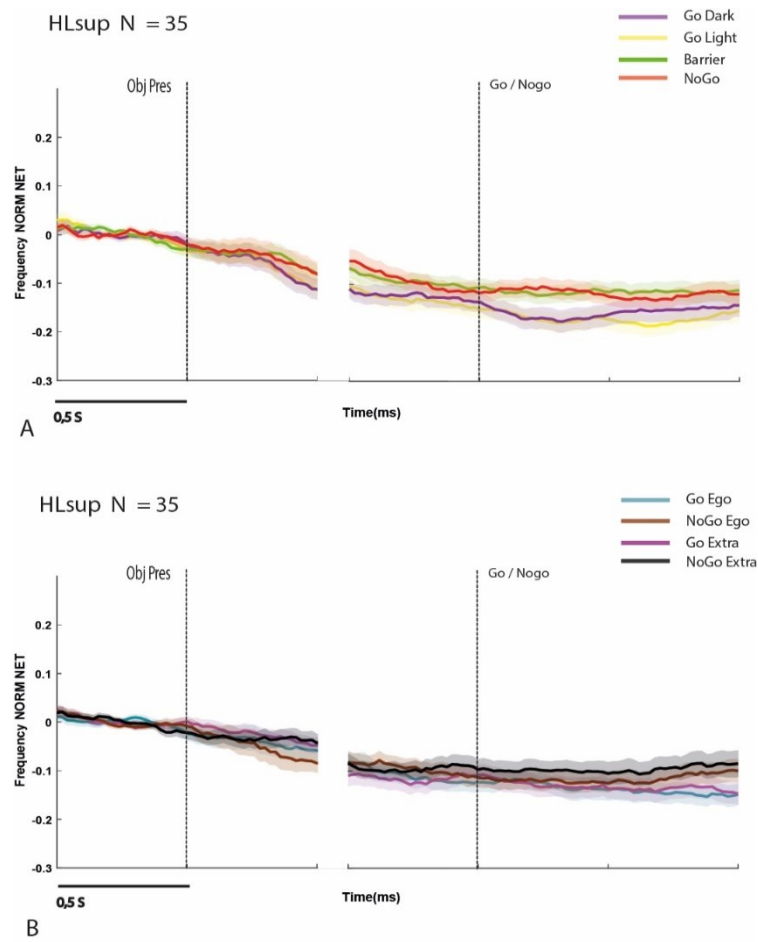
*Figure 16: time course on the net normalized population activity between conditions. **A**: suppressed population during both high and low sound; conditions: Go dark, Go light, Barrier, No-go (VMT). **B**: suppressed population during both high and low sound; conditions: Go ego, No-go ego, Go extra, No-go extra. . The shading around each area represent 1 standard error.*

Population Lsup

This population is made of visuo-motor neurons with a response of suppression during No-go conditions. This population do not show any response during the condition of Go-light and Go-dark in any epochs relative to baseline. In the VMT, during the No-go condition, the population is suppressed in Epoch 3 and 4 ($p < 0.05$). In the Barrier condition there is a suppression of the activity in the same epochs (Epoch 3 and Epoch 4: $p < 0.05$). Between No-go and Barrier conditions there is a difference between epochs 3 and 4 (No-go vs Barrier: Epoch 3: $p = 0.026$; Epoch 4: $p = 0.022$) (Fig. 17A).

In OT in the extrapersonal space there is a suppression of the activity during the epoch of object presentation and also in Epoch 3 and 4 of both conditions (Go OTe: Epochs 2, 3, 4: $p < 0.001$; No-go Ote: Epochs 2, 3, 4 $p < 0.001$). In OT in peripersonal space the activity is suppressed in Go (Epoch 3: $p = 0.020$; Epoch 4: $p = 0.002$) and in the No-go trials (Epochs 3, 4: $p < 0.001$) with a higher suppression during the No-go compared to the Go trials (Epoch 3: $p < 0.001$; Epoch4: $p < 0.001$) (Fig. 17B).
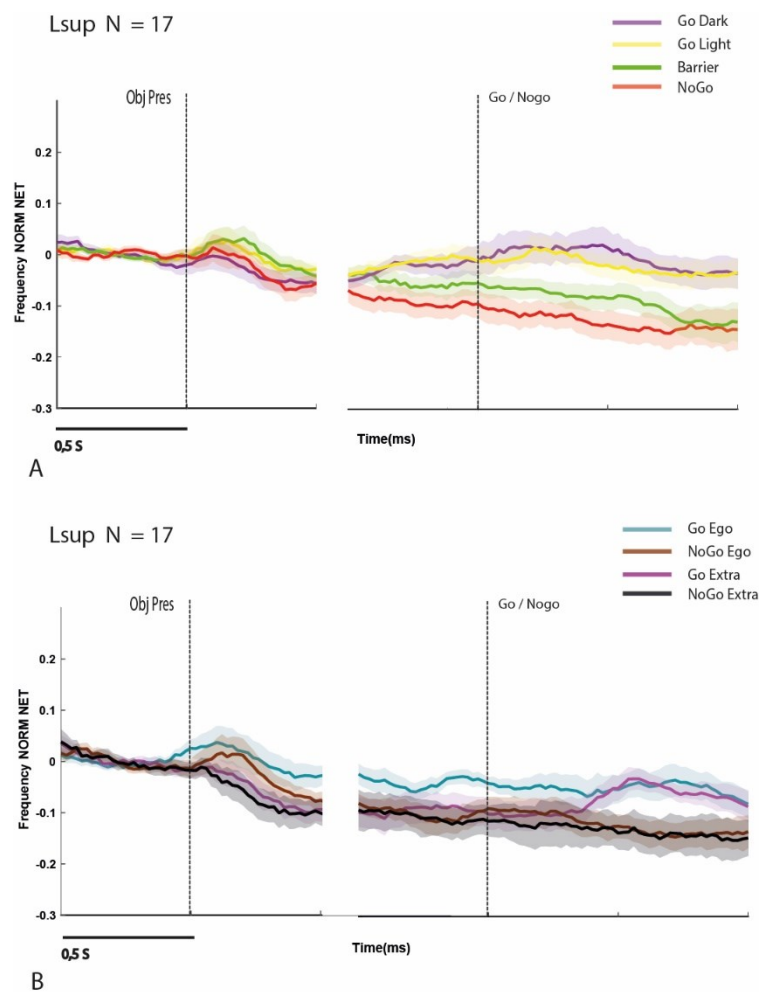

*Figure 17: time course on the net normalized population activity between conditions. **A**: suppressed population during low sound; conditions: Go dark, Go light, Barrier, No-go (VMT). **B**: suppressed population during low sound; conditions: Go ego, No-go ego, Go extra, No-go extra. The shading around each area represent 1 standard error.*

6.   DISCUSSION

In the present study we characterized the properties of area F6 single unit of two macaques. We tested neuronal responses during planning and execution or observation of visually-guided reaching-grasping actions, as well as their withholding. By considering the execution task, we found that about half of the recorded neurons showed a facilitated response (56%) whereas the remaining (46%) exhibited a suppressed discharge. Interestingly, both facilitated and suppressed neurons in the execution task showed a coherent behaviour when comparing Go and No-go conditions: in particular, neurons showed an opposite modulation in the two sets of conditions, regardless of whether the modulation was positive (facilitated neurons) or negative (suppressed neurons). Most interestingly, the same neuronal population response tested during observation tasks showed either no modulation or a coherent dissociation of the conditions in which an action was performed or withheld, although with much lower discharge intensity relative to the execution task.

The present findings highlight a marked difference with respect to area F5, where the same task was previously employed (Bonini et al. 2014). Indeed, whereas F5 neurons could encode withheld actions with the very same discharge profile and firing pattern displayed during the correspondent action execution condition, with additional specificity for executed/withheld actions of self or others, area F6 essentially dissociates executed and withheld actions of the self. These findings emphasize the role of this area in orchestrating initiation and braking of voluntary goal directed actions, regardless of whether the available contextual information derives from an arbitrary contextual signal (the cue sound), the presence of another agent (e.g. the experimenter, gating action execution especially when acting in the monkey's

peripersonal space), or a physical barrier (the transparent plastic screen). These findings are in line with previous neurophysiological studies pointing to a role for F6 in releasing and stopping of planned actions (Lang et al., 1989; Rizzolatti et al., 1990; Matsuzaka et al., 1992): in addition, our neuronal data evidence a mutual role of facilitated and suppressed neurons in this function. Of course, we still ignore which type of cells are mostly included in these neuronal populations: they may be predominantly excitatory pyramidal neurons (Kraskov et al. 2009) or inhibitory interneurons (Boudrias et al., 2009; Kaufman et al., 2013). Further analysis considering, for example, the through-to-peak amplitude and repolarization time of identified neurons may help to clarify the functional classification of the isolated cells, providing hints on the intrinsic circuitry underlying the triggering/stopping of planned action in F6. For example, the functional interpretation may be very different if facilitated neurons are predominantly inhibitory interneurons and suppressed neurons are excitatory pyramidal cells, or viceversa. Future studies should tackle this issue to shed further light on local circuitry for action organization in area F6.

An apparently contrasting finding relative to the previous literature is the absence of a facilitated response during the observation task. Indeed, several previous studies showed the presence of a large set of neurons with clear responses to the observation of others' action (Yoshida et al. 2012; Falcone et al., 2017; Livi et al. 2019). The lack of such a response in the present data set essentially derives from the fact that the focus has been put on the visuomotor task. Hence, the possible opposite sign of action observation response and the typical brief and phasic discharge of F6 neurons in all tasks relative to those of F5 (Lanzilotto et al. 2016) may have likely prevented us from appreciating population responses to the observation of others' action.

Although preliminary, the present findings also suggest the possibility that neurons with inaction-like properties (Bonini et al. 2014; Maranesi et al. 2015) may be hindered in HLexc population. Additional analysis are required to investigate this possibility more carefully at the single neuron level, but the overall emerging picture so far described appears to be more compatible with a role of F6 in coordinating the execution or withholding of one's own action rather than to provide a conceptual-like representation of self/other actions. The strong anatomical link between F6 and F5 (Matelli et al. 1986; Luppino et al. 2003; Gerbella et al 2011) may provide the basis for a functional coupling and local specialization of these two nodes of the extended cortical grasping network, in which the contribution of F6 may enable area F5 to encode motor representation at a more abstract level.

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