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NEURONAL SELECTIVITY FOR SPONTANEOUS YAWNING IN THE MONKEY VENTRAL PREMOTOR CORTEX

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SELETTIVITÀ NEURONALE PER LO SBADIGLIO SPONTANEO NELLA CORTECCIA PREMOTORIA VENTRALE DELLA SCIMMIA

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ABSTRACT – ENGLISH

Yawning is an involuntary and paroxysmal breathing movement mostly characterized by the sequential opening and closing of the mouth. Studies over the past 40 years have shaded light on some brain areas that might be involved in the production of this behavior, and have led to several hypothesis concerning its possible functions. Nonetheless, many aspects of yawning remain still unknown.

In the present exploratory study, using wireless recordings and multi-camera systems, we recorded single neurons activity in ventral premotor cortex (PMv) while the monkey yawned and freely moved in a large cage (the NeuroEthoRoom, NER).

We found that while most neurons responded during both yawning and other mouth-involving behaviors (yawn-other units), some neurons responded uniquely during yawning (yawn-units). The response of these yawn-units, although being often tuned with the peak in mouth aperture, did not appear to be related to the kinematic of maximal mouth aperture, as instead happened for some yawn-other units.

On a population level, neuronal activity during yawning was clearly distinguishable from that related to other observed behaviors.

Our findings suggest that in PMv there is a specific encoding for yawning behavior, which might be involved in the at least partial voluntary modulation according to contextual and social factors of a behavior that would otherwise be largely automatic and dependent from subcortical circuits.

ABSTRACT – ITALIANO

Lo sbadiglio è un movimento respiratorio involontario e parossistico caratterizzato principalmente dall'apertura e conseguente chiusura delle fauci. Studi condotti negli ultimi 40 anni hanno fatto luce su alcune aree cerebrali che potrebbero essere coinvolte nell'espressione di questo comportamento, e hanno portato alla formulazione di diverse ipotesi sulle sue possibili funzioni. Ciò nonostante, molti aspetti dello sbadiglio rimangono tuttora ignoti.

In questo studio esplorativo, attraverso l'uso di registrazioni wireless e sistemi multi-camera, abbiamo registrato l'attività di singoli neuroni nella corteccia premotoria ventrale (PMv) durante gli sbadigli di una scimmia libera di muoversi in un'ampia struttura (la NeuroEthoRoom, NER).

Abbiamo trovato che mentre la maggior parte dei neuroni risponde sia durante lo sbadiglio sia durante comportamenti che coinvolgono la bocca (yawn-other units), alcuni neuroni rispondono unicamente durante lo sbadiglio (yawn-units). La risposta di queste yawn-units, per quanto spesso abbia un timing legato al picco di apertura della bocca, non risulta essere legata alla cinematica della massima apertura della bocca, come invece accade per alcune yawn-other units.

A livello di popolazione, l'attività neurale durante lo sbadiglio è risultata essere chiaramente distinguibile rispetto a quella legata ad altri comportamenti osservati.

I risultati indicano che in PMv esiste una codifica specifica del comportamento di sbadiglio, che potrebbe essere coinvolta nell'almeno parziale modulazione volontaria in base a fattori contestuali e sociali di un comportamento altrimenti largamente automatico e dipendente da circuiti sotto-corticali.

1. INTRODUCTION.

1.1 YAWNING IN A COMPARATIVE PERSPECTIVE.

Spontaneous yawning is a very commonplace phenomenon both in our everyday life and, more generally, in the animal kingdom. Yet, most of our knowledge on this subject comes from studies carried out only in the last 40 years and is still very limited concerning the causes and mechanisms of yawning. So, what do we currently know about yawning?

Yawning is often defined as an involuntary and paroxysmal breathing movement, sometimes associated with the act of stretching (Fraser, 1989; Provine, Hamernik, et al., 1987). A very detailed characterization of the human yawning sequence comes from Barbizet (1958), who describes it as a three-phase motor-respiratory sequence, with each phase having an estimated duration ranging from 1.5 to 4 seconds. First there is an active and slow opening of the mouth, then a peak of mouth aperture and muscle contraction, and finally a passive and quicker mouth closing. The first two phases are typically accompanied by a deep inspiration with the dilatation of the pharynx, the larynx, the trachea, and the thorax, with a partial or total occlusion of the eyes, unitedly with nose wrinkling, eyebrows lifting, and head tilting. The last phase presents a sudden interruption of the inspiration, with a quick expiration and the gradual reduction of the pharynx, the larynx, the trachea, and the thorax to their normal dimensions.

The three-phased mouth movement of yawning and yawn-like behaviors have been documented across different species and classes in the vertebrate group e.g., (Baenninger, 1987; Bakkegard, 2017; Enokizu et al., 2021, 2022; Hartzell et al., 2017; Massen et al., 2021; Palagi et al., 2019; Rasa, 1971; Sauer & Sauer, 1967). For instance, Deputte (1994) and Hadidian (1980) described yawning in macaques as a three-stages behavior in a very similar way as the human yawn described by Barbizet (1958). As we can see from Figure 1, first, the mouth opens slowly to a small oval, with the teeth remaining covered, while the head begins to tilt back, and an inspiration occurs. Then, in the acme, the mouth is rapidly opened to its fullest extent, uncovering the teeth, while the head is tilted back and there's a deep inhalation. Finally, the mouth is closed rapidly, the head is lowered, and there's a quick exhalation.

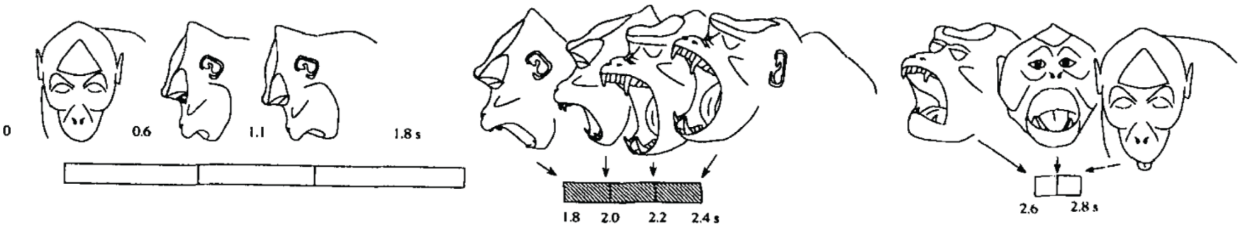


Figure 1. Schematic drawing showing the dynamics of the three stages of yawning in macaques. (Adapted from Deputte, 1994)

Once initiated, the yawn inevitably goes to completion, with minimal influence of sensory feedback. Such characteristics have led to the definition of yawning as a "fixed action pattern" (FAP) (Provine, 1986). Contributing to this definition of yawning as a FAP is the fact that spontaneous yawning might have a genetic base, since it can already be observed in humans from the 12th week of gestational age (Walusinski et al., 2005), and its frequency has been artificially selected in Sprague-Dawley rats, creating sublines of high-yawning and low-yawning rats (Urbá-Holmgren et al., 1990). However, although highly stereotyped, the yawn is not invariant in its duration, frequency, and form. For instance, Provine (2005) reported the possibility to perform normal yawns while closing the nose, clenching teeth or closing lips, although in these last two conditions people referred the unfulfilling sensation of being stuck in mid-yawn, suggesting that mouth aperture, rather than respiration, is an essential component. In addition, yawning frequency can be modified by means of operant conditioning in monkeys (*Macaca Tonkeana*: Anderson & Wunderlich, 1988; *Macaca Nemestrina*: Louboungou & Anderson, 1987), suggesting that it might be under at least partial voluntary control. Accordingly, ethological observation on non-human primates (NHPs) revealed the existence of different types of yawns, mostly differentiated by the degree of canines exposure (Baenninger, 1987; Leone et al., 2014; Zannella et al., 2017).

It is yet unclear which releasing stimulus triggers the yawning FAP, because it was observed in many different contexts, and there is evidence suggesting that it can be affected by several physiological, environmental, and social factors. For instance, yawning has a clear circadian pattern in many species, being more frequent in light transitions and consistently producing a bimodal distribution of frequency, with higher frequency shortly before sleeping and after waking (Anías et al., 1984; Deputte, 1994; Provine, Hamernik, et al., 1987; Sauer & Sauer, 1967). Moreover, in humans, differences in sleep-waking rhythms of different chronotypes affect yawning frequency trend across the day (Zilli et al., 2007). Age seems to influence yawning too, since elder people tend to yawn less frequently than younger individuals (Zilli et al., 2008).

Interestingly, Provine (1986, 2005) showed that even individual, cognitive activities that can recall the concept of yawning, such as, reading or thinking about yawning, can trigger the behavior.

In captive animals subjected to regular feeding time, yawning was observed consistently before eating (Baenninger, 1987; Fureix et al., 2011; Holmgren et al., 1991), not as a sign of hunger but rather as a sign of "food expectancy" and related arousal, stress or anxiety (Holmgren et al., 1991). Another link with arousal is given by the fact that people tend to yawn more when exposed to uninteresting rather than interesting stimuli (Provine & Hamernik, 1986), and while doing monotonous activities (e.g., driving, sitting in class; Greco et al., 1993). In different species yawning is typically, but not necessarily, associated with an increase in motor activity (Baenninger et al., 1996; Enokizu et al., 2021; Giganti et al., 2002; Vick & Paukner, 2010), is mostly observed during resting (Campos & Fedigan, 2009; Deputte, 1994; Palagi et al., 2019; Sauer & Sauer, 1967), and tends to increase with ambient temperature (Campos & Fedigan, 2009; Deputte, 1994; Gallup et al., 2009, 2010).

Interestingly, in sexually dimorphic species of NHPs in which males have larger canines than females, yawning is typically more frequent in males than females (Hadidian, 1980; Leone et al., 2014; Schino & Aureli, 1989; Troisi et al., 1990), and in some species of NHPs, the highest ranking adult male tend to yawn more frequently (*Macaca nigra:* Hadidian, 1980; *Theropithecus Gelada:* Leone et al., 2014; *Macaca fascicularis:* Troisi et al., 1990). In addition, in NHPs, yawning tends to increase for stressful events such as hostile social situations (Deputte, 1994; Hadidian, 1980; Leone et al., 2014; Maestripieri et al., 1992; Troisi et al., 1990), and there is also evidence of stress-induced yawning increase in budgerigars (Miller et al., 2010) and rats (Kubota et al., 2014, 2023).

Moreover, it is known (or at least believed) that in humans the yawn is contagious, suggesting that one releasing stimulus for the yawn FAP might be the exposure to the yawn itself (Provine, 1986). Indeed, people seem to yawn after seeing (Provine, 1989; Provine & Hamernik, 1986) or hearing (Massen et al., 2015; Norscia et al., 2020) someone else yawn. Recently, both field observations and laboratory studies have investigated the yawn contagion in different species, showing its possible existence in some of them but not in others (see Gallup, 2022). Experiments on contagious yawning have, however, received critics both for methodological issues in the quantification of yawn contagion and for the functional interpretation of yawn contagiousness (Campbell & Waal, 2010; Kapitány & Nielsen, 2017; Massen & Gallup, 2017).

Concerning spontaneous yawning, researchers are currently trying to unveil its evolutionary primitive function(s) and neurobiological substrate(s), integrating data coming from animal models (both laboratory experiments and wild ethological observations), neurological patients, and experiments with normal subjects.

1.1.1 Neurobiology of yawning.

Still today, there is no clear evidence for the specific neuroanatomical location of a hypothetical yawning motor center. However, several studies have shed light on some brain regions involved in the production and control of yawning.

Observations on anencephalic newborns showed that, although they were totally or partially lacking the telencephalon and diencephalon, they were still able to yawn (Catel & Krauspe, 1930; Gamper, 1926; Peiper, 1932). This suggests that the yawning production is mainly a prerogative of lower brain structures, probably located in the brainstem, where the central pattern generators (CPG) for oral and respiratory control are located. Accordingly, reports on neurological patients show that, although rare, excessive yawning can occur from the altered functionality of brainstem centers (Bayri et al., 2015; Cattaneo et al., 2006; Postert et al., 1996; Saura et al., 2014; Wicks, 2007); furthermore, patients with loss of, or impairment in, voluntary control of facial and pharyngeal movements can still be able to yawn, showing automatic-voluntary dissociation (Bauer et al., 1980; Higashi et al., 1977; Weller, 1993). In rats, stimulation of the ventral tegmental area consistently induced yawning (Sanna et al., 2012). Therefore, different hypotheses have been made to identify the specific brainstem location of the yawning center, with Heusner (1946) locating it near the respiratory and vasomotor centers in the medulla oblongata, Askenasy (1989) near the reticular activating system, Gamper (1926) in the periaqueductal grey, and Walusinski (2006) among the ponto-medullary CPGs. Still nowadays, no conclusive experimental evidence is available to support one against the others of these alternatives.

Even though the brainstem could be the location for the yawning motor center, evidence suggest that it is not the only region involved in yawning, since excessive yawning can occur also for dysfunction of supratentorial regions (Kuba et al., 2010; Singer et al., 2007; Specchio et al., 2011). Lesion in the insula and in the caudate nucleus correlates with the duration of excessive yawning period in stroke patients (Krestel et al., 2015). Stimulation studies showed that yawning can be induced in rats with stimulation of the paraventricular nucleus (PVN) in the hypothalamus, of the hippocampus, and of the posterior and central nuclei of the amygdala can induce yawning in rats (Kubota et al., 2019, 2023; Melis et al., 1986;

Sanna et al., 2012; Sato-Suzuki et al., 1998). In humans, stimulation of the putamen in one epileptic patient consistently induced yawning (Joshi et al., 2017).

Therefore, it has been proposed that the brainstem center for yawning is under top-down modulatory control of higher supratentorial areas, and removal of such control might be the cause of pathological yawning (Cattaneo et al., 2006; Williams, 2000).

In this network, several neurotransmitters, neuropeptides and neurohormones can influence the induction and regulation of yawning, including acetylcholine, dopamine, glutamate, serotonin, oxytocin, GABA, opioids, adrenergics, nitric oxide, ACTH and α-MSH (Argiolas & Melis, 1998; Krestel et al., 2018). Many of these molecules (e.g., dopamine, oxytocin, excitatory amino acids, opioids, but not serotonin) affect yawning by interacting with oxytocinergic neurons in the PVN, which therefore seems to be the main pathways for yawning modulation. Altogether, four distinct neural pathways were hypothesized as forming the yawning network (Collins & Eguibar, 2010): 1) the oxytocinergic pathway from the PVN to the pons, the medulla oblongata, and the spinal cord; 2) the oxytocinergic pathway from the PVN to the cholinergic neurons in the hippocampus; 3) the pathway that indirectly connects the ACTH/α-MSH activated neurons in the PVN to the cholinergic neurons of the hippocampus via cholinergic neurons in the medial septum; 4) the still unknown pathway for serotonin-mediated yawning, which recently has been proposed to include the insula (Krestel et al., 2015).

1.1.2 Functional hypotheses for yawning.

Yawning and yawn-like behaviors have been reported across different species and lineages, suggesting that it might have an adaptive function. Although the high prevalence across species does not necessarily imply any functional significance (Gould & Lewontin, 1979), several observations in humans unitedly with comparative considerations suggest that yawning might have an adaptive function. For instance, in human yawning fulfillment is associated to pleasant feeling (Provine, 1986), is observable from the fetal stage (Walusinski et al., 2005), and in some individuals can lead to jaw injuries (McCorkell, 1985; Tesfaye & Lal, 1990). In a comparative perspective, Miller and colleagues (2010) suggest that yawning could conflict with immediate antipredatory behaviors, since the individual is temporarily detached from the surrounding environment. Moreover, there's evidence for a strong positive correlation between yawn duration and brain weight, brain size, and cortical/pallium neurons density (Gallup et al., 2016; Massen et al., 2021), supporting the adaptationist interpretation.

Several hypotheses have been proposed to explain the potential adaptive role(s) of yawning in its phylogenetic history, even though, in Baenninger words, "behavior leaves no fossils so unambiguous evidence on how and why yawning evolved is impossible to obtain" (1997, p. 200). Among these are the respiration hypothesis, the brain cooling hypothesis, the arousal hypothesis, and the communication hypothesis.

According to the respiration hypothesis, yawning is a mechanism for re-equilibration of blood oxygen levels, protecting the organism from cerebral hypoxia and hypercapnia. The main prediction that comes from this hypothesis is that yawning rate and/or duration should depend on blood oxygen levels. However, this prediction hasn't received empirical support, and strong lines of evidence firmly disconfirm this hypothesis, since yawning rate and/or duration is not altered in young healthy students by breathing a gas mixture with high levels of CO_2 or with 100% O_2, nor by doing physical exercise that doubled their breathing rate (Provine, Tate, et al., 1987); furthermore there is no correlation between yawn duration and inter-yawn interval, because people that perform shorter yawns do not compensate by yawning more frequently and vice versa (Provine, 1986). In their recent analysis of the literature, Doelman and Rijken (2022) propose that yawning is a mechanism that allows long-term oxygenation via repositioning of muscles around the airway. However, the validity of this work (but not of the physiological variations described therein) has received severe critics from Massen and Gallup (2022).

The brain cooling hypothesis claims that yawning is a compensatory mechanism for brain temperature reduction (Gallup & Gallup, 2007, 2008). As predicted by this hypothesis, yawning is triggered by hyperthermia and produces a measurable cooling effect (Shoup-Knox, 2011; Shoup-Knox et al., 2010). According to Gallup and Eldakar (2013) yawning might lower brain temperature in three ways: 1) by increasing the cooler arterial blood flow to the head via cardiovascular modifications occurring during the deep inspiration and the powerful jaws stretching; 2) by cooling the blood flowing through the internal carotid artery at the level of the cavernous sinus via powerful inspiration of cooler ambient air; 3) by ventilating the sinus system and promoting the evaporation of the sinus mucosa via a powerful flexing of the sinus walls due to the pterygoid muscles contraction occurring during yawning jaw activity. Moreover, the thermoregulatory hypothesis proposes the thermal window model, according to which yawning as a brain cooling mechanism is adaptive only when ambient temperature rises within a small range over brain temperature, whereas becomes dangerous or counterproductive when ambient temperature gets excessively hot or cold (Massen et al., 2014). The thermoregulatory hypothesis currently remains debated since, although several critics have been proposed (Elo, 2010, 2011; Guggisberg et al., 2010; Guggisberg et al., 2011; Walusinski, 2013), no experimental evidence seems yet to exist in contrast to its main predictions (Gallup, 2011a; Gallup & Eldakar, 2013).

Many hypotheses have been formulated suggesting that yawning is a behavior that leads to an increase in arousal and vigilance. For instance, Askenasy (1989) proposes that yawning is an arousal defense reflex that reverses drowsiness induced by brain hypoxia, with a yawning center located in the brainstem near the Reticular Activating System. In his review of the literature, Baenninger (1997) concludes that yawning is an adaptive behavior for the maintenance or increase of arousal and wakefulness. Matikainen and Elo (2008) propose that yawning increases arousal, alertness and wakefulness by altering blood flow through mechanical stimulation of the carotid body. More recently, Walusinski (2014) proposes that yawning disengages the Default Mode Network, which is active during resting state, and promotes the onset of a more attentive and vigilant state by accelerating the circulation of the cerebrospinal fluid, thereby leading to an increased clearance of hypnogenic substances there contained (e.g. prostaglandin PGD2), resulting in a reduced propension to sleepiness. Although experimental and observational evidence supports the idea that yawning occurs during drowsiness or low vigilance states, researchers still have to provide an unequivocal prove for an arousing effect of yawning, since variations of physiological parameters associated to an arousal increase (e.g. EEG, skin conductance, heart rate) have not been observed consistently before, during or after yawns (Guggisberg, Mathis, & Hess, 2010).

Finally, the communication hypothesis argues that yawning acts as a social cue that informs the viewer about the performer's internal state, favoring the behavioral synchronization of a group (Guggisberg et al., 2010). Different hypotheses were formulated, proposing that yawning might be a signal for the yawner's drowsiness (Provine, Hamernik, et al., 1987) or a state of uneasiness (e.g. hunger, stress) (Deputte, 1994), helping synchronizing the physiological and behavioral state of a group, with the yawn itself being classified as a displacement activity. Alternative hypotheses considered yawning a mean to signal a mildly to moderately unpleasant - but not threatening - state in the yawner (Guggisberg et al., 2010: but see Gallup, 2011b) or the end of a stressful experience or of a period of sustained concentration in the yawner (Dourish & Cooper, 1990). Recently, Gallup and Meyers (2021) proposed the group vigilance hypothesis, which states that spontaneous yawning is informative of the reduced alertness of the yawner and might induce neurophysiological changes that enhance the vigilance of the observers, thereby maintaining or improving the group vigilance to compensate for the reduction experienced by the yawner. Concerning group synchronization, only one observational study on wild African lions (Casetta et al., 2021) showed that yawn contagion increases the likelihood that the observer replicates the motor patterns of the yawner, favoring the collective switching of behavior towards a new status (e.g. from resting to moving or vice versa).

Overall, these hypotheses are not mutually exclusive, and yawning is most likely a multifunctional behavior, especially when considered in a cross-species perspective. Given its presence in non-social animal species, the most phylogenetically primitive function of yawning is most likely a physiological one, and possible social-communicative functions are phylogenetically more recent features derived from that original trait (Gallup, 2011b).

1.2 MOUTH ACTIONS IN THE VENTRAL PREMOTOR CORTEX.

The motor cortex is the caudal portion of the frontal cortical territory that is included laterally between the central sulcus and the arcuate sulcus, while extending mesially to border the cingulate cortex. Based on cytoarchitecture, Brodmann (1909) subdivided this agranular cortex into the caudal Area 4 (BA4) and the rostral Area 6 (BA6), distinguishing them by the different distribution of Betz' giant pyramidal cells in layer V. Accordingly, Fulton (1935) proposed that the motor cortex could be functionally divided into a primary motor cortex (BA4) and a premotor cortex (BA6), with the latter being functionally higher than the former.

Modern research has refined our understanding of motor cortex organization, showing a far more complex subdivision of the monkey premotor cortex into a multiplicity of structurally and functionally distinct areas, named from F2 to F7 (with F1 being the primary motor cortex, see Figure 2), each having different afferent and efferent connections and playing partially distinct roles in the planning and control of motor actions (Belmalih et al., 2007; Rizzolatti et al., 2014).

These areas can be grouped into mesial (i.e., F3 and F6), dorsal (i.e., F2 and F7) and ventral areas (i.e., F4 and F5). For the purpose of this work, we will focus on the inferior portion of BA6, known as the ventral premotor cortex (PMv), having a strong representation of mouth acts and movements (e.g., jaw, tongue) and being involved in their control and planning (Maranesi et al., 2012; Yoshino et al., 2000).

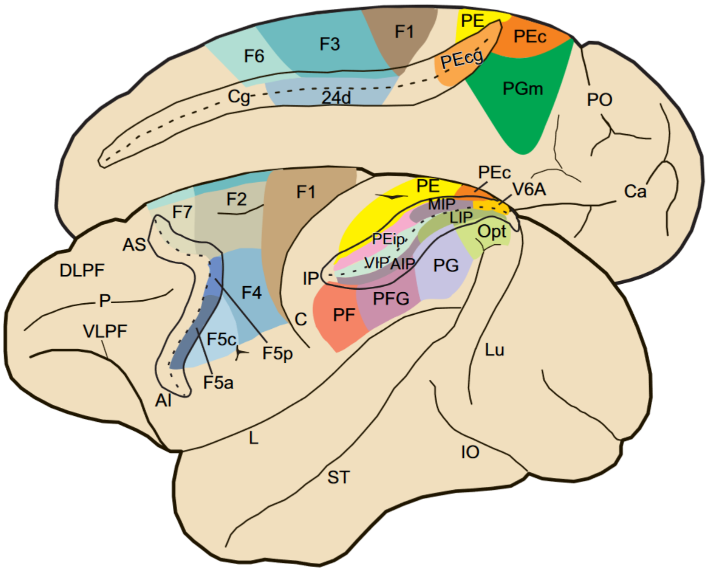


Figure 2. Lateral and mesial view of the monkey brain showing a detailed anatomical parcellation of the agranular frontal cortex and the posterior parietal cortex. Intraparietal, arcuate, and cingulated sulci are shown unfolded. Nomenclature of frontal areas as defined in text, other parcellations are not reported. Anatomical landmarks: Ca, calcarine fissure; DLPF, dorsolateral prefrontal cortex; VLPF, ventrolateral prefrontal cortex; AI, inferior arcuate sulcus; AS, superior arcuate sulcus; C, central sulcus; Cg, cingulated sulcus; IO, inferior occipital sulcus; IP, intraparietal sulcus; L, lateral fissure; Lu, lunate sulcus; P, principal sulcus; PO, parieto-occipital sulcus; ST, superior temporal sulcus. (Rizzolatti et al., 2014)

1.2.1 Mouth representation in F4.

Area F4 represents the caudal part of PMv, occupying the precentral gyrus just rostral to the precentral area F1, and contains a partial somatotopy, showing a representation of arm, neck, face, and mouth movements (Fogassi et al., 1996; Gentilucci et al., 1988). In fact, as shown by Maranesi and colleagues (2012), intracortical microstimulation of this area produces forelimb and face movements in its dorsal section (F4d), while in the ventral section (F4v) produces tongue and jaw movements. Accordingly, in their study they also report that neural activity in F4d is related to goal-directed forelimb and mouth motor acts, while activity in F4v is mostly related to simple, non-goal-directed movements (Figure 3a).

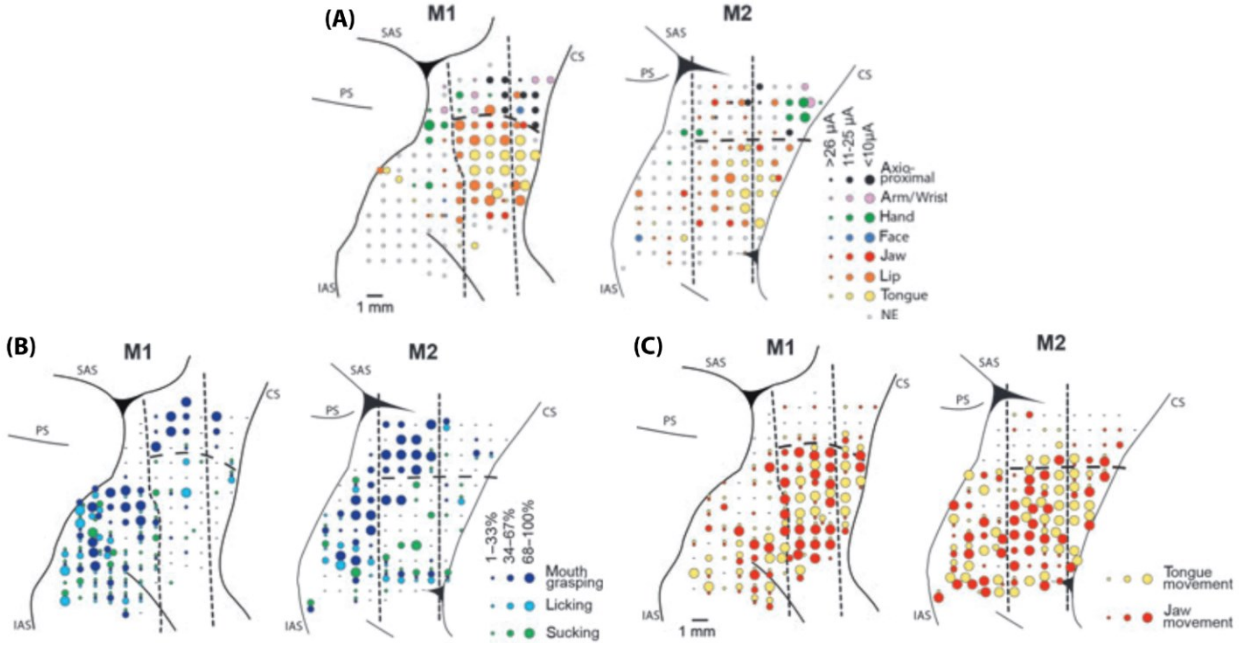


Figure 3. Stimulations and recordings in PMv and F1v as recorded by Maranesi et al., 2012. A) Localization of movements evoked by ICMS; circles' colors identify the activated body part, whereas the size of the circles represents the activation threshold. Small white circles represent penetrations not electrically excitable (NE) with the parameters employed in the study. B) and C) show the distribution of respectively mouth motor acts and simple movements; circles' colors identify the effector, whereas the size of the circles represents the percentage of responses recorded for that behavior in that site. Small white circles represent non-responding sites. Overall, horizontal dashed lines indicate the anatomo-functional borders between F5, F4d, F4v, F1vd, and F1vv. (Adapted from Maranesi et al., 2012)

A large majority of neurons in F4 also respond to sensory stimuli, with unimodal neurons responding either to visual or somatosensory stimuli and bimodal neurons responding to both stimuli (Fogassi et al., 1996; Gentilucci et al., 1988; Graziano et al., 1994). The tactile receptive fields (RFs) of these neurons are predominantly located on the face, neck, trunk, and arms, whereas the visual RFs extend three-dimensionally in the space around a certain body part and are only activated by three-dimensional moving stimuli entering that space. Figure 1 shows an example of bimodal neurons with RF covering or including the mouth. The vast majority of these visual RFs is anchored to the tactile field in somatocentric rather than retinocentric coordinates, that is, their position around a body part is independent of eye position but moves as the corresponding body part moves (Fogassi et al., 1996; Graziano et al., 1994), and can expand in depth proportionally to the increasing velocity of the approaching stimulus (Fogassi et al., 1996). Therefore, researchers hypothesized that F4 plays an important role in encoding of peripersonal space not in geometric but in motor terms, providing a dynamic encoding of space that allows the preparation of the appropriate motor acts for interacting, or avoiding to interact with, stimuli near the body (Bufacchi & Iannetti, 2018).

F4 has important connections with F1. According to Maranesi and colleagues (2012), the dorsal portion of F4 and of ventral-dorsal F1 (respectively F4d and F1vd) form a functionally unitary cluster involved in the control of goal-directed motor acts within the body-centered peripersonal space, whereas the ventral portion of F4 and of ventral-ventral F1 (respectively F4v and F1vv) form a functionally unitary cluster mostly related to the control of tongue and oro-facial simple movements; accordingly, neurons in F4d share very similar sensorimotor properties with F1vd, while those in F4v are similar to F1vv.

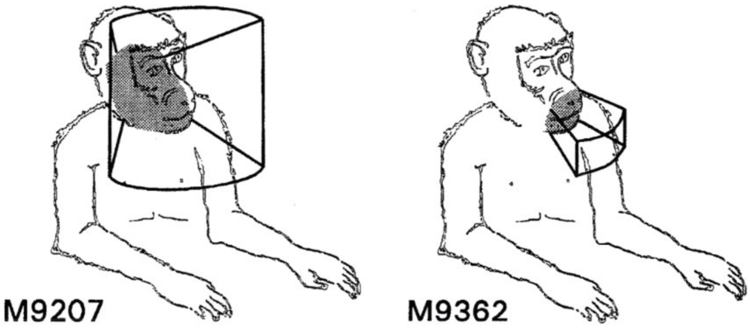


Figure 4. Bimodal neurons. Both neurons were recorded from the left emisphere. In both pictures the shaded area on the monkey represents the body location of the tactile RF, while the area protruding tridimensionally correspond to the visual RF. Neuron M9207 has a wide RF covering the contralateral hemi-face, including half of the mouth. Neuron M9362 has a smaller RF covering the entirety of the mouth and including also the nose. (Adapted from Fogassi et al., 1996)

1.2.2 Mouth representation in F5.

Area F5 covers the postarcuate convexity cortex just rostral to F4 and the posterior bank of the inferior arcuate sulcus, and contains partially overlapping representations of hand and mouth (Maranesi et al., 2012; Rizzolatti et al., 1988).

Although being less excitable than F4, electrical stimulation of F5 can produce hand and mouth movements (e.g., jaw, tongue, lips movements; Figure 3) (Coudé et al., 2011; Maranesi et al., 2012).

Like in F4, some neurons are purely motor neurons (Figure 3), here discharging during the execution of specific hand and/or mouth goal-directed motor acts (particularly hand and mouth grasping), coding the goal of such acts rather than the sequence of movements that form them (Rizzolatti et al., 1988; Umiltà et al., 2008), and some even showing a selectivity for the final goal of the action in which the coded acts are embedded (Bonini et al., 2010, 2011). Moreover, these neurons exhibit a selectivity for the type of grip (e.g., precision grip, whole hand prehension) with which the grasping is executed and show different discharging timing during the execution of that motor act (Bonini et al., 2012; Maranesi et al., 2012; Rizzolatti et al., 1988).

Interestingly, Coudé and colleagues (2011) reported a peculiar group of neurons that discharge during the production of voluntary vocalization, suggesting that F5 can, at least partially, extend its control over the larynx for voluntary vocalization.

Another class of neurons present in F5 are visuomotor neurons, that is, neurons that respond both to motor and visual inputs: they have been distinguished in “canonical” and “mirror” neurons. Canonical neurons discharge during both grasping and observation of a three-dimensional object, supposedly encoding the potential motor act congruent with the pragmatic physical properties of that object (Murata et al., 1997; Raos et al., 2006). Mirror neurons (MNs), on the other hand, discharge both for the execution of a goal-directed motor act and for the observation of the same or a similar act - with variable degree of congruence between observation and execution - supposedly for the recognition of the observed motor act (Gallese et al., 1996; Rizzolatti et al., 1996). Notably, Ferrari and colleagues (2003) reported a group of MNs responding to mouth actions related to ingestive functions (e.g., mouth grasping, sucking), and a smaller group of MNs that also respond to communicative mouth gestures (e.g., lip smacking, tongue protrusion), suggesting a possible involvement of area F5 in communicative functions.

1.3 THE NEUROETHOLOGICAL APPROACH.

Neuroethology is a rather recent interdisciplinary approach to the study of the neural bases of natural behavior that integrates theories and techniques from the fields of neurophysiology and ethology.

On the one hand, the classical ethological approach is based on the detailed and systematic observation and description of a species-specific behavior occurring in ecologically valid conditions (either in the field or in laboratory), in order to understand its ultimate causes (i.e., its adaptive function and phylogeny) and its proximate causes (i.e., its mechanisms and ontogeny) (Tinbergen, 1963). Some examples of behaviors described and explained by ethologists with this classical approach are the brood parasitism of the common cuckoo (Schulze-Hagen et al., 2009), the reproductive behavior of the three-spined stickleback (FitzGerald, 1993), and the infanticide in lions (Pusey & Packer, 1994).

On the other hand, in the classic neurophysiological approach used in neuroscience the animal is passively exposed to stimuli and/or must produce very limited, stereotyped and repeated movements during specific over-trained tasks, while recordings and/or alterations of the neural activity occur to infer functional properties and functional roles of the neural substrates. This classic laboratory approach allows for a high level of control over precisely identified, independent variables and possible covariates, but lacks in ecological validity, since the animal behavior is limited, as well as the variety of contexts in which each behavior can take place, because of the laboratory setting, thus it may not be representative of the animal's wider and more complex behavioral repertoire (Jackson et al., 2007). In fact, at least for larger species (e.g., primates), the animal behavior is typically restricted due to physical constraints (e.g. the primate chair for macaques) necessary both for the correct functioning and protection of the tethered recording equipment and for the reduction of undesired movements that might undermine the inference about the relationship between the desired behavior and the neural activity. Nonetheless, our current knowledge about the structure and functions of the central nervous system greatly derives from results obtained with this method of investigation, allowing researchers to gain pivotal insights such as the description of simple and complex receptive fields of V1 neurons (Hubel & Wiesel, 1962), the role of MT/V5 neurons in motion perception (Newsome & Pare, 1988), and the different coding of movements force and direction in M1 neurons (Evarts, 1968; Georgopoulos et al., 1982).

Combining these two classic approaches, neuroethology has its basis in the bidirectional interactions between proximate and ultimate causes, and investigates neural activity during the expression of species-specific behaviors, relying on the premise that neural circuits of different species are refined by evolution to support a specific function with adaptive significance (Testard et al., 2021). For instance, tethered recordings in freely moving small animals allowed researchers to gain interesting insights on the neural basis of more naturalistic behaviors, such as planar spatial navigation in rats (place cells: O'Keefe, 1976; grid cells: Hafting et al., 2005; border cells: Solstad et al., 2008), in squirrel monkeys (*Saimiri sciureus:* Ludvig et al., 2004) and in marmosets (*Callithrix jacchus:* Courellis et al., 2019), or conspecific conversation in marmosets (Nummela et al., 2017). Unfortunately, although possible (Hazama & Tamura, 2019), freely moving tethered recording of naturalistic behavior is not easily doable in larger species due to the higher risk of damage to the recording system. However, recent development of data logging, wireless recording, and automatic behavioral tracking systems has overcome these limitations (Testard et al., 2021), allowing researchers to freely unleash the animal and to observe and study behaviors that would otherwise be impossible to study in restrained conditions. One example is the production of vocalizations in marmosets, which can only be studied with wireless recording because they are severely inhibited when the animal is restrained (Roy & Wang, 2012). Concerning bigger animals, wireless recordings were recently used in freely moving macaques to study neural correlates of foraging strategy (Shahidi et al., 2022), of planar spatial navigation (Mao et al., 2021), of reaching and walk-and-reach behaviors planning and execution (Berger et al., 2020), and of wakefulness (active and quiet) and rest (Milton et al., 2020).

Lastly, wireless technology represent an important step forward in the implementation of the Refinement principle in research with laboratory animals, enhancing animal wellbeing and data quality and validity (Bonini, 2019).

2. AIMS OF THE STUDY

Our knowledge on the encoding of mouth behaviors in PMv is grounded in neuroscientific studies utilizing constrained conditions during which the monkey must perform well-structured, repetitive and often overly-learned tasks. Yawning behavior represents an example of a spontaneous behavior that could be hardly investigated with such approach, since the restrictions might impair the mouth and head movements that characterize yawning.

Therefore, by adopting wireless recordings synchronized with a multi-camera system, this exploratory study aims to investigate the possible coding of yawning behavior and its pattern of mouth aperture/closure in the PMv of a freely-moving macaque.

3. MATERIALS AND METHODS.

3.1 ETHICAL STATEMENT.

Every part of the experimental protocols, from animal handling to surgical and experimental procedures, comply with the European law on the humane care and use of laboratory animals (Directive 2010/63/EU) and with the Italian laws in force on the protection of animals used for scientific purposes (D.lgs 26/2014). They were approved by the Veterinarian Animal Care and Use Committee of the University of Parma (Prot. 52/OPBA/2018) and authorized by the Italian Ministry of Health (Aut. Min. 802/2018-PR).

3.2 SUBJECTS.

Experimental protocols were carried out on one adult male *Macaca Mulatta* (9 years, 13 Kg). The recording sessions have been preceded by training through positive reinforcement that allowed to instruct the monkey to spontaneously sit in a primate chair and to be moved from its home cage in the animal facility to the laboratory.

After training completion, the monkey underwent two surgical interventions. The first operation allowed to implant the headpost, a titanium cylinder with a 4 feet base shaped according to a 3D rendering of the cranial bone curvature previously derived from MRI scan of the monkey's head. The second operation allowed the implantation of 4 floating 32-channels microelectrode arrays in the monkey's Ventral Premotor Cortex of the left hemisphere (see Figure 5). Both operations followed the same anaesthetic procedures, being carried out in deep anaesthesia and followed by post-surgical pain medication. A 0.03mg/kg atropine administration preceded by 15 minutes the anaesthetic induction, which in turn was carried out with a 4.5 mg/kg ketamine (Lobotor) and a 0.05 mg/kg medetomidine hydrochloride (Domitor) administration, and then maintained via inhaled isofluorane

(IsoFlo, 100% p/p). The neural recording sessions started after fully recovery of the monkey (three weeks after the last operation).

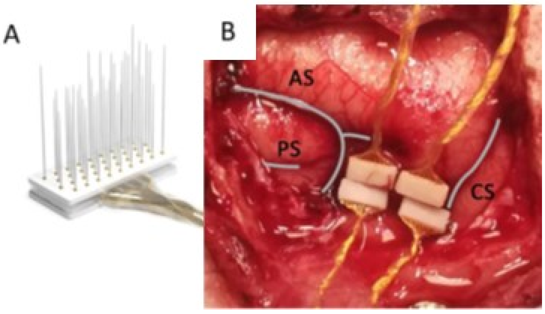


Figure 5. A) A schematic representation of one FMA. B) Location of the four FMAs implants. Anatomical sites: AS, Arcuate Sulcus; PS, Principal Sulcus; CS, Central Sulcus.

3.3 APPARATUS AND EXPERIMENTAL DESIGN.

The recordings required the execution of motor acts during unconstrained freely-moving conditions in the NeuroEthoRoom (NER), a custom-made transparent plexiglass enclosure (Width: 208cm; Height: 205cm; Depth: 181cm) equipped with a system of 8 color cameras for the monkey behavioral recordings (see Figure 6). One of the four side walls of the NER consists of two large doors that allow the experimenter to enter and prepare the environment before and after each session. Each door in turn contains a smaller opening with a vertical sliding gate through which the monkey passes from its chair to the NER at the beginning of every session.

The NER allows to be equipped with different enrichment items (see Figure 6), such as a wooden structure mounted on the floor, a rope hanging high in one of the walls, and some climbing holds mounted on the walls. Food was also displaced on several levels of the NER, that is, on the floor, on the wooden structure, on the climbing holds, and could also be lowered from the roof through four different hooks, each connected to a nylon thread externally operated by the experimenter. This allowed us to study hand behaviors such as grasping the food and placing the food to the mouth, which were repeated for right and left limb.

Solid and liquid reward were also given directly into the monkey's mouth (respectively with a stick and with a syringe) using appropriate holes and openings in the NER structure (see Figure 6); this allowed us to study mouth behavior such as the consumption of solid or liquid reward).

Such food and non-food enrichment, unitedly with the NER structure itself, made it possible to study not only the same behaviors that classically have been studied in restrained conditions with the primate chair (e.g., grasping, solid or liquid reward consumption), but also a large variety of ethologically relevant behaviors in the macaque's repertoire (e.g., yawning) which are otherwise impossible to investigate with classical neurophysiological methods.

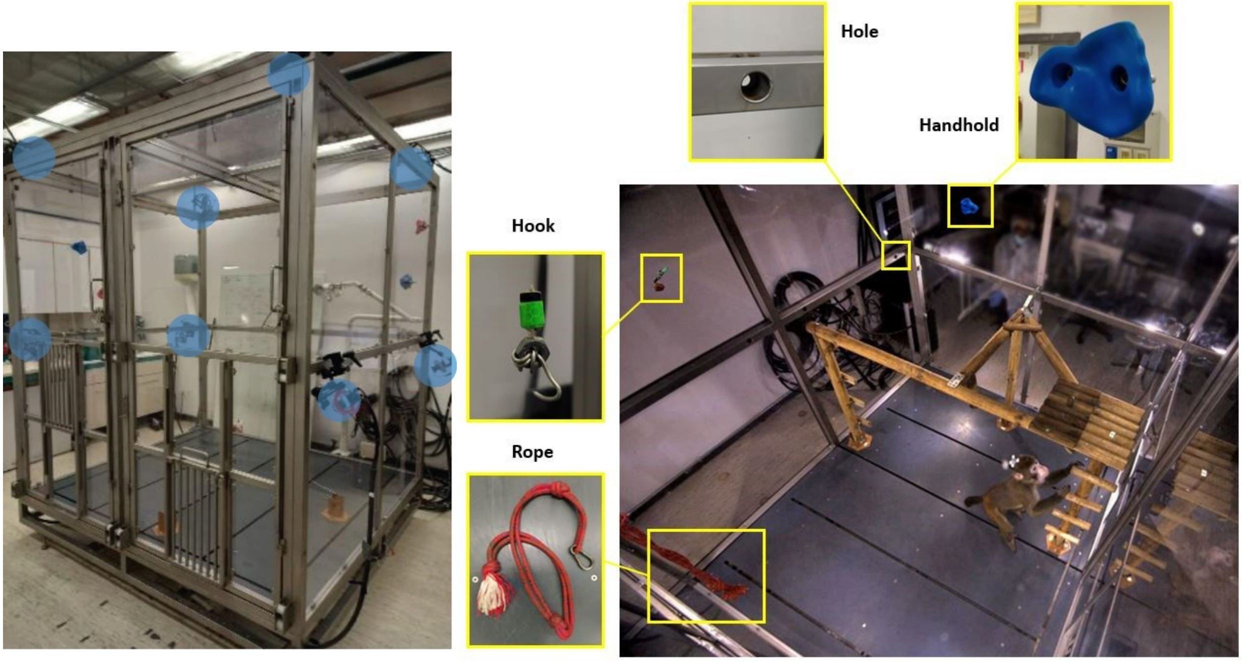


Figure 6. On the left, a picture from the outside of the NER structure, with the 8 cameras (in the blue circles) in the corners of the structure, and the doors for the experimenters and the smaller doors for the primate access on the front. A picture of the inside of the NER with the set of enrichment items: holes, handholds, hooks, the wooden structure mounted on the floor, and a rope.

3.4 BEHAVIORAL DATA ACQUISITION AND ANALYSIS.

3.4.1 Behavior recordings.

Data acquisition for the monkey behavior was accomplished with a system of eight high-resolution synchronized cameras mounted at two different levels on movable arms attached to the four external corners of the NER (see Figure 6). Such cameras are Dual Gigabit Ethernet Machine vision cameras (mvBlueCOUGAR-XD, Matrix Vision) with a 1936x1214 resolution, set to 50Hz, and equipped with a global shutter with sensor size 1/2" format (5.86μm pixel), a manual C-Mount Lenses with 5 mm focal length (CCTV Lens, KowaOptical Products Co., Ltd) and LEDs ring lights. Each camera has two RJ-45 Gigabit Ethernet connectors with screw-locking and two Industry standard 12-pin locking connectors to provide transmission of images and signals to the Windows computer, and to synchronize all cameras through a synchronization box connected to both cameras and computer. SIMI Motion Capture is the dedicated and commercially available software for 3D motion data acquisition and analysis used to capture and visualize the monkey’s behavior.

3.4.2 Ethogram.

The ethogram used for behavioral analyses was defined considering the functional properties of the ventral premotor recorded region (see Introduction, paragraph 1.2). We considered a total of 9 behaviors categorized as instantaneous events (point events) and operationalized as described in Table 1.

We chose mouth behaviors such as threat, mouth grasp, and solid or liquid reward consumption in order to differentiate neurons with a broad tuning to moth movements from those with selectivity for specific motor acts, such as yawn, threat, or ingestive behaviors. In addition, the choice to include threat behavior was made considering its similarity to yawning in the mouth gaping movement. Since macaques have a wide range of threatening behaviors, for “threat” we refer to the open-mouth threat behavior, in which the monkey stares directly to the object of the threat with its mouth open and tense, with the lips covering the teeth. Therefore, threat behavior was included to discriminate among neurons that genuinely respond to yawning from those related to large mouth gaping movement. Given their similarity in mouth movement, for yawn and threat behavior we decided, instead of considering the whole behavior in its temporal unfolding, to consider it as three subsequent instantaneous events, that is, the opening, the peak in aperture, and the closure of the mouth.

Moreover, we also included hand behaviors such as grasping and placing the food to the mouth as controls in order to distinguish neurons that respond both to mouth and hand actions. For these hand behaviors we differentiated whether it was executed with the left and/or with the right hand, in order to identify neurons with unilateral or bilateral receptive fields.

Behavior	***Event type***	***Operational description***
Active food to the mouth – Right	Point event	Monkey actively places the food into the mouth with the right hand. Start when the right hand reaches the mouth. If bimanual add a comment.
Active food to the mouth - Left	Point event	Monkey actively places the food into the mouth with the left hand. Start when the left hand reaches the mouth. If bimanual add a comment.
Grasp food – Right	Point event	Monkey grasps food pieces with the right hand. Also if it grasps food in the mouth or placed on the hooks. Start when hand touches food. Grasp in a hole: start when the finger enters the hole. If bimanual add a comment.
Grasp food - Left	Point event	Monkey grasps food pieces with the left hand. Also if it grasps food in the mouth or placed on the hooks. Start when hand touches food. Grasp in a hole: start when the finger enters the hole. If bimanual add a comment.
Mouth grasp	Point event	Monkey eats food with its mouth (it doesn't pick it up with hands). Start when mouth and food get in contact.

Liquid reward	Point event	Monkey receives passively liquid reward with syringe (directly in the mouth). Start when the mouth touches the syringe.
Solid reward	Point event	Monkey receives passively solid (fruit pieces, raisins, ...) reward directly in the mouth. Start when the food touches the mouth. Only if the experimenter gives it.
Yawn peak	Point event	The mouth is at the maximum opening. Start the frame before the monkey begins to close the mouth.
Yawn start	Point event	Mouth opening. Start the frame the mouth starts opening.
Yawn stop	Point event	Mouth closure. Start the frame the mouth is completely closed.
Threat peak	Point event	The mouth is at the maximum opening. Start the frame before the monkey begins to close the mouth.
Threat start	Point event	Mouth opening. Start the frame the mouth starts opening.
Threat stop	Point event	Mouth closure. Start the frame the mouth is completely closed.

Table 1. The ethogram with the 13 behaviors of interest, each one as a point event with its operational description for the scoring.

3.4.3 *Behavioral scoring.*

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

The kinematic of mouth aperture and closure during each yawn was tracked using SIMI Motion Capture by manually marking monkey’s upper and lower lips in frame-by-frame modality. The final output of SIMI Motion Capture was a set of 3D coordinates for the upper and lower lips from which we calculated the Euclidean distance in order to obtain the distance between the lips in every frame.

3.5 NEURAL DATA ACQUISITION AND ANALYSIS.

3.5.1 Neural activity recordings.

Recording of neural activity was conducted with 32-channel FMAs, with alternated electrodes of 4 and 2.5 mm, implanted in the PMv of the left hemisphere. Each FMA was connected through an Omnetics connector to the recording system, a wireless 128 channel neural data logger (http://deuterontech.com/) synchronized along the whole session to the rest of the recording devices. The logger used a radio signal to communicate with the transceiver, updating the internal clock and allowing the synchronization of the neural recording with the video acquisition using a unique 50Hz digital signal generated by a LabView based software and transmitted via a BNC cable.

For each channel, the neural signal was grounded and referenced using dedicated low impedance electrodes in each FMA and recorded at a conversion rate of 32 kHz with a band-pass filter ranged 2-7000Hz, thereby being able to sample single and multi-unit activity and Local Field Potentials. Neural signals were amplified, digitized, and stored in a MicroSD memory card (64 GB) to prevent any possible transmission error. The device was powered by a small external battery connected with a short cable. After linking the logger device to the electrode arrays into the chamber, all the components were sealed within a cover screwed on top of the chamber (see Figure 7). In addition, the logger had a magnetic on-off switch, so that it could be switched on and off also when the device was sealed into the protective chamber, with no need to physically touch the animal or remove any component.

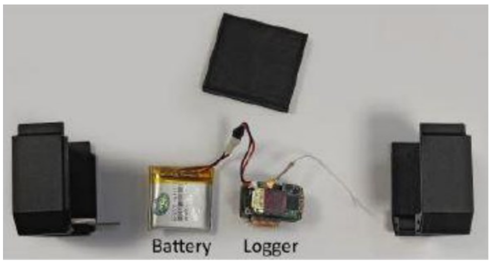


Figure 7. The logger and the battery, with the black protective cover of the chamber.

3.5.2 *Single units' extraction.*

All formal signal analyses were conducted offline on the whole dataset collected in the session. Spike sorting was performed with MountainSort, an open-access software with a fully automated spike sorting algorithm (Chung et al., 2017), setting a 3 standard deviation threshold of signal-to-noise ratio for each channel as a parameter for single units detection. Classification of units into single-units or multi-units used a noise overlap threshold of 0.15. The noise overlap parameter varies between 0 and 1, and only units with values below the threshold were considered as single-units, while all the remaining waveforms with higher noise overlap formed the multi-units signal. In addition, visual inspection of the Inner Spike Interval distribution and the waveform shape was conducted to further verify the isolation of single-units.

Lastly, possible artefacts were removed by visual inspection and all the remaining waveforms that could not be classified as single-units were included in the multi-units activity.

3.5.3 *Single units' selectivity.*

After units' classification and behavioral scoring, we studied the firing of the recorded units in relation to those scored behaviors occurring at least 7 times.

To investigate the single neuron's modulation, we defined a 2s epoch around the behavior of interest and binned the activity in 200 ms windows slit forward in 20 ms step (*Sliding Windows* procedure); one-sample right-tailed t-tests were used to compare the mean firing rate in each window with the average firing rate of the neuron across the whole recording session. Since the number of occurrences of different behaviors was variable in the session - ranging from 12 to 60 - we randomly extracted 10 trials within the pool of these behaviors and repeated this extraction 100 times (*Bootstrap method)*. The median of the 100 p-values obtained this way, multiplied by 2 (Bhattacharya & Habtzghi, 2002; Vovk & Wang, 2020) was then considered as an estimate of the corrected p-value for significant differences in each window, and if at least 5 consecutive windows had a corrected p-value lower than 0.05 (significance window), then the single-unit was considered positively modulated by the reference behavior. To avoid erroneous attribution of a significance window, we removed the attribution to a behavior if in at least half of the trials another behavior was closer to the center of the significance window than the behavior currently analyzed.

3.5.4 Responses during yawning.

Single units' response during yawning was also analyzed by extracting their peak in firing rate. We first smoothed the firing rate of each neuron with a 40ms Gaussian kernel and then defined the peak of firing rate as the maximal value of firing rate in the time window extending from 0.5 seconds before yawn start to 0.5 seconds after yawn stop, in order to include possible modulations occurring just before the start or just after the stop.

To investigate if the peaks in firing rate of units responding during yawning could be related to the extension of the mouth aperture, we correlated the values of the peaks in firing rate of a unit with the correspondent extent of maximal mouth aperture in all yawns.

Due to the exploratory nature of this study, we adopted a permissive approach with no correction for multiple testing, opting for an overly inclusive rather than exclusive approach, as suggested by Streiner and Norman (2011).

3.5.5 Population analysis.

To investigate the neuronal population activity in each behavior, we applied the Uniform Manifold Approximation and Projection (UMAP, McInnes et al., 2020) technique for non-linear dimensionality reduction of high-dimensional datasets. Our dataset consisted of the average firing rate of all the extracted single-units, regardless of their tuning, calculated in a 1s epoch around the behavior of interest.

From this firing rate dataset, the UMAP compute a low-dimensional representation of the data that best approximates the structure of the original dataset. By relying on the entire population of neurons at once, this analysis could identify clusters of neural activity having some behavioral relevance (Bagi et al., 2022). The UMAP was applied to the average firing rate dataset considering all the scored behaviors that occurred at least 7 times.

4. RESULTS

The analyses are based on a single recording session in the NER, where the monkey performed a total of 12 yawns. Overall, of the 9 behaviors considered in the ethogram, only 6 were observed at least 7 times as necessary for the analysis (see Table 2).

We isolated 60 single units, recorded from 128 electrodes of all the four chronic FMAs implanted.

Behaviors	***Frequency***
Yawning	12
Liquid reward	23
Active food to the mouth - Right	18
Active food to the mouth - Left	60
Grasping – Right	19
Grasping - Left	57

Table 2. The six observed behavior during NER condition, with the number of times they occurred.

4.1 YAWN KINEMATIC.

Each yawn had a different duration, ranging from 2s to 3.7s and with a mean duration of 2.67 ± 0.51s (see Figure 8a), in line with previous behavioral findings in macaques (Deputte, 1994). Despite this variability, once aligned to the peak of mouth opening, the dynamic pattern appears consistent across yawns, with a mean value of maximal mouth aperture of 9.51 ± 0.32cm (see Figure 8b). The time interval from the start of mouth aperture to the peak of mouth aperture (1.69 ± 0.38s) was systematically longer across yawns with respect to the time interval from the peak of mouth aperture to subsequent mouth closure (0.98 ± 0.31s), showing that mouth aperture is slower than mouth closure. This is line with Deputte's finding of the pre-peak phase lasting more than the after-peak phase in macaques (1994).

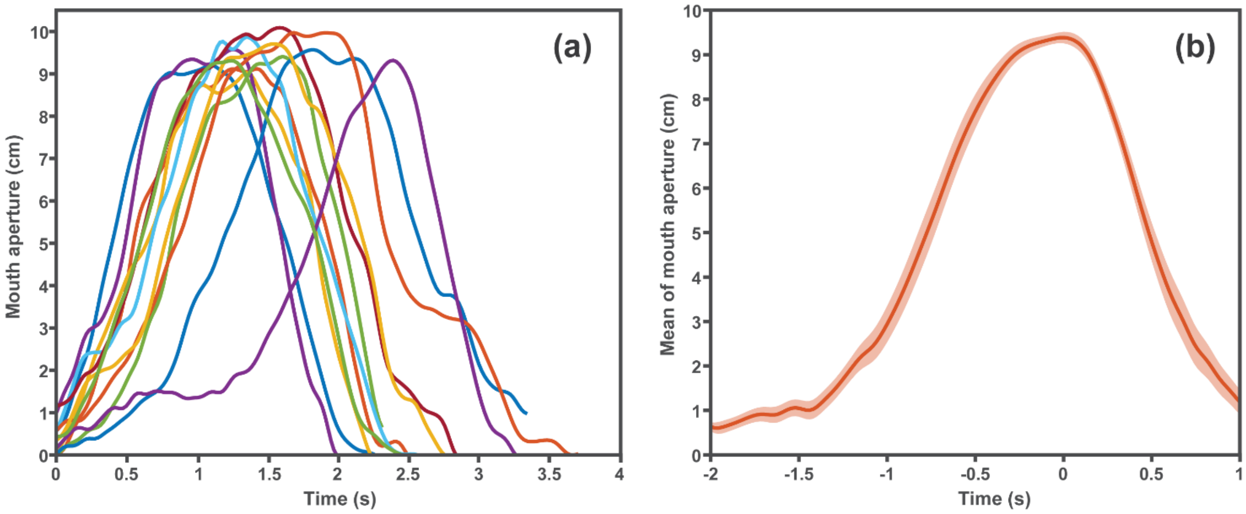


Figure 8. A) All 12 yawns aligned to the start. B) Mean values of mouth kinematic in every instant aligned to the peak of mouth aperture (at zero) and shown in an interval of 2s before the peak and 1s after the peak: shaded area corresponds to 1 standard error.

4.2 SINGLE UNITS' ACTIVITY.

After single units' extraction, we analyzed the positive modulation of each unit to each observed behavior in the considered time window of 2 seconds around that behavior (see Materials and methods). Among the 60 recorded units, 41 (68.3%) were positively modulated during at least one behavior. Of these, 26 were modulated during yawning, with 8 units (19.5%) responding uniquely during yawning and 18 (43.9%) responding during both yawning and at least another behavior. We will refer to units selective for yawn as "yawn-units", to units modulated during yawning and at least another behavior as "yawn-other units", and to units not modulated during yawning as "other-units" (Table 3).

Among the yawn-other units, all of them responded during behaviors involving to some extent the mouth, alone or in combination with other effectors (i.e., hand/arm). The same happened for all but one the other-units (Table 3).

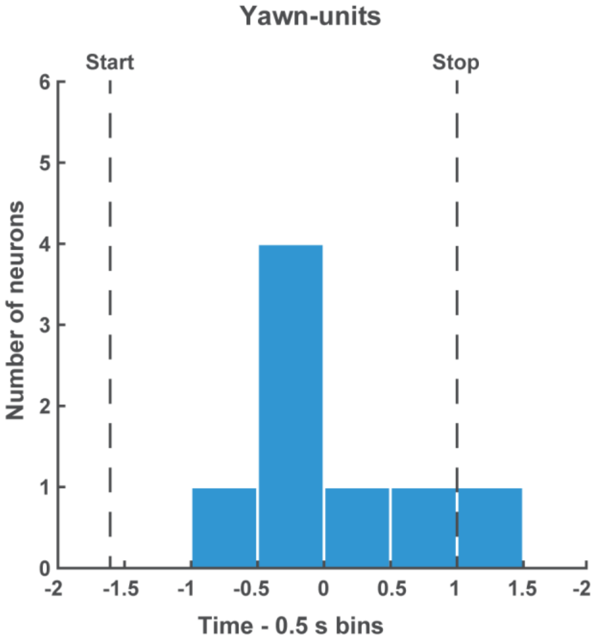
Behaviors	***N (%)***	***Unit class***	***N (%)***
Yawning	8 (19.5)	Yawn	8 (19.5)
Yawning + A.F.M	3 (7.3)	Yawn-other	18 (43.9)
Yawning + L.R.	4 (9.8)		
Yawning + A.F.M. + Grasping	2 (4.9)		
Yawning + L.R.+ A.F.M	4 (9.8)		
Yawning + L.R.+ A.F.M + Grasping	5 (12.2)		
Grasping	1 (2.4)	Other	15 (36.6)
Liquid reward (L.R.)	4 (9.8)		
Active Food to the Mouth (A.F.M.)	4 (9.8)		
L.R.+ A.F.M	1 (2.4)		
L.R.+ A.F.M. + Grasping	5 (12.2)		

Table 3. The number of units with a positive modulation for one or more behaviors and their grouping into different classes depending on their selectivity to yawning. For exposition purpose, the left-right distinction for "grasping" and "active food to the mouth" is not reported.

4.3 Responses during yawning.

We analyzed the timing of the neuronal responses during yawning of both yawn and yawn-other units to investigate whether they exhibit a different tuning for yawn start, peak, or end. For each unit we calculated the average timing of the peak in firing rate. In a time window ranging from 0.5 s before to 0.5 s after the mean duration of yawning, using time bins of 0.5s, we identified, for each unit, in which bin the average peak of firing rate was located. We obtained the histogram in Figure 9, showing the number of neurons discharging maximally in each time bin. Given the different number of units in the two groups, and given the low number of yawn-units, this data has only a descriptive value for the sampled units. As can be seen from Figure 9, the majority of both yawn and yawn-other units discharge around the peak of mouth aperture.

Neuron 1 (Figure 10) and Neuron 2 (Figure 11) exemplify this tuning to the peak in yawn and yawn-other units, respectively.



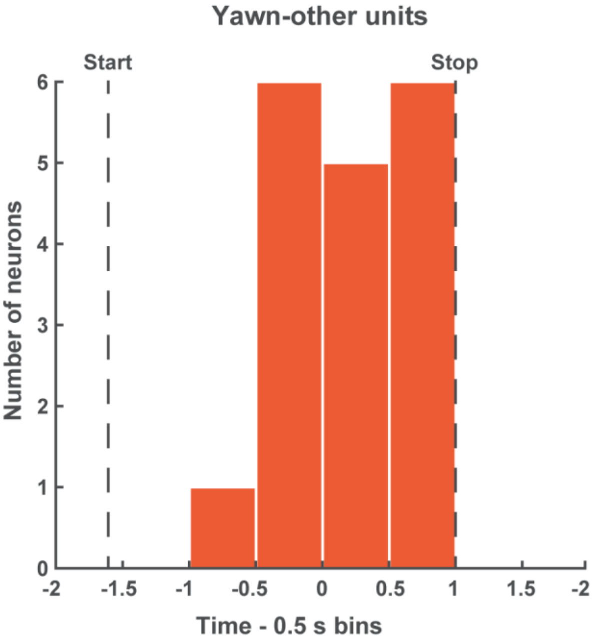


Figure 9. Histograms showing the average timing of peaks in firing rate in both yawn (on the left) and yawn-other (on the right) units. Each column shows the number of units discharging in that time bin. At zero the peak in mouth aperture, whereas yawn start and stop are indicated by dashed lines.

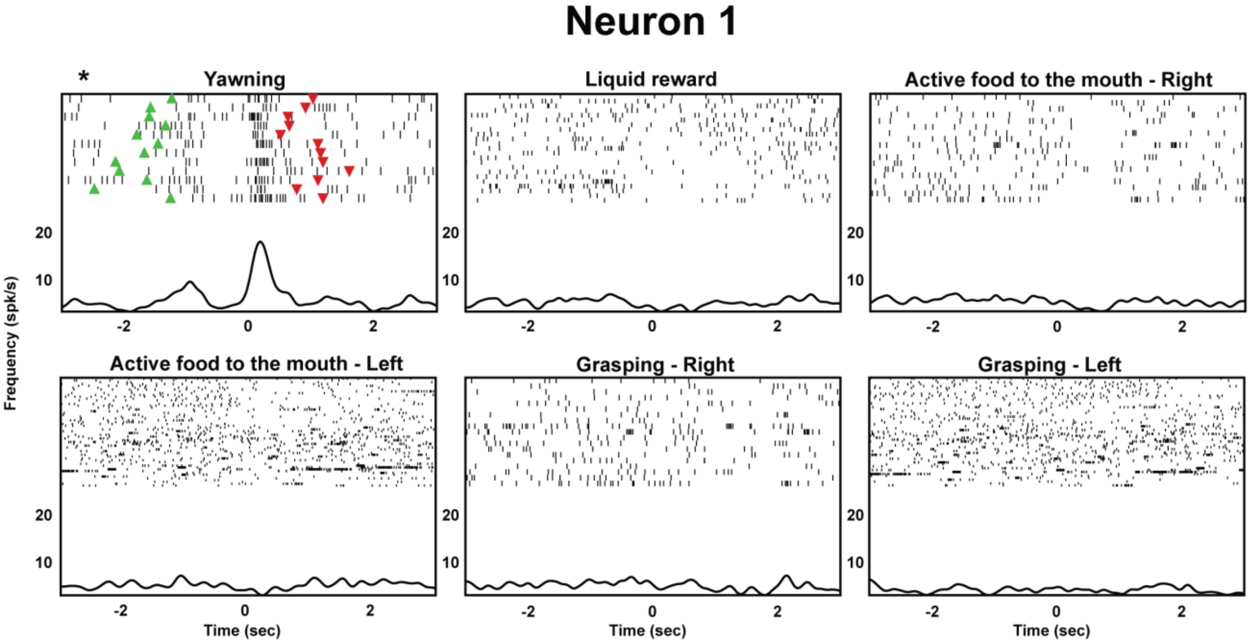


Figure 10. Neuron 1. Example of a yawn-unit exhibiting a phasic discharge right after the yawn peak. The raster plots for the six considered behaviors are presented. Each raster plot is aligned on the above specified behavior. Yawning is aligned on the maximal mouth opening, with yawn start and stop indicated by the green and red triangles, respectively. The asterisk above the raster plots indicates p<0.05 significance of the response relative to the baseline (see Methods).

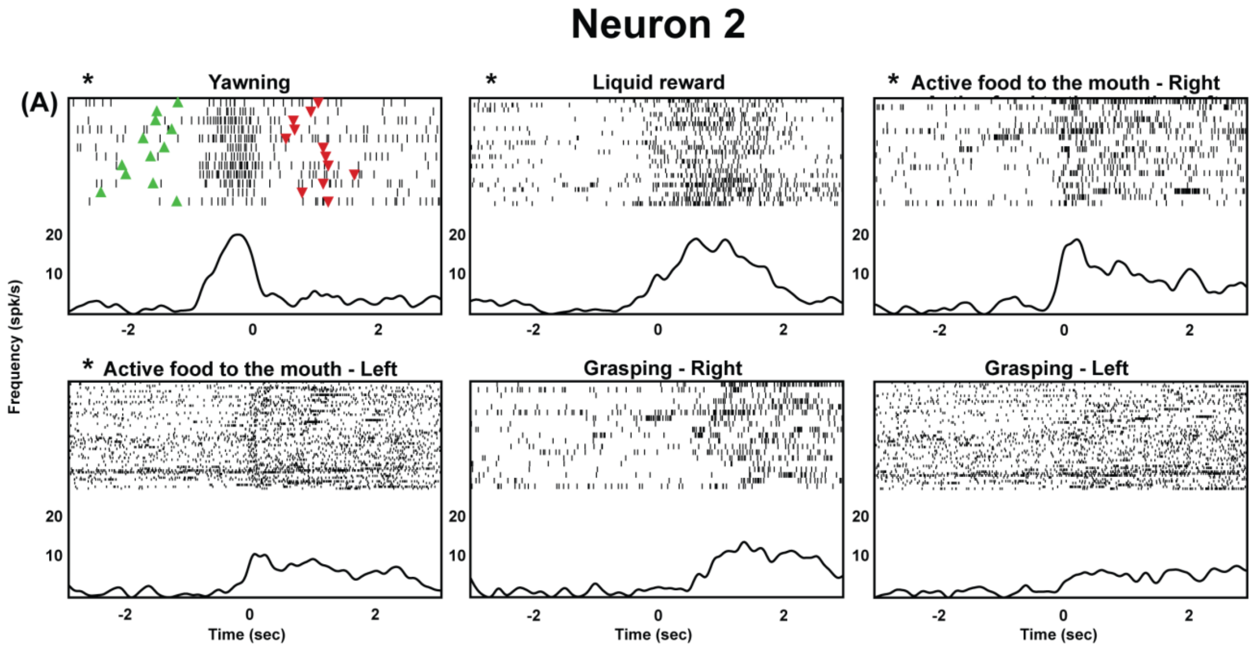


Figure 11. Neuron 2. Example of a yawn-other unit exhibiting a phasic discharge during the yawn peak. This unit also respond when the monkey sucks liquid reward from a syringe and places food in the mouth. Conventions as in Figure 10.

Given this tuning to the yawn peak, we investigated whether yawn-unit selectivity could be explained by specific kinematic parameters, such as the extension of the mouth aperture. If this were the case, yawn-units may simply be cells that respond during maximal mouth opening, which does not occur during other behaviors, and their discharge should exhibit a correlation between firing rate and mouth aperture. In contrast, we found that none of the yawn-unit had its peak in firing rate correlating with these values, suggesting that their selectivity does not depend on the kinematic parameter of the extension of mouth aperture. Notably, the only units that correlate with the maximal mouth extension are 3 yawn-other units that respond during “active food to the mouth”, a behavior during which the monkey inserts food in its mouth, hence requiring the opening of the mouth. Neuron 3 exemplifies the behavior of one of these 3 yawn-other units.

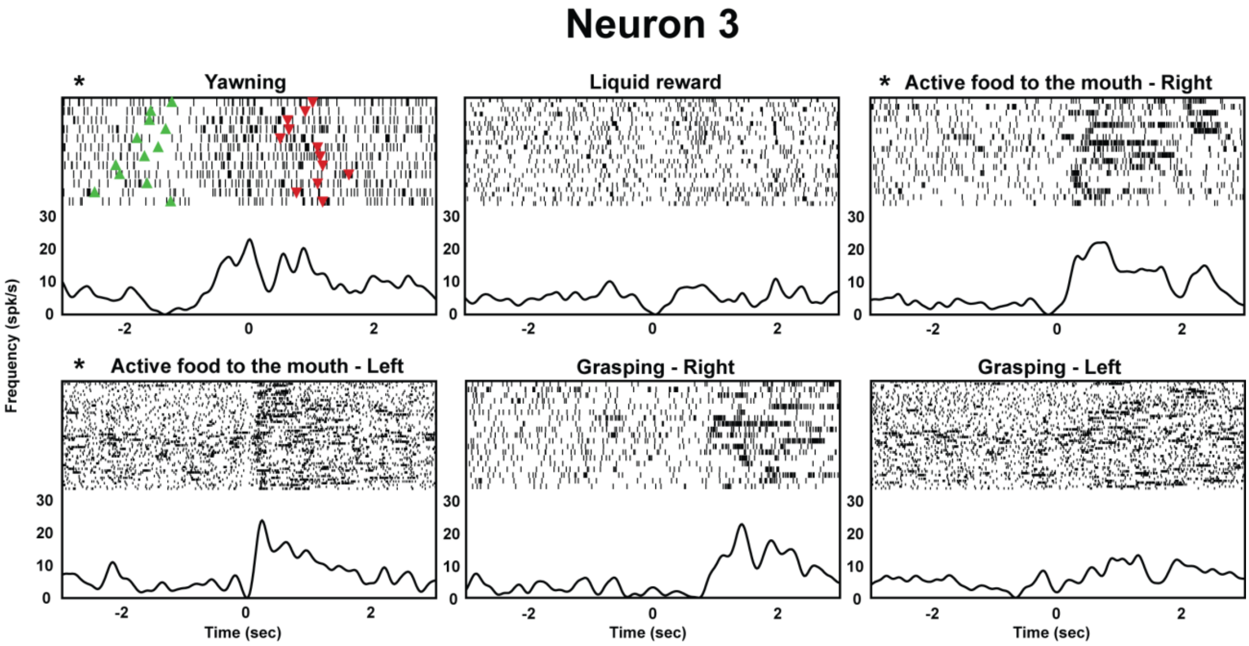


Figure 12. Neuron 3. Example of a yawn-other unit with its discharge being positively correlated to the maximal mouth aperture occurring during yawning (r = 0.72; p < 0.01). This unit results positively modulated during yawning and "active food to the mouth", with no effector preference. Conventions as in Figure 10.

4.4 POPULATION ANALYSIS.

To better understand the encoding of yawning on a population level in the ventral premotor recorded region we applied the UMAP analysis, plotting the average firing rate of all 60 simultaneously recorded units to obtain a two-dimensional neural space for the behaviors reported in. In this analysis, yawning is considered in its three events in order to better study possible differences in clustering for each event. As we can see from Figure 13, neural data concerning yawning behavior is significantly clustered, with each yawn event forming an easily identifiable unitary group that stands distant from other behaviors. A clear grouping can be observed also for liquid reward behavior, whereas no clusters are identifiable for the remaining hand behaviors. Therefore, although being all behaviors involving the mouth, yawning and consuming liquid reward and taking food into the mouth can be easily distinguished on a population level.

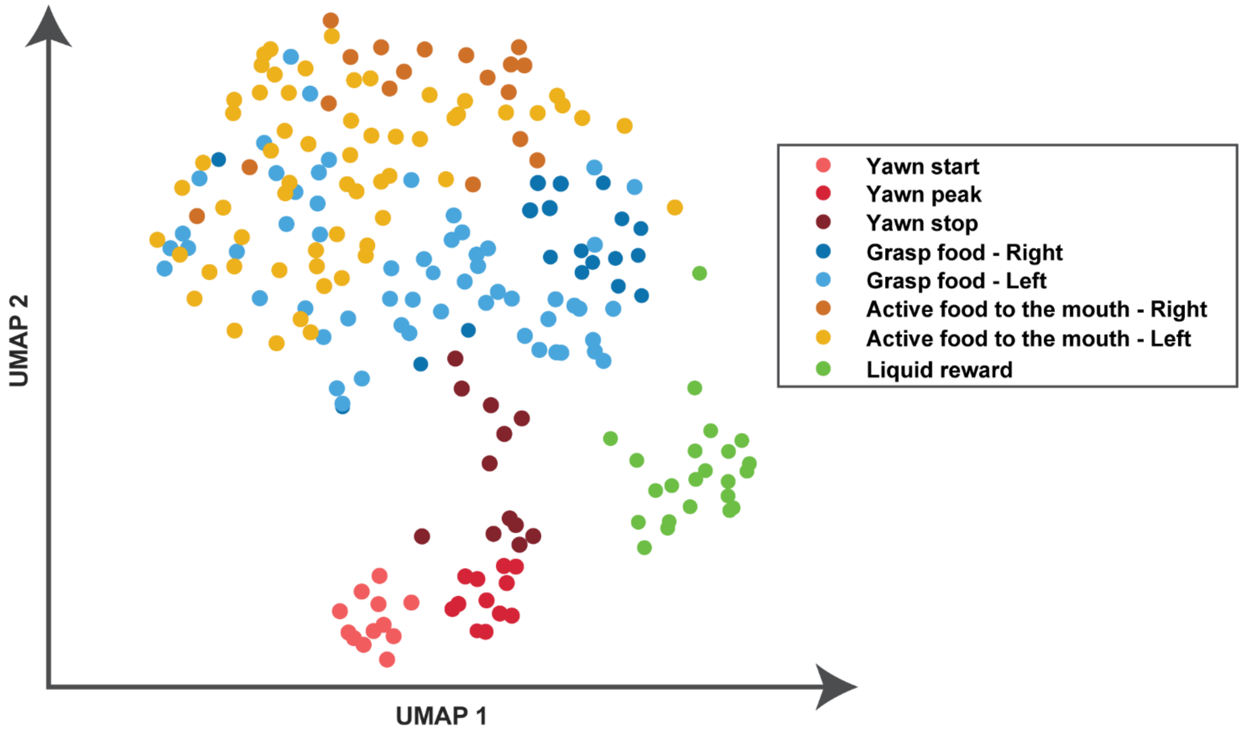


Figure 13. A 2D UMAP showing the neural data from the entire population of 60 single units. Behaviors defined according to Table 1, with yawning being considered as a unique behavior rather than three distinct events.

5. DISCUSSION

In the present study, we explore the possible selectivity of neurons in the ventral premotor cortex (PMv) during yawning behavior. However, yawning is most likely controlled by a network of subcortical structures, with the brainstem playing a key role (Krestel et al., 2018). Why then should we study PMv activity during yawning? Yawning is an involuntary and paroxysmal breathing movement mostly characterized by the sequential opening and closing of the mouth, and we know from previous studies that PMv neurons can respond during jaw, and more generally mouth, movements (Maranesi et al., 2012; Yoshino et al., 1998, 2000). Moreover, previous studies have shown that some neurons in the monkey's PMv also respond during communicative mouth behaviors (Ferrari et al., 2003), and yawning, mostly for its mouth aperture-closure pattern, have been hypothesized to hold a communicative function (Guggisberg et al., 2010; Zannella et al., 2017). Therefore, it appears at least plausible to hypothesize that some neurons in PMv might respond also or selectively during yawning.

Given the behavioral complexity of yawning, the main challenge to the aim of this study was the physical restrictions of the classical neurophysiological setup. Although there is currently no evidence that head fixation and physical constraint of the primate chair have any influence on yawning in monkeys (as instead happens for other behaviors, such as vocalizations in marmosets; see Roy & Wang, 2012), it is not difficult to imagine how these restrictions might impair the expression of such behavior. For instance, the gorget neck restriction might limit the extension of mouth aperture during yawning, especially when the monkey has its head fixated, which prevents the typical head tilting that occur during yawning. Removing at least some physical constraints seems therefore a basic precondition to study the neural activity occurring during spontaneous yawning. Therefore, following the recent trend in neuroscientific literature to study brain correlates of behavior under more ecological conditions (Testard et al., 2021), in this study we adopted wireless recordings synchronized with multi-camera system to study spontaneous yawning in an unconstrained, freely moving monkey.

Our findings on yawning kinematic show that, albeit the variability in duration, the overall kinematic profile remains consistent across trials, with a slow mouth aperture during the longer pre-peak phase and a quicker closure in the shorter post-peak phase. This is in line with the few reports explicitly accounting for kinematic details in the description of yawning in macaques (Deputte, 1994), in humans (Barbizet, 1958; Walusinski et al., 2005) and in other species (e.g., Enokizu et al., 2021, 2022; Sauer & Sauer, 1967), supporting the hypothesis that yawning might indeed be a FAP (Provine, 1986) and a phylogenetically old behavior shared across different species (Baenninger, 1997).

The main finding of this study is that PMv can selectively encode yawning both at population and at single neuron level. Applying an unsupervised analysis (UMAP) to the average activity of all simultaneously recorded neurons we found clusters of activity showing behavioral relevance, as previously found by Bagi and colleagues (2022) with different behaviors. Among our unitary clusters of population activity, yawning resulted easily identifiable among other behaviors involving the mouth. On the other hand, in our sample some single neurons resulted uniquely activated during yawning (yawn-units). To our knowledge, there are no previous studies reporting such activation. We also found a higher number of units responding during both yawning and at least another behavior (yawn-other units), which most of the times involved the mouth, either alone or in combination with other effectors. Most of both these units discharge tuned to the yawn kinematic peak. Therefore, since previous studies showed that PMv neurons can respond during mouth aperture movements (Maranesi et al., 2012; Yoshino et al., 1998, 2000), one might argue that yawn-units selectivity derives from the large mouth aperture occurring during yawning but not during other behaviors that we considered as controls. However, our findings disprove this hypothesis, showing that yawn-units discharge does not seem to be related to the extension of mouth aperture, as instead happens for some yawn-other units. The fact that the yawn-other units whose activity correlates with the kinematic of mouth aperture also respond when the monkey opens its mouth to place food in it suggests that yawn-other units rather than yawn-units' activity is more likely to reflect the pattern of mouth aperture occurring during yawning.

Given its exploratory nature, the present study has several limitations. Firstly, since we decided not to apply any correction for multiple testing, preferring an over-inclusive approach rather than an over-exclusive one given the exploratory nature of this investigation, we cannot exclude the possibility that the difference between yawn-and yawn-other units in the encoding of mouth aperture depends on Type I error. Moreover, the low number of units and the unbalanced number of yawn and yawn-other units does not allow for a solid inference but only for a description of these units in our sample. Future studies with larger neuronal samples and a less unbalanced number of yawn and yawn-other units could easily resolve these problematics.

Moreover, even though our data show that the yawn-units in our sample do not encode the extension of mouth aperture, they do not allow us to exclude the possibility that these units selectivity could be related to other low-level parameters of yawn kinematic. In fact, yawning is a complex behavior that goes beyond the mere jaws gaping, including the tilting of the head, the contraction of the whole face musculature, and postural and respiratory adjustments. All these components are most likely absent in the behaviors that we took as control for the selectivity, and therefore should be considered as possible explanatory variables for the observed selectivity. In addition, we must specify that our measurement of mouth aperture was limited to the extension of lips aperture. This is relevant since, as reported by Deputte in macaques (1994) and by Provine in humans (2005), the lips movement does not necessarily reflects the undergoing jaw movement. Thus, the actual effect of jaws movements on yawn-units discharge remains to be tested. Future studies may adopt EMG to record activities from the masseter, temporalis, and anterior digastric muscles, as previously done by Yoshino and colleagues (1998, 2000) in their studies on PMv activity during jaw movements. Another opportunity for future studies is to employ software for markerless motion capture, simplifying the movement tracking on a whole body scale (Berger et al., 2020).

Beyond the identification of yawn-units, further studies will have to clarify the nature of their response during yawning. From our recordings, we could not identify any visual or tactile stimulation occurring systematically during yawning, which therefore leads us to speculate that yawn-units response is most likely due to either motor or proprioceptive information.

If our findings were confirmed by future studies, we could speculate that neurons discharging selectively during yawning could be involved in the modulation and in the at least partial voluntary control of this behavior according to contextual and social factors. This would be in line with studies on primates showing firstly that yawning can have different morphologies likely for different functions (Leone et al., 2014; Vick & Paukner, 2010), and secondly that monkeys could be able to voluntarily control the expression of yawning for social goals (Anderson & Wunderlich, 1988; Louboungou & Anderson, 1987).

In conclusion, the present exploratory investigation represents an important step forward toward the implementation of a neuroethological approach to the study of spontaneous behaviors like yawning. In fact, previous studies of yawning in macaques were limited to either behavioral observations (Deputte, 1994; Hadidian, 1980; Troisi et al., 1990) or to neuropharmacological studies on yawn induction (e.g., Code & Tang, 1991), whereas registration of brain activity only took place in rats during pharmacologically induced yawning (e.g., Sato-Suzuki et al., 1998; Seki et al., 2002). The more ecological conditions under which brain activity during this behavior can now be studied using wireless recordings in unconstrained conditions could lead to interesting insights on both the neuronal substrate and the function of yawning.

Beyond basic research, the possibility to apply these techniques in NHPs and the possibility to synchronously record from different areas could have important translational applications, since a broader knowledge of the brain structures and neural dynamics underpinning yawning could lead to a better understanding of its pathological expression in many neurological conditions as diverse as stroke, epilepsy, tumors, and neurodegenerative disease (Krestel et al., 2018).

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