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Behavioral and contextual modulation of yawning in macaques: the possible role of ventral premotor neurons

Modulazione comportamentale e contestuale dello sbadiglio nei macachi: il possibile ruolo dei neuroni della corteccia premotoria ventrale

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"... Seeing a dog and horse and man yawn, makes me feel how much all animals are built on one structure."

Charles Darwin, 1838, Notebook

Abstract – English

Yawning, often considered a stereotyped behavior, involves paroxysmal and involuntary movement primarily affecting oral and nek motor districts. While subcortical structures are thought to primarily regulate its motor patterns, much of our current knowledge stems from rat experiments, with research on non-human primates (NHPs) remaining limited and heavily relying on behavioral observations and clinical studies. Yawning exhibits a complexity beyond common perception, influenced by both endogenous and social dynamics, and potentially subjected to partial voluntary control. In NHPs, yawning likely serves communicative functions, possibly signaling an individual's physical and mental state and/or coordinating behavior within a group.

We employed wireless neural recording systems to monitor single neuron activity in the ventral premotor cortex (PMv) of freely moving monkeys in two different settings: the NeuroEthoRoom (NER), a plexiglass enclosure for neuroethological studies in NHPs, and the animals' home-cage. We identified PMv neurons that responded either exclusively to yawning (yawn-selective neurons) or to yawn in addition to different mouth and mouth-forelimb behaviors used as controls (yawn-related neurons). Importantly, we observed that these neurons did not exhibit a correlation with the kinematic parameters of yawning, such as mouth opening and head elevation. To further validate our findings, we integrated physiological data with the outcomes of Intracortical Microstimulation (ICMS), revealing a positive association between the cortical sites where yawn-responsive neurons were identified and their causal link with ICMS evoked mouth movements. Moreover, we discovered yawn-responsive neurons that exclusively responded in the NER but not in the home-cage, while others displayed consistent responses across both contexts. This contextual selectivity could not be attributed to variations in yawning displays across contexts. Even when considering yawns with consistent displays (e.g. teeth exposure), there was no difference in the duration, yet variations persisted in neuronal responses. These findings shed light

on the cortical mechanisms underlying yawning in NHPs and highlight potential contextual influences on its regulation by top-down neural signals.

Abstract – Italiano

Lo sbadiglio, spesso considerato un comportamento stereotipato, coinvolge movimenti parossistici e involontari che interessano principalmente i distretti motori orali e del collo. Nonostante si ritenga che le strutture sottocorticali siano le principali responsabili della regolazione dei pattern motori dello sbadiglio, gran parte della nostra conoscenza attuale deriva da esperimenti sui ratti, mentre la ricerca sui primati non umani (NHPs) è limitata e si basa principalmente su osservazioni comportamentali e studi clinici. Lo sbadiglio mostra una complessità che va al di là della percezione comune, è influenzato sia da fattori endogeni che sociali, ed è potenzialmente soggetto a un parziale controllo volontario. Nei primati non umani, lo sbadiglio assolve probabilmente funzioni comunicative, segnalando lo stato fisico e mentale di un individuo e/o coordinando il comportamento all'interno di un gruppo.

In questo studio abbiamo utilizzato sistemi wireless di registrazione neurale per monitorare l'attività di singoli neuroni della corteccia premotoria ventrale (PMv) di scimmie in libero movimento in due ambienti diversi: la NeuroEthoRoom (NER), uno spazio in plexiglass per studi neuro-etologici nei NHPs, e la gabbia dell'animale. Abbiamo identificato neuroni che rispondevano esclusivamente allo sbadiglio e altri che rispondevano allo sbadiglio insieme a diversi comportamenti di bocca e mano-bocca utilizzati come controlli. È importante notare che questi neuroni non hanno mostrato una correlazione con i parametri cinematici dello sbadiglio, quali l'apertura della bocca e l'elevazione della testa. Per validare ulteriormente i nostri risultati, abbiamo integrato i dati fisiologici con i risultati della Microstimolazione Intracorticale (ICMS), rivelando un'associazione positiva tra i siti corticali dove sono stati identificati neuroni responsivi allo sbadiglio e il loro legame causale con i movimenti della bocca evocati dall'ICMS. Inoltre, abbiamo scoperto neuroni responsivi allo sbadiglio che rispondevano esclusivamente nella NER ma non nella gabbia dell'animale, mentre altri mostravano risposte consistenti in entrambi i contesti. Questa selettività contestuale non poteva essere attribuita a variazioni nelle manifestazioni dello sbadiglio

tra i contesti. Infatti, anche considerando esclusivamente gli sbadigli con precise manifestazioni (ad esempio l'esposizione dei denti), non c'era differenza nella durata, ma persistevano le variazioni nelle risposte neuronali. Questi risultati gettano luce sui meccanismi corticali alla base dello sbadiglio nei primati non umani ed evidenziano le potenziali influenze contestuali sulla sua regolazione da parte dei segnali neurali top-down.

1. Introduction

1.1 What is a yawn?

Everyone has experienced one for sure but, perhaps, no one has ever thought about why we yawn, when it happens and whether this curious behavior is universal across animal species. An operational definition always provides a solid starting point for a clear understanding of the phenomenon under discussion.

Barbizet depicts yawning as "an involuntary, paroxysmal breathing movement" (Barbizet, 1958), while Deputte, years later, describes yawning as "a common, species-typical, behavior in vertebrates, generally associated with transition from sleep to wakefulness" (Deputte, 1994). The motor component of yawning consists of a specific sequence of respiratory acts: the first one is a phase of long inspiration followed by a peak where the mouth is at its maximum aperture, and finally a rapid expiration. This motor sequence is accompanied by a coordinated motor pattern that involves the opening and closure of the jaw, usually accompanied by closure of the eyes, and a contraction of facial muscles followed by stretch of the trunk, neck and arms (Krestel et al., 2018). This complex motor pattern seems to be modulated mostly by subcortical structures (Krestel et al., 2018).

This very precise behavior is often pictured as stereotyped or reflex-like due to the fact that once this response begins, it is difficult to stop (Lehmann, 1979). The use of the term "reflex-like" is not arbitrary; rather, it suggest that a yawn, even though it's quite stereotyped, can exhibit some sort of variability in its parameters such as duration, the peak of the mouth aperture and the frequency (Deputte, 1994).

1.2 Who yawns?

The intriguing aspect of yawning lies in its widespread occurrence across various classes of vertebrates. It is logical to consider an ancient phylogeny for this behavior, possibly evolving with the appearance in the evolutionary scene of jawed fishes (Gallup, 2022).

However, as Baenninger pointed out, "behavior leaves no fossils, so unambiguous evidence on how and why yawning evolved is impossible to obtain. Skulls of extinct species can tell us only whether yawning was possible, not whether it occurred" (Baenninger, 1997). Despite these challenges, we can propose that yawning, given its persistence across species, represents an adaptation with significant functionality and a potential evolutionary advantage. Indeed this behavior is observed in most classes of vertebrates from fetal stages (Guggisberg et al., 2010) to old age (Baenninger, 1987; Bakkegard, 2017; Enokizu et al., 2022; Enokizu et al., 2021; Hartzell et al., 2017; Massen et al., 2021; Palagi et al., 2019; Rasa, 1971; Sauer & Sauer, 1967). Nevertheless, when considering both the respiratory and gaping component, the yawning observed in mammals differs from the gaping of fishes, reptiles and amphibian, ultimately being considered as a behavior that is merely analogous among these species (Deputte, 1994; Sauer & Sauer, 1967). The homology of yawning between mammals, birds and herbivores remains controversial (Heusner, 1946). Finally, yawning in rodents, carnivores and non-human-primates can be consider without doubt a homology of the same behavior that we can observe in humans (Deputte, 1994).

Despite the homology of this behavior across different animal classes remains uncertain, the similarity of its motor pattern is unquestioned. Indeed, Darwin described it as "a deep inspiration, followed by a long and forcible expiration; and at the same time almost all the muscles of the body are strongly contracted, including those around the eyes. During this act tears are often secreted, and I have seen them even rolling down the cheeks" (Darwin, 1872). This basic motor sequence is present in all major classes of vertebrates and the whole sequence is reported to last 4-7 seconds (Barbizet, 1958).

A more recent study carried out a phylogenetically analysis, considering yawns from 101 different species (55 mammals and 46 birds), revealing a positive correlation between yawn duration (using the definition by Barbizet mentioned in paragraph 1.1) and brain mass (Massen et al., 2021). Once again, we observe the widespread prevalence of this behavior across vertebrates, and the figure below provides examples of yawning in various animal species (Figure 1).

Figure 1. Examples of yawns from several mammals and birds species. Above: Mammals, from left to right: Chimpanzee (Pan troglodytes), Ring-tailed lemur (Lemur catta), Southern sea lion (Otaria flavescens), Lion (Panthera leo), and Capybara (Hydrochoerus hydrochaeris). Below: Birds, from left to right: Greylag goose (Anser anser), Common raven (Corvus corax), Snowy owl (Nyctea scandiaca), Diamond dove (Geopelia cuneata), and Budgerigar (Melopsittacus undulatus) (Casetta et al., 2021; Massen et al., 2021).

Focusing on primates, several studies have analyzed different types of yawns based on the situations in which they occur. For example, Palagi's group demonstrated that different types of yawns in *Macaca tonkeana* can be associated with distinct functional contexts (Zannella et al., 2021). They categorized three types of yawns following Deputte's motor pattern definition (Deputte, 1994). During phase 1, the head raises and moves backward while the mouth starts to open forming an oval shape and, in this phase, teeth are still covered. Phase 2 involves further head movements, while the mouth reaches a maximum opening showing teeth, especially canines, and often eyes are closed. Finally, in phase 3, the head returns to its normal position, and the mouth closes, covering the teeth. It's worth noting that not all three phases are consistently present, leading to the identification of three types of yawns: one with covered teeth and two with exposure of the canines (Figure 2).

Hence, it appears that in non-human primates, yawning may be a more complex behavior and may have multiple functions depending on the context in which it occurs (to have a more detailed explanation of how different yawns may serve different functions see paragraph 1.4).
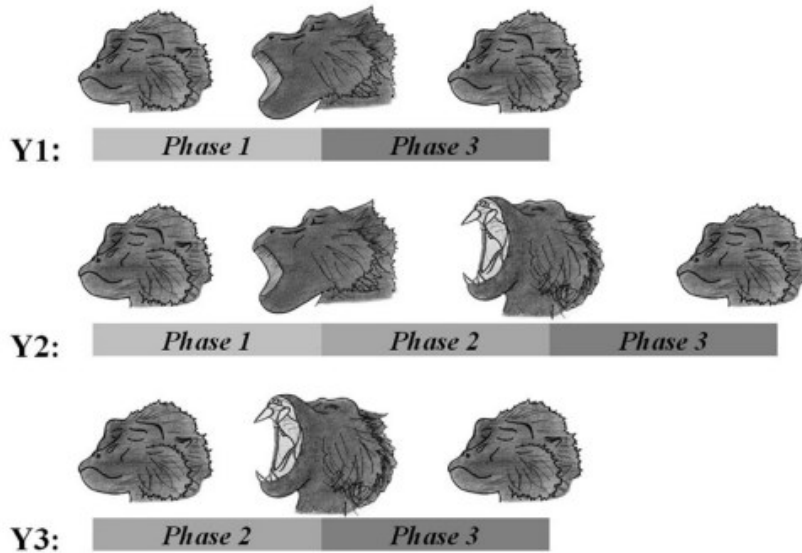

Figure 2. Illustrations of different types of yawning according to the phases that they include: Yawn 1 (long yawn with covered teeth) including Phases 1 and 3, Yawn 2 (long yawn with uncovered teeth) including Phases 1–3 and Yawn 3 (short yawn with uncovered teeth) including Phases 2 and 3 (Zannella et al., 2021).

1.3 Why do we yawn?

One of the renowned researchers who investigate yawning was Darwin, who studied it among both humans and animals and used it to conceive the natural selection theory (Smith, 1999). The omnipresence of yawning across the animal kingdom suggests an important functionality of this behavior, considering that it survived natural selection. Nevertheless, there are various theories considering the potential role of yawning. The most widely accepted theories are the respiratory hypothesis, the brain-cooling hypothesis, the arousal hypothesis, and the communication hypothesis.

The respiratory hypothesis is the oldest, dating back to Hyppocrates (4th century BC), who believed it to be an act to remove bad air from the lungs and intensify the respiration act (Guggisberg et al., 2010). Today, this hypothesis states that yawning is related to changes in the respiratory function or brain perfusion along with the theory that this behavior is connected with

medical conditions like anemia or insufficient brain perfusion (Krestel et al., 2018). This hypothesis lacks strong experimental support, and the experimental evidence attempting to refuse it is also weak and controversial. The first piece of evidence against it comes from observations of yawning-like behaviors, such as mouth movements, grimaces, and tongue protrusions in human and rodents' fetuses. Despite the physiological need for oxygen and the expulsion of carbon dioxide, fetuses do not engage in pulmonary respiration. Since they do not breathe through the lungs, these movements, do not serve a respiratory function. Instead, they seem to constitute a motor pattern whose activation may serve to prepare newborns to produce coordinated and integrated yawns shortly after birth (Sherer et al., 1991; Van Woerden et al., 1988; Walusinski, 2010). The second line of evidence against it involves the examination of individuals after a physical effort that led to a significant breath increase, yet did not affect yawning expression and frequency (Provine et al., 1987). The third piece of evidence concerns the observation that periods of apnea are not followed by yawning in a significant way (Baenninger, 1997). Although these lines of evidence are not univocally considered sufficient to categorically confute the respiratory hypothesis, there is also poor experimental support favoring it, thereby rendering it a weak explanation of this behavior.

The second theory regarding the functional role of yawning is the brain cooling hypothesis. Gallup, the author who strongly supports this thesis, has conducted extensive research to validate it (Gallup & Eldakar, 2013). Three processes have been described to explain how yawning could cool down the brains of mammals and birds. The forceful extension of the jaw during yawning leads to heightened blood flow in the neck, head, and facial areas. Additionally, the deep inhalation that accompanies yawning results in a substantial downward flow of cerebrospinal fluid and an elevation in blood flow in the internal jugular vein. The second process views yawning as mechanism to exchange air, taking advantage of cooler ambient air for thermoregulation (Gallup et al., 2009). The last one is about the jaw activity during yawning, where the pterygoid muscle contract causing a sinus wall bending. This movement promotes a system ventilation and the evaporation of the sinus mucosa, therefore creating brain cooling effect (Baker, 1982; Gallup &

Eldakar, 2013). This hypothesis is further supported by a cross-species study that explored the idea that animals with larger brains require longer yawns in order to better serve the brain cooling function. The study found a positive correlation between yawn duration, brain mass and total number of neurons (Massen et al., 2021). This hypothesis is not without critics (Guggisberg et al., 2010, 2011; Walusinski, 2013), especially because the duration of many automatic or rhythmic behaviors, like walking or chewing (Biewener & Patek, 2018), is usually positively correlated with body mass that, in turn, tends to correlate with brain mass (Massen et al., 2021). Yet there are no evidences against it, probably because it's challenging to design an experiment to verify it and obtain positive support for it (Gallup, 2011a; Gallup & Eldakar, 2013).

One of the first supporters of the arousal hypothesis was Askenasy, who described yawning to have an "antisleep effect". This behavior, indeed, is favored by neurotransmitters like serotonin and dopamine, usually known to have an activating role (Askenasy, 1989). Later, Baenninger proposes that yawning serves as a mechanism to regulate cortical arousal in many animal species, especially when the environment is momentarily unstimulating, therefore a low level of arousal could be dangerous for the animal's survival (Baenninger, 1997). More recently it was suggested that yawning is important in the switching between the default-mode network, active during rest periods, and the attentional network activating it. This process seems to be done through the cerebrospinal fluid acceleration that causes the clearance of somnogenic substances with the consequence of reducing the individual's sleepiness (Walusinski, 2014).

Finally, according to the communication hypothesis, yawning is portrayed as a form of non-verbal communication that serves the function of synchronizing the behavior of a group (Daquin et al., 2001; Deputte, 1994; Guggisberg et al., 2010). It is well known that yawning represents sleepiness; in fact, is frequently observed during transitions between sleep and awake states (Baenninger, 1997; Govern, 1993). In this case, yawning seems to serve the function of increasing alertness and creating a condition in which the animal is able to adjust its behavior during unexpected situations (Casetta et al., 2021; Provine, 2005). It also signals a state or boredom, and it

occurs more often in the presence of repetitive or uninteresting stimuli (Govern, 1993; Provine & Hamernik, 1986). Yawning can also express a threat or stress, but in this case is more prevalent in the animal kingdom (Baenninger, 1987, 1997).

One phenomenon that supporters of this hypothesis cite, is the contagious nature of yawning. Contagious yawning is defined as a behavioral response that is elicited automatically by viewing or listening to another individual performing the same act (Provine, 2005). Contagious yawning has been observed in humans (Cordoni et al., 2021) and other animals species like primates (Guggisberg et al., 2010), wild lions (Casetta et al., 2021), wolves (Romero et al., 2014), domestic dogs in response to the owner's yawn (Joly-Mascheroni et al., 2008), and, in general in very social species. These observations support the hypothesis according to which yawning serves the function of synchronizing the behavior of a group like we reported earlier in this paragraph.

In conclusion we do not have enough data to exclude one hypothesis and firmly accept another; therefore yawning can be better described as a multifunctional behavior, that probably emerged with the first jawed vertebrates, fishes (Baenninger, 1987). The initial function of this behavior is probably physiological, from which a more social and communicative aspect evolved (Gallup, 2011b).

1.4 When do we yawn? Focus on non-human primates

Our daily life experience tells us that we may feel the urge to yawn in a variety of contexts, in some of which it may even be inappropriate to freely display this behavior; nevertheless, this urge suggests us that it is an extremely stereotypical act, extremely difficult to suppress, and hence assumed to be consistent and without significant variations in its parameters. Nonetheless, various observations, especially within the order of primates, indicate that yawning displays changes associated with the context in which the animal yawns, likely involving some form of top-down regulation. Indeed, "not all yawns tell the same story" (Zannella et al., 2021).

We have previously explored three different types of yawns identified in non-human primates (see paragraph 1.2 and Figure 2). In this study the researchers observed that usually yawns with teeth exposure are typically performed in tense situations, leading them to conclude that in these circumstances, a yawn serves as a threat signal (Zannella et al., 2021). Additionally, they noted variations in the degrees of mouth opening and yawn duration. The longest yawns (Y1 and Y2) occurred predominantly in relaxed or social situations, and interestingly, they could occur in both sitting and laying positions, while shorter yawns (Y3) occurred mostly during tense situations (Zannella et al., 2021). Among the three identified types of yawns, only Y2 and Y3 displayed significant differences in duration. The animal's state, influenced by previous aggression, affected the frequency of Y3, suggesting a connection to the animal's arousal state (notably, Y3 shows a frequency peak during the first minutes after a conflict). On the other hand, Y2 was more frequent when the animal engaged in low motor activities, indicating a relaxed context (Zannella et al., 2021). Curiously, despite teeth exposure in Y2, this type of yawn did not show any variation in frequency following a conflict (Zannella et al., 2021).

Another aspect that may exhibit variability in the yawning motor pattern is the opening and closing of the eyes. When investigating the connection between yawning and eye movement, there are limited observations available, often providing conflicting information. Charles Darwin made observations regarding the eyes during threat-like displays, such as yawning in sexually dimorphic non-human primates. He noted that in these instances, a dominant male would open his mouth while staring at a subordinate and displaying canines (Darwin, 1872). This differs from "true yawns", characterized by elevation of the head and closing of the eyes, while during threat-like yawns the yawner stares at the opponent to monitor the effect of the threat (Deputte, 1994). In fact, Deputte (1994) considers yawning a potential threat display but suggests that it might be less direct and controlled than other threat signals in primate social interactions. This is because yawning involves the interruption of ongoing activities, causing the yawner to lose track of their partners' reaction caused by head rising and/or eye closure (Deputte, 1994). In carnivores, primates, and

domestic animals two types of yawns have been identified: a passive yawn associated with a relaxed, bored or hungry state, characterized by closed eyes, and an active yawn, characterized by open eyes and exposed teeth, considered threatening (Daquin et al., 2001; Deputte, 1980). Furthermore, a study on chimpanzees observed two different yawns: a full yawn and a modified yawn with reduced mouth aperture. This suggests a partial voluntary control of the facial muscles and indicates multiple functions of yawning based on context. Interestingly, no significant pattern of eye closure in relation to these yawning types has been identified (Vick & Paukner, 2010).

We already introduced the fact that yawning can serve an additional communicative function beyond its more common purposes conveying a threat or a stressful message from the yawner to an observer. In a notable ethological study, Deputte (1994) observed two types of yawns in two primate species (two groups of *Cercocebus albigena* and one group of *Macaca fascicularis*): *rest yawns* (90%) during the transition from rest to activity and *emotional yawns* (10%). Both males and female exhibited rest yawn with no discernible differences based on age. However, emotional yawns were more common in adult males, and the frequency within this category increased with age. Authors noted another distinction involving teeth exposure and yawning duration. Short yawns with uncovered teeth are followed by intense motor activity, often after stressful events (indicating an increase in arousal level). Longer yawns, with or without teeth exposure, are associated with a relaxed state in non-human primate (Leone et al., 2014; Zannella et al., 2021; Zuckerman, 2013). Additionally, emotional yawn is predominantly performed by high-ranking males and is characterized by an exposure of the canines (Leone et al., 2014).

A study comparing Japanese macaques (*Macaca fuscata*) described as despotic, and Tonkean macaques (*Macaca tonkeana*) described as tolerant, revealed intriguing findings (Zannella et al., 2017). Despotic species, characterized by low social tolerance, often exhibit a pronounced kin-centric power imbalance between dominants and subordinates (Kutsukake & Castles, 2001; Zannella et al., 2017). Conversely, egalitarian species are less influenced by social rank and kinship relationships (Butovskaya, 2004; Zannella et al., 2017). Tonkean macaques showed a higher

frequency of uncovered teeth yawns, primarily during high-tension situations like feeding. This supports the communication redundancy hypothesis, suggesting different functions for covered and uncovered teeth yawns, with the latter carrying a threat-like meaning, particularly in tolerant species where extensive communicative skills are required (Zannella et al., 2017). Tolerant species, characterized by high variability in group interactions, require a more sophisticated communicative repertoire to mitigate the risk of misunderstanding due to unpredictable social interactions (Scopa & Palagi, 2016; Zannella et al., 2017). Finally, as the authors of the article state, "The more evident the signal, the clearer the message" (Zannella et al., 2017).

1.5 Where is the yawn center?

If we want to find a precise and secure location of the so-called yawn center, we still have to dig up into the brain. Currently, the neuroanatomical location that controls the yawning respiratory-motor patterns remains unknown, although there are various hypothesis (Krestel et al., 2018).

The first hypothesis follows the observation of two cases of anencephalic infants who, despite the lack of the whole telencephalon or of some parts of it, could still perform the act of yawning (Catel & Krauspe, 1930; Gamper, 1926). Consequently, it was concluded that the yawn center should be located in the medulla oblongata, a region close to the vasomotor and respiratory centers (Heusner, 1946). Later, it was proposed a location in the brainstem close to the RAS (reticular activating system). This hypothesis emerged from observation of patients who had their corticobulbar pathways interrupted because of a tumor, but nevertheless retained the capacity to yawn (Askenasy, 1989). The last consideration involves the pontomedullary central pattern generator, structures that serve some fundamental survival functions, such as cough, swallowing and breathing; each one of them is responsible for repetitive motor patterns such as yawning (Walusinski, 2006).

All these pieces of evidence point towards a significant role of the brainstem. However, other structures have also been identified as having a role in the yawning act. One of these structures is the hypothalamic paraventricular nucleus (PVN), often depicted as the supratentorial control of the yawning center in the brainstem (Krestel et al., 2018). This comes from studies that observed yawn induced in anesthetized rats through chemical and electrical stimulation of the PVN (Sato-Suzuki et al., 1998). Finally, some authors suggested an emotional aspect of yawning involving the central nucleus of the amygdala (CeA), a core cerebral region for emotion's expression. They performed stimulation and lesions of this region and found a role of the CeA in the yawning behavior, therefore they suggest that emotional-yawning may involve a neural pathway connecting the PVN and the CeA (Kubota et al., 2023; Kubota et al., 2019; Melis et al., 1986; Sanna et al., 2012; Sato-Suzuki et al., 1998).

In human the case of an epileptic patient has been reported in which the stimulation of the putamen produced yawning (Joshi et al., 2017). Additionally, studies in patients with ischemic stroke into the anterior circulation (AC), particularly affecting the posterior insula and the caudate nucleus, reported abnormal yawning (Cattaneo et al., 2006; Krestel et al., 2015; Singer et al., 2007)[1].

These findings suggest that a variety of substrate can contribute to induce yawning, possibly through different pathways and mechanisms. However, in addition to the identification of relevant anatomical centers is also important to elucidate the neurochemistry underlying yawning. There are several neurotransmitters, neurohormones and neuropeptides that seem to play a role in this behavior. One proposal focuses on oxytocinergic neurons of the PVN, one of the main structures suggested to be a key yawn center (Krestel et al., 2018), as it projects to other areas relevant for yawning and take part to the yawning modulation (Argiolas & Melis, 1998). Essentially, when neurons in the PVN are activated by dopamine receptor agonists, oxytocin and excitatory amino

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[1] Abnormal yawning is more frequent than normally perceived, compulsive and/or triggered by stimulus that are not into the category of the ones that normally trigger a yawn such as sleepiness, boredom or induced by observation of others. The frequency of yawn is considered abnormal if it surpasses the range from 2 yawns/10 min to 30 yawns/10 min.

acids, yawning is elicited. On the contrary, the inhibition of these neurons by opioids, suppresses the yawning response (Argiolas & Melis, 1998; Krestel et al., 2018).

In conclusion, until now the research has primarily identified subcortical structures associated with yawning. It is noteworthy that, to date, data on spontaneous yawning relied on indirect and few direct studies involving human patients or investigations conducted on rats, while research in non-human primates is still poor or mainly based on behavioral data.

1.6 Possible cortical modulation of subcortical yawn-related regions

As mentioned in the preceding paragraph, research has predominantly associated subcortical structures with yawning creating a circuit composed by the PVN and its projections to the brainstem. Other structures connected to this circuit have been identified through studies on human patients and rats. In non-human primates, behavioral studies reveal a complexity in yawning that appears to serve various functions, suggesting a plausible level of cortical control over this behavior.

Considering the motor representations of facial and mouth actions within the ventral premotor cortex (PMv), along with the existence of neurons possessing both sensorimotor and social functional properties in this region, our hypothesis is that PMv itself might be the cortical region that modulates yawning displays for social and communicative purposes.

1.6.1 The ventral premotor cortex

For a very long time it was believed that the agranular frontal cortex of the macaque monkey, also known as the areas 4 and 6 according to Broadmann classification (Brodmann, 1909), included two functionally distinct areas: the primary motor cortex (area 4), where body movements are precisely represented, and the supplementary motor cortex (area 6), where more complex sequence of movements are represented in terms of action goals (Rizzolatti & Sinigaglia, 2019).

Further studies (Rizzolatti et al., 2014; Rizzolatti et al., 1996) revealed that this cortical region can be divide into seven different areas, all identified by the letter F (meaning frontal) followed by a number. F1 correspond to the primary motor cortex (Brodmann's area 4), Broadmann's area 6 is now divided into three regions according to their anatomical localization: a mesial, a dorsal and a ventral region, in turn partitioned in a rostral (anterior) and a caudal (posterior) sub-sector.

In a caudo-rostral direction, the mesial region includes areas F3 and F6 (respectively corresponding to SMA and pre-SMA); the dorsal PMd includes F2 and F7 (respectively corresponding to PMd and pre-PMd); the ventral region includes areas F4 and F5 (Matelli et al., 1985) (Figure 3).
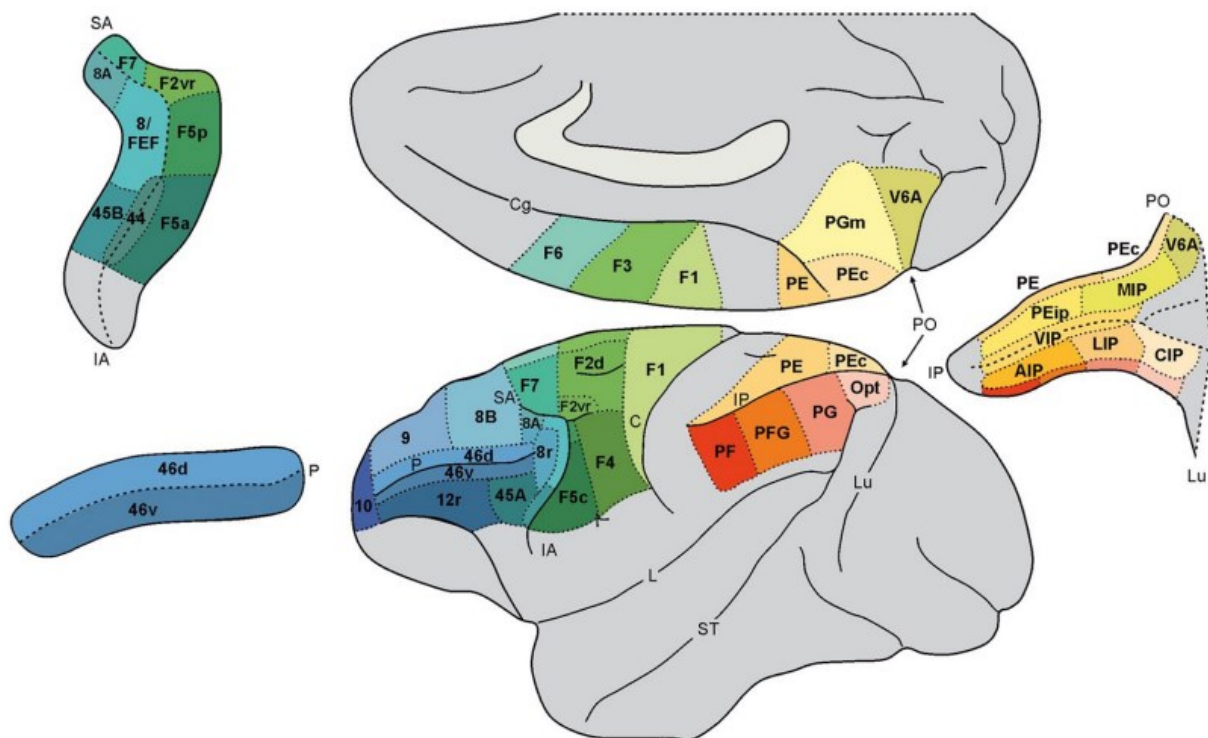

Figure 3. Lateral and mesial views of the macaque brain showing parcellation of the frontal and posterior parietal cortex. The areas located within the arcuate and the principal sulci are shown in an unfolded view of the sulci in the left part of the figure, and the areas located within the intraparietal sulcus are shown in the right part of the figure. Dashed lines indicate the architectonic borders. Key: C central sulcus, Cg cingulate sulcus, IA inferior arcuate, L lateral fissure, Lu lunate sulcus, P principal sulcus, PO parieto-occipital sulcus, SA superior arcuate, ST superior temporal sulcus (Gerbella et al., 2017).

1.6.2 *Representation of mouth motor acts in area F4*

Area F4 covers the dorso-caudal part of the PMv, occupying the precentral gyrus in a rostral position relative to F1 (as illustrated in Figure 3). This ventral area is mutually connected with the Ventral Intraparietal Area (VIP), located into the intraparietal sulcus (Rizzolatti & Sinigaglia, 2006), and with F1.

Area F4 contains representations of the nek, mouth, and arm motor acts (Fogassi et al., 1996; Gentilucci et al., 1988). More recent studies (Kurata, 2018; Maranesi et al., 2012) explored the somatotopy of this area by combining the recording of neuronal activity with short-train intracortical microstimulation (ICMS). These investigations revealed that F4 can be divided into a dorsal and a ventral part due to their different functional properties. The stimulation of F4d (dorsal) produced forelimb and face movements, and neuronal activity related to forelimb and mouth goal-directed motor acts. This area also shows somatosensory and visual properties: some sites responded to visual stimuli moving within a space reachable by the monkey. Conversely, F4v (ventral) is more excitable, and the evoked movements are simple rather than goal-directed ones and related to the tongue and the jaw. In this ventral area, visual responses are nearly absent, while somatosensory ones are present (Maranesi et al., 2012). F1 and F4 share several features and this suggest that F4v and F1vv form two interconnected cluster, that contribute to the organization of tongue and oro-facial simple movements while F4d and F1vd forma cluster for the control of harm and hand motor acts (Maranesi et al., 2012). A more detailed schematic visual representation is presented in Figure 4.
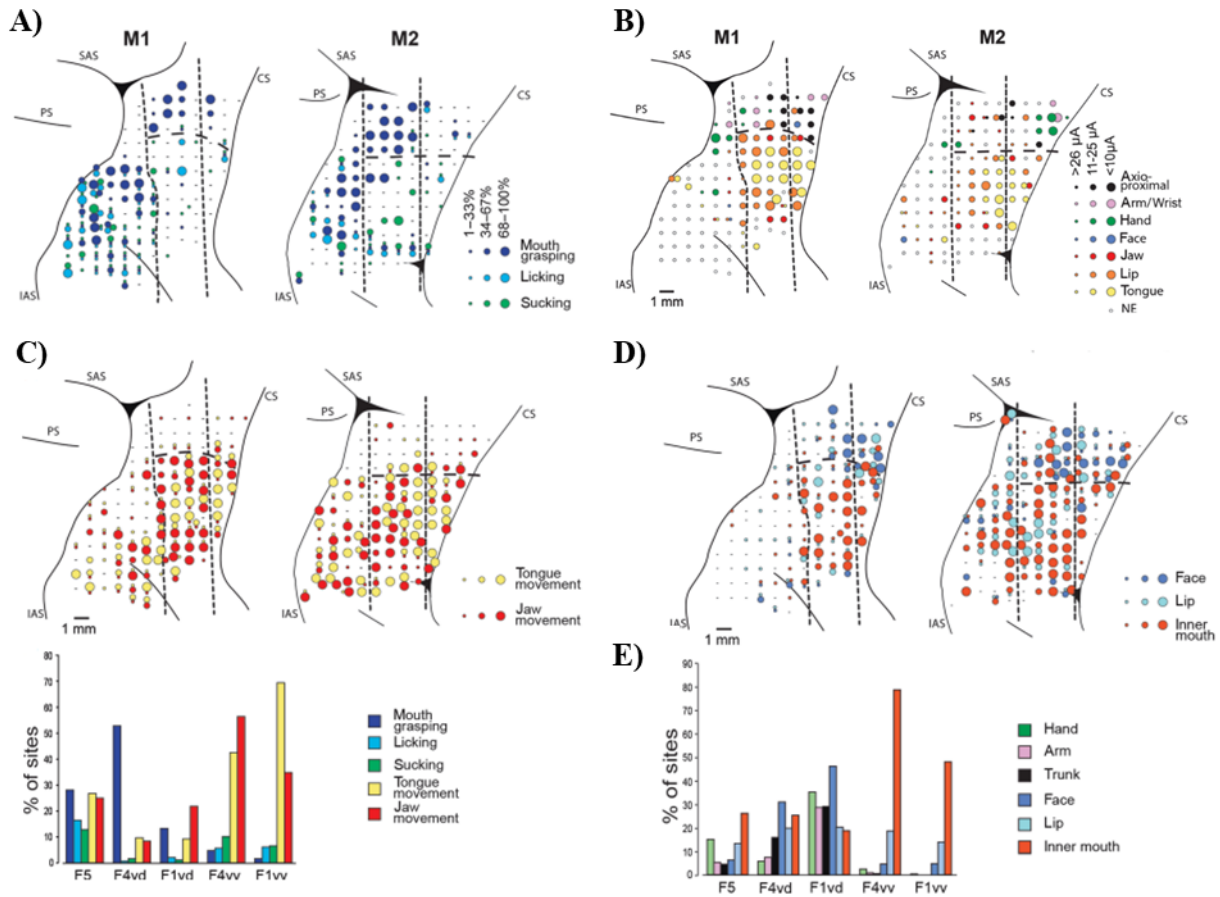
Figure 4. Stimulations and recordings in PMv and F1v as recorded by Maranesi et al., 2012. A) Localization of movements evoked by ICMS; circles' colors identify the activated body part, whereas the size of the circles represents the activation threshold. Small white circles represent penetrations not electrically excitable (NE) with the parameters employed in the study. B), C), D) 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. E) Proportion of the different tactile fields in the investigated areas (Maranesi et al., 2012).

1.6.3 Mouth acts representations in F5

Area F5 is located in the anterior portion of PMv. It was pointed out that this area is not uniform; on the contrary, it is composed by three subareas that can be separately identified based on their cytoarchitecture (Luppino et al., 1999). Specifically, the portion of F5 located into the cortical convexity is labeled as F5c, while F5a (anterior) and F5p (posterior) are located into the posterior part of the arcuate inferior sulcus (Belmalih et al., 2009; Rizzolatti & Sinigaglia, 2019).

Through studies of extracellular recording and intracortical microstimulation experiments of F5, researchers have successfully identified representations of hand and mouth movements. These

studies reveal a partial overlap of motor maps and comparing it with F4, F5 demonstrates lower excitability (Coudé et al., 2011; Maranesi et al., 2012; Rizzolatti & Gentilucci, 1988).

Another property of F5 is that the majority of its neurons do not encode individual movements; instead, they encode hand and mouth motor acts, defined as coordinated movements aimed at achieving a certain goal (Gentilucci et al., 1988; Rizzolatti et al., 1988; Rizzolatti & Gentilucci, 1988). These neurons are termed "purely-motor" and are deemed to the action final goal, the specific effector (left or right hand, or the mouth) used to achieve the goal (Bonini et al., 2011; Rizzolatti et al., 1998) or the precise sequence of muscle activation (Umiltà et al., 2008).

In F5 there are also visuo-motor neurons that discharge to both motor and visual inputs. They have been divided into two groups: "canonical neurons" and "mirror neurons". Canonical neurons exhibit responses to the execution of specific motor action towards an object, and also respond to the simple visual presentation of the same object. (Murata et al., 1997). Mirror neurons become active both during the execution of a certain action and when observing the same action performed by someone else (Gallese et al., 1996). Initial research proposed that mirror neurons exclusively existed in area F5c, while canonical neurons were located mostly in F5p. However, more recent studies (Bonini et al., 2014; Rizzolatti & Sinigaglia, 2019) revealed the presence of both neuron types in both areas. It's crucial to understand that these categories (purely-motor, canonical and mirror) should not be considered as strictly segregated, as a single neuron may express multiple functional properties (Bonini et al., 2014). During the recording of F5 mirror neurons' activity, Ferrari and his team observed not only the conventional mirror neurons but they also identified what they named "communicative mirror neurons". These neurons exhibit activation in response to communicative actions, such as lip-making and tongue protrusion, which are not directed toward an object. An example of the activity of these neurons can be seen in Figure 5 (Ferrari et al., 2003). This is important if we consider the fact that a subset of F5 neurons responds to voluntary vocalization's execution. This finding suggests a hypothetical role of this region in the origine of language as we know it in humans, considering the shared traits between F5 in macaque

monkeys and area 46 in humans. These traits include neural activation and motor maps, anatomical

location and participation in the mirror circuit (Coudé et al., 2011; Rizzolatti & Sinigaglia, 2006).

An intriguing aspect about this area is its inclusion of face and mouth movements and seems to be

linked to a communication purposes. Given that yawning is a mouth act and can also serve a

communicative function, it is conceivable that this area may play a crucial role in the cortical

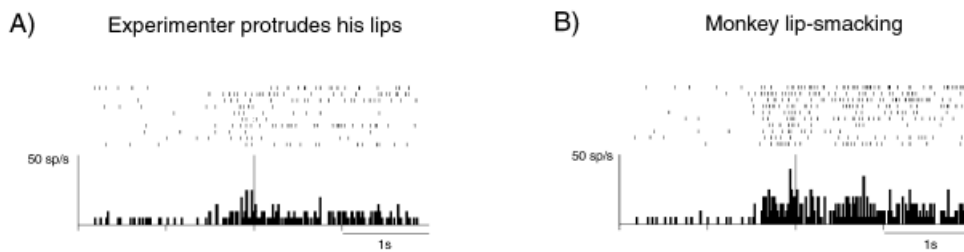control of this behavior.

Neuron 33

Figure 5. Example of communicative mirror neuron: Neuron 33, lips protrusion mirror neuron. (A) The experimenter protrudes his lips looking at the monkey; (B) during the experimenter lips protrusion the monkey respond almost simultaneously to the experimenter gesture by making a lip-smacking action.(Ferrari et al., 2003).

2. Aims of the study

Yawning has long been considered a stereotyped behavior whose respiratory motor patterns seems primarily orchestrated by subcortical structures. Nevertheless, yawning has greater complexity than commonly perceived, and despite its highly stereotyped nature, it can manifest in various forms influenced by endogenous and social factors, likely mediated by cortical influences, and can also be partially under voluntary control. Given the motor representation of facial and oral movements in the ventral Premotor Cortex (PMv) and the discovery of neurons with sensorimotor and social functional properties in this region, we hypothesize that PMv may host neurons relevant to top-down regulation of yawning.

To investigate this hypothesis, our first goal is to characterize PMv neurons based on their neural responses to yawning relative to other spontaneous mouth and mouth-forelimb behaviors used as controls. Due to the variability observed in yawning displays, we conducted the experiments in two distinct contexts: the NeuroEthoRoom (NER) and the home-cage during the night. Furthermore, we leveraged Intracortical Microstimulation (ICMS) to pinpoint the potential motor output of the recorded cortical sites, in order to proof their possible causal relevance in modulating yawning behavior.

3. Materials and methods

3.1 Ethical statement

All experimental protocols comply with the European (Directive 2010/63/EU) and Italian laws on the protection of animals used for scientific purposes (D.lgs 26/2014). They were approved by the Veterinarian Animal Care and Use Committee of the University of Parma and authorized by the Italian Ministry of Health.

3.2 Experimental subjects and surgical procedures

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

After training completion, each monkey underwent a first surgical intervention in deep anesthesia, followed by post-surgical pain medication, to implant the head fixation system (headpost). The headpost is a titanium cylinder with 4 feet shaped according to the 3D reconstruction of the cranial bone curvature based on a previously taken MRI scan of the monkey's head. Monkeys were prepared for the anesthesia with atropine administration (0.03 mg/kg) 15 minutes before the induction of anesthesia. Next, anesthesia was induced with ketamine (Lobotor, 4.5 mg/kg) and medetomidine hydrochloride (Domitor, 0.05 mg/kg) and maintained by inhaled isoflurane (IsoFlo, 100% p/p). Then, monkey's head was shaved, and skin and muscles were cut. Once the headpost was positioned and the screws fixed along the feet of the headpost, muscles and skin were separately sutured so that only the cylinder required to fix the monkey's head protrudes.

Finally, the monkey was awakened by administering atipamezole hydrochloride (Antisedan, 0.05 mg/kg), a synthetic α2-adrenoreceptor antagonist. A second operation was performed to implant 4 floating 32-channels microelectrode arrays (FMAs) in Mk1 and 6 in Mk2 (Figure 6A and C), to record the neural activity from the left premotor and motor cortices in both subjects. A third operation was carried out on Mk1 to implant six 32-channels FMAs on the right premotor cortex (Figure 6B). Surgical and anesthetic procedures were the same as the described above, but in this case a craniotomy was performed and a part of the brain (chosen according to magnetic resonance image) was exposed and the microelectrode arrays were positioned and slowly lowered in the cortical tissue. Dura mater was sutured, and the bone flap repositioned and fixed with dental cement and micro bone screws to the skull. The chamber was fixed to the skull with bone screws and dental cement and the Omnetics connectors positioned in their recess on top of it, before sealing it with the protective cap. The muscles and skin were sutured, and the monkey was awakened after the appropriate pharmacological treatment. The monkey was allowed to fully recover for three weeks before starting the neural recording sessions.
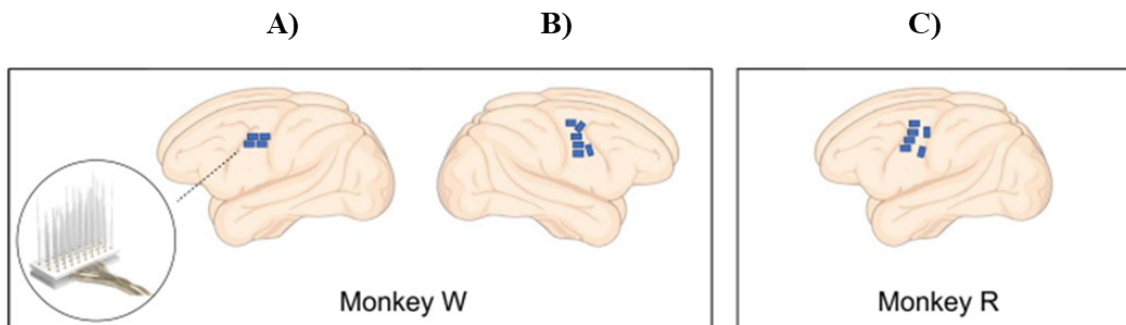

Figure 6. Schematic representation of the FMAs implanted in the premotor and motor cortices: A) left implant in Mk1; B) right implant in Mk1; C) right implant in Mk2.

3.3 Apparatus and experimental design: The NeuroEthoRoom and home-cage contexts

Especially in primates, we know that yawning can have different meanings and functions according to the context in which it is displayed (see for more details paragraph 1.4); considering that yawning is a spontaneous behavior, we created different contexts in which yawning could have different

purposes. In particular, we recorded the neural activity during yawning in two different conditions for both monkeys tested separately: a laboratory conditions and a home-cage condition. Although both monkeys were previously accustomed to the laboratory environment, there is still the possibility that it represents a stressful condition for the animals, providing an opportunity to explore instances of emotion-induced yawning, commonly referred to as stress yawning. The second condition involved neural recording at late afternoon and night inside the home-cage of the monkeys, providing an opportunity to study yawning associated to periods of transitions between wake and rest states.

The recordings required the execution of species-specific behavior during unconstrained freely moving conditions in the NeuroEthoRoom (NER) and inside the home-cage.

Figure 7. Picture of the NER where all the equipment is visible: the set of 8 cameras, the two large doors with the small opener that allows the monkey to enter, climbing holds, hooks on the roof and on the floor the places where the wooden structure is mounted.
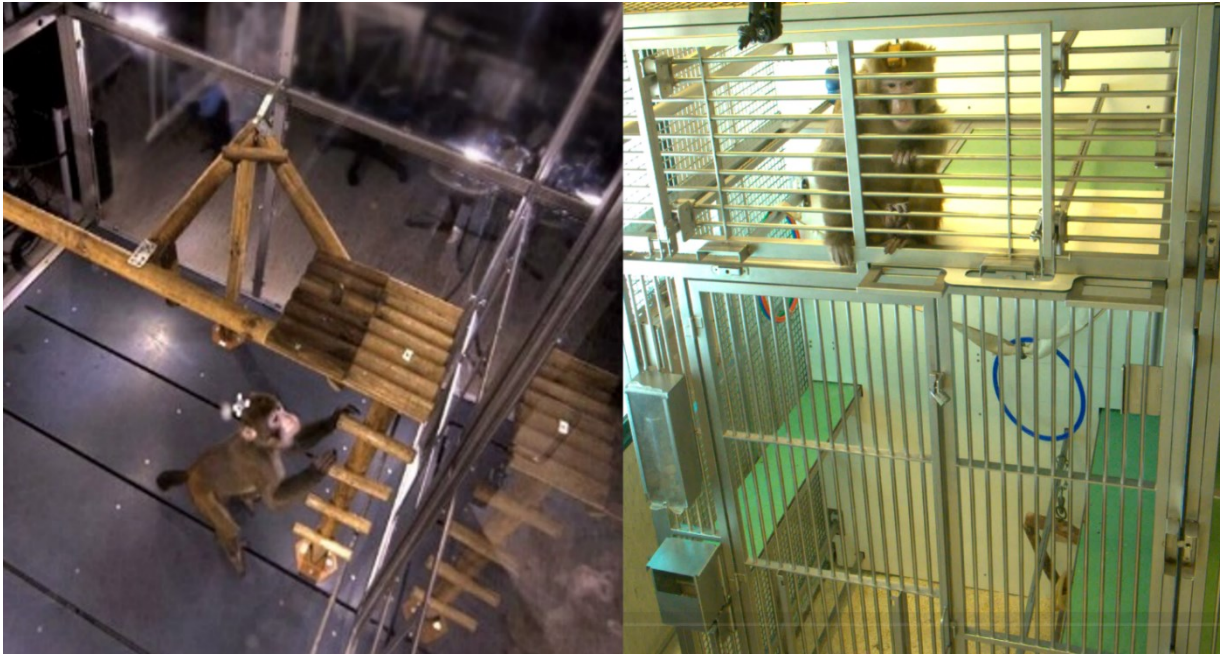
Figure 8. The picture on the left represents Mk1 inside the NER with the wooden structure mounted, while the picture on the right represents Mk2 inside its home-cage.

The NER is 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 (Figure 7). 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 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, or vice versa at the end of the session. In the NER we used different enrichment items to create an ecological condition in order to elicit spontaneous species-specific behaviors. In particular, we mounted climbing holds on the walls and a wooden structure through which the monkey could climb, walk and rest. To elicit foraging the experimenter could introduce food from the outside by means of openings of the NER walls, or by nylon-threaded hooks hanging from the roof. Moreover, the transparent plexiglass walls allowed the experimenter to interact with the monkey and induce communicative affiliative (e.g. lip-smacking) or aggressive (e.g. threat) behaviors. All behaviors (spontaneous or induced by stimulus presentation) were recorded in both the NER and the home-cage conditions. The only difference

between the two conditions, is represented by yawning occurring during the night. Pictures of both conditions can be seen in Figure 8.

3.4 Behavioral data acquisition and analysis

3.4.1 Video acquisition in the NER

Monkey behavior was recorded through with a system of eight high resolution synchronized cameras placed around the NER (see Figure 7). We used 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 Video acquisition in the home-cage

The experimental apparatus was composed of three portable computers, an infrared camera (Basler acA2040-120um, 2048x1536 pixels resolution) used to record the behaviors in all tasks and an audio interface (Behringer UMC-404HD) that received audio signals from a pair of stereo microphones (Thomann tbone sc140). The system was controlled via NIMH MonkeyLogic (Hwang et al., 2019), a MATLAB based package. Via an USB National Instrument DAQ board (NI USB-6001) the software sent: transistor-transistor logic (TTL) signals to the neural activity recording software via a transceiver and a 5V square wave with a frequency of 100Hz to the video recording

system. For the purposes of the video recordings, two infrared LED lights were mounted during the acquisition at 2 meters from the cage.

3.4.3 Ethogram

The ethogram used for behavioral analyses was defined considering the functional properties of the ventral premotor recorded region (see Introduction, paragraph 1.6). We considered a total of 28 behaviors categorized as instantaneous events (point events) and operationalized as described in Table 1 and 2.

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. 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. We also included all the parameters that could change in a yawning display: we tracked yawns that were performed in a laying or in a sitting position and we also considered if there was a display of the canines or not.

We also recorded and described mouth behaviors such as mouth grasp, sucking and chewing (respectively throughout giving liquid and solid reward to the monkeys) to differentiate neurons with a yawn selectivity from those discharging during several mouth movements.

Finally, we included forelimb-mouth behaviors such as bringing the food to the mouth as controls in order to distinguish neurons that respond to both mouth and hand actions. For these behaviors we considered the ones executed with the forelimb contralateral to the implanted hemisphere.

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Yawning behaviors

</div>

Behavior	Event type	Operational description
Yawn start laying	Point event	The monkey is yawning. Track the frame when the mouth starts to open. The monkey is in a laying position.
Yawn peak laying	Point event	The monkey is yawning. The mouth is at the maximum opening. Track the frame before the monkey begins to close the mouth. The monkey is in a laying position.
Yawn stop laying	Point event	The monkey is yawning. Track the frame when the mouth is completely closed. The monkey is in a laying position.
Yawn start sitting	Point event	The monkey is yawning. Track the frame when the mouth starts to open. The monkey is in a sitting position.
Yawn peak sitting	Point event	The monkey is yawning. The mouth is at the maximum opening. Track the frame before the monkey begins to close the mouth. The monkey is in a laying position.
Yawn stop sitting	Point event	The monkey is yawning. Track the frame when the mouth is completely closed. The monkey is in a sitting position.
Yawn start teeth	Point event	The monkey is yawning. Track the frame when the mouth starts to open. The yawn is done with a full display of the canines.
Yawn peak teeth	Point event	The monkey is yawning. The mouth is at the maximum opening. Track the frame before the monkey begins to close the mouth. The yawn is done with a full display of the canines.
Yawn stop teeth	Point event	The monkey is yawning. Track the frame when the mouth is completely closed. The yawn is done with a full display of the canines.

Yawn start no teeth	Point event	The monkey is yawning. Track the frame when the mouth starts to open. The yawn is done only with the opening of the mouth and without a display of the teeth.
Yawn peak no teeth	Point event	The monkey is yawning. The mouth is at the maximum opening. Track the frame before the monkey begins to close the mouth. The yawn is done only with the opening of the mouth and without a display of the teeth.
Yawn stop no teeth	Point event	The monkey is yawning. Track the frame when the mouth is completely closed. The yawn is done only with the opening of the mouth and without a display of the teeth.

Table 1. Complete list of the 12 yawning epochs of interest, each one scored as a point event, followed by the operational description used for the scoring.

Control behaviors

Behavior	**Event type**	**Operational description**
Threat start	Point event	The monkey is threatening the experimenter. Track the first frame the mouth opens during the threat.
Threat peak	Point event	The monkey is threatening the experimenter. Track the frame before the monkey starts to close the mouth after its maximum opening.
Threat stop	Point event	The monkey is threatening the experimenter. Track the frame the mouth is completely closed.
Bring to mouth L start	Point event	The monkey is foraging and actively places food pieces into the mouth with the left hand. Track the first frame the mouth is starting to open while the monkey is bringing the

34

		food to the mouth.
Bring to mouth L	Point event	The monkey is foraging and actively places food pieces into the mouth with the left hand. Track the frame the food is completely into the monkey's mouth; the left hand is attached to the monkey's mouth.
Bring to mouth L stop	Point event	The monkey is foraging and actively places food pieces into the mouth with the left hand. Start the frame when the left hand is no longer in contact with the mouth after the act of placing the food into the mouth.
Bring to mouth R start	Point event	The monkey is foraging and actively places food pieces into the mouth with the right hand. Track the first frame the mouth is starting to open while the monkey is bringing the food to the mouth.
Bring to mouth R	Point event	The monkey is foraging and actively places food pieces into the mouth with the right hand. Track the frame the food is completely into the monkey's mouth; the right hand is attached to the monkey's mouth.
Bring to mouth R stop	Point event	The monkey is foraging and actively places food pieces into the mouth with the right hand. Start the frame when the right hand is no longer in contact with the mouth after the act of placing the food into the mouth.
Sucking open	Point event	The monkey receives passively liquid reward (juice or water) given from the experimenter by means of a syringe. Track the first frame the mouth opens while the monkey is approaching the syringe.
Sucking closure	Point event	The monkey receives passively liquid reward (juice or water) given from the

		experimenter by means of a syringe. Start the frame when the monkey's lips are completely closed on the syringe.
Sucking stop	Point event	The monkey receives passively liquid reward (juice or water) given from the experimenter by means of a syringe. Start the frame when the monkey has stopped the act of sucking, therefore the syringe is no longer in contact with the lips.
Biting open	Point event	The monkey receives passively solid reward (food pieces) given from the experimenter by means of a stick. Track the first frame the mouth opens while the monkey is approaching the stick.
Biting closure	Point event	The monkey receives passively solid reward (food pieces) given from the experimenter by means of a stick. Start the frame when the monkey's lips are completely closed on the solid reward.
Biting stop	Point event	The monkey receives passively solid reward (food pieces) given from the experimenter by means of a stick. Start the frame when the monkey has taken the solid reward and moves the head away.
Mouth grasp	Point event	Monkey eats food with its mouth (it doesn't pick it up with hands). Track the frame when mouth and food get in contact.

Table 2. Complete list of the 16 control behaviors of interest, each one each one scored as a point event, followed by the operational description used for the scoring.

3.4.4 Behavioral scoring

All videos from both conditions 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 described in Table 1 and 2 in order of appearance, each one with the precise timestamp at which they occurred.

3.4.5 Kinematics analysis

Yawning is a complex synergic behavior that implies enormous movements of mouth and head if compared with other mouth behaviors. Thus, we tracked, by means of a dedicated software (Simi Reality Motion Systems GmbH, Unterschleissheim, Germany) the mouth opening and the head elevation kinematics to investigate if neurons' responses to yawn could account for this low-level kinematics parameters. In particular, we used a customized head-post cover equipped with 4 retroreflective markers to track the monkey's head position in the NER in each yawn occurrence. The same procedure was also used to track the mouth opening during yawning marking the upper and lower lips.

The final output of SIMI Motion Capture was a set of 3D coordinates for the front and back markers and for the upper and lower lips. For the mouth kinematics, we calculated the Euclidean distance between upper and lower lip in every tracked frame (measured in centimeters). For the head kinematic we calculated the degrees of head elevation (Figure 9).

After assessing the normality of the distribution using the Royston test, we investigated the potential correlation between the neurons peak firing rate and the maximum mouth opening and

head elevation during yawning. In each yawn occurrence, we extracted the maximum values of mouth aperture and head elevation, and the peak firing rate of neurons within a specific time window: 500ms prior to the start of the yawn until its conclusion. We then correlated for each neuron the peaks in firing rate with the corresponding value of maximal mouth aperture and maximum head elevation in each yawn occurrence (*values correlation*).

Given the long nature of yawning and the possibility of neuronal responses occurring prior and subsequently to the peak of the head and mouth kinematics, we also carried out correlation between the time at which the peak firing rate occurred (in each yawn occurrence), and the time of the maximum mouth opening and head elevation *(time correlation)*. In cases where there were multiple peaks in the firing rate within one trial, we considered the peak closest to the mean peak of the firing rate among yawn occurrences in that session. Subsequently, to mitigate the risk of family-wise error rate, we applied the Bonferroni-Holm method (1979) to correct the p-values for the number of comparisons. Finally, to statistically determine if there was a correlation between the peak firing rate and both head and mouth kinematics, we used the Kendall rank correlation coefficient. Specifically, we used the critical values for Kendall's rank correlation coefficient tau according to our α level and sample size ($\alpha = 0.05$; N = 5-20) (Kendall, 1938).
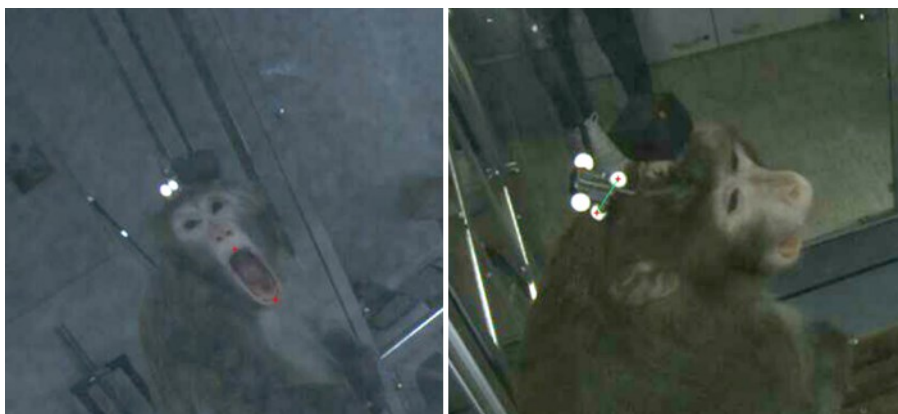

Figure 9. In the left picture is represented a video frame showing an example of the tracking of the up and lower lip of the monkey while yawning; in the right picture is represented a video frame showing an example of the tracking of the front and back retroreflective markers.

3.5 Neural data acquisition and analysis
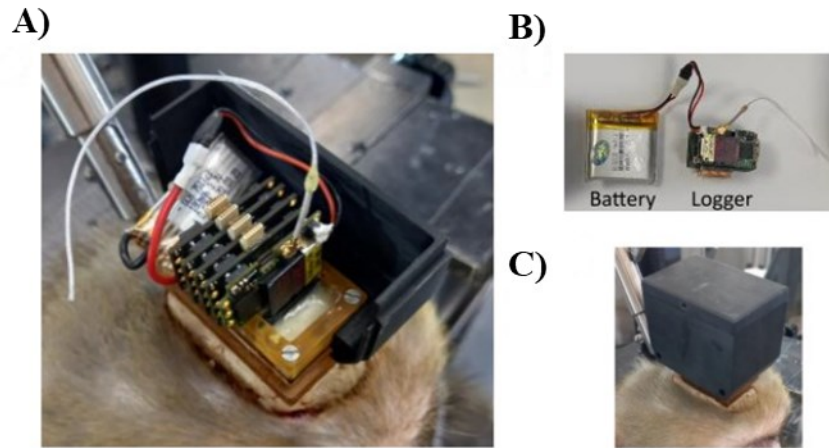
3.5.1 Neural activity recordings

Figure 10. The recording chamber divided in its components. A) the recording chamber open; B) the battery connected with the recording system (RatLog-128 from Deuteron technologies); C) the cover sewed around recording components.

The neural recordings were carried out through 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. For each channel, the neural signal was grounded and referenced using dedicated low impedance electrodes in each FMA and recorded at a conversion rate of 32 kHz with a bandpass filter ranged 2-7000Hz, thereby being able to sample not only Single (SUA) and Multi-unit (MUA) activity, but also Local Field Potentials (LFP). Neural signals were amplified, digitized, and stored in a MicroSD memory card to prevent any possible transmission error. The device was powered by a small external battery placed inside the cover. 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 (Figure 10). 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.

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 SUA or MUA used a noise overlap threshold of 0.15. Units were distinguished into SUA and MUA using the noise overlap, a parameter that can vary between 0 and 1, with units with a value below 0,1 considered as SUA, while all the remaining waveforms with higher noise overlap considered MUA. In addition, visual inspection of the ISI (Inter Spike Interval) distribution and the waveform shape was conducted to further verify the isolation of single-units, and to remove possible artefacts.

3.5.3 Single units' selectivity

After units' classification and behavioral scoring, we studied the firing of the recorded units in relation to the behaviors of interest that occurred at least 7 times. To investigate the single neuron's modulation, we defined a 3 s epoch, ranging from -1.5 s to 1.5 s around the behavior of interest, and binned the activity in 250 ms windows not overlapping. One-way repeated measures ANOVA was used to evaluate a possible main effect of the factor Epochs (250 ms), thus revealing a significant modulation of the neuron for the behavior of interest.

3.5.4 ICMS

After the conclusion of the recording sessions, Intracortical Microstimulation (ICMS) experiments were carried out on the left FMAs implant of Mk1. Monopolar, biphasic trains of cathodic square wave pulses were administered using a constant current stimulator (PlexStim, Plexon) with the following parameters: total train duration of 500 ms, single pulse width of 0.2 ms, and pulse

frequency of 200 Hz. Current intensity (100 or 150 µA) was monitored on an oscilloscope by measuring the voltage drop across a 10-kΩ resistor in series with the stimulating electrode. During these procedures, ICMS was administered at each site while the monkey was in a calm and relaxed state. The monkey was situated in a primate chair without any head fixation. The detection of induced movements relied on the consensus of two experimenters observing the animal during pulse delivery. The stimulation protocol started with a current intensity of 100 µA for 500 ms, then increased to 150 µA for 500 ms. If each stimulation administered at the same intensity reliably elicited the same movement (typically three out of three consecutive stimulations with the same parameters), then the site was considered associated with a specific effector. At the conclusion of the stimulations, two maps corresponding to current intensity were generated where each site was associated with the single or multiple effector movements evoked. To compare the functional properties and the results obtained through ICMS, we applied a $\chi2$-test to investigate if there was a significant distribution of yawn-responsive and no-yawn neurons among the excitable and non-excitable sites. Then, through the same test, we evaluated the presence of a significant association between the FMAs' channels where yawn-related neurons were identified and those where motor responses of specific effectors were evoked via ICMS.

4. Results

4.1 Single units' activity

The analyses were based on 8 sessions recorded in the NER, during which the two monkeys exhibited a total of 93 yawns, along with other behaviors previously outlined in the ethogram (see Table 1 and 2 for a comprehensive list of behaviors).

We isolated a total of 236 single units and categorized them into three classes using the one-way repeated measures ANOVA (see paragraph 3.5.3) in order to identify modulated neurons. We defined as "yawn-selective" neurons exhibiting significant modulation exclusively during yawning and not for other behaviors such as threat, sucking, biting and bringing to mouth (example in Figure 11A). Neurons were considered "yawn-related" if there was a significant modulation for yawning and at least one other behavior (example in Figure 11B). Lastly, we categorized neurons as "no-yawn" if the significant modulation did not involve yawning but did include at least one of the other behaviors (example in Figure 11C). In the following paragraphs we will label both yawn-selective and yawn-related neurons as "yawn-responsive" neurons when considered together.
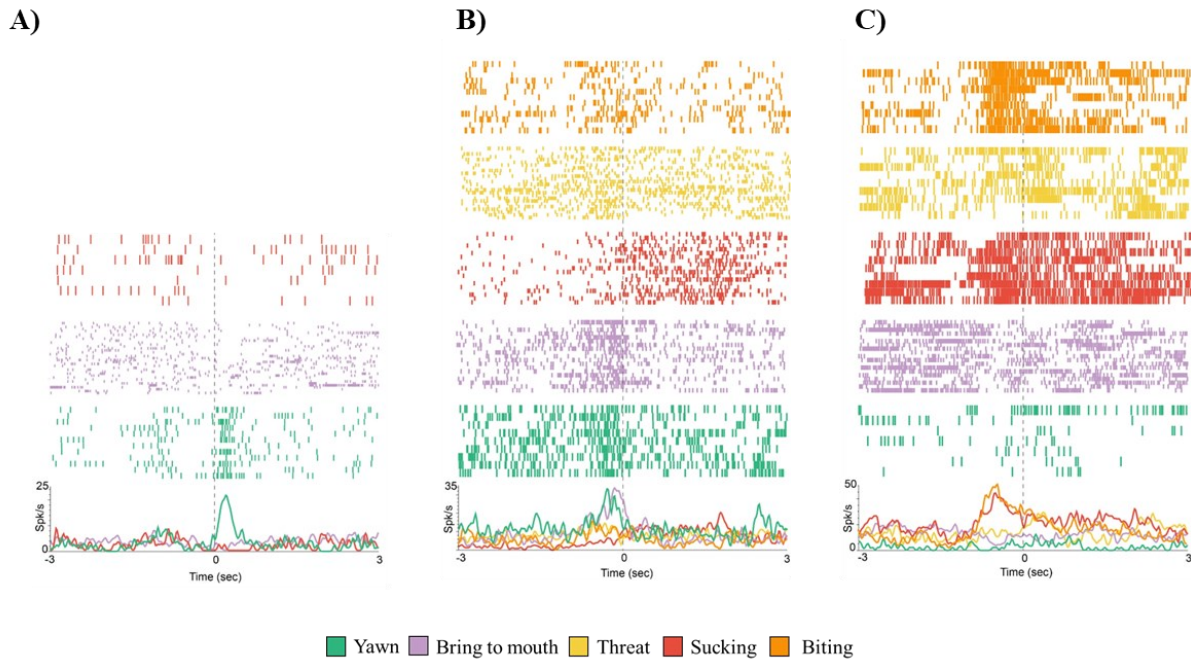
Figure 11. Examples of neurons for each of the three identified classes are shown. Each curve represents the mean firing rate of the neuron among all trials of a behavior (behaviors are indicated in the legend) for which it was tested throughout the entire session. A) yawn-selective neuron exhibiting a significant response solely during yawning. B) yawn-related neuron exhibiting significant responses to yawning and bringing to mouth. C) no-yawn neuron exhibiting significant responses to sucking and biting. Each behavior is represented by its central moment: for yawning and threat, the peak of mouth opening is plotted; for sucking and biting, the moment of mouth closure around the syringe/food is depicted; for bringing to mouth, the instant when the mouth opens as the hand approaches is shown.

Out of the total number of neurons identified, 147 neurons (62%) responded to at least one of the behaviors of interest (Figure 12). Except for the yawn-selective neurons that exhibited a significant response only during yawning, neurons in the other two classes showed various response patterns to different combinations of behaviors. For both yawn-related neurons (n = 54) and no-yawn neurons (n = 61), Table 3 lists all the combination of behaviors to which a certain set of neurons was modulated, namely, yawning (Y), threat (T), sucking (S), biting (B), bringing to mouth (BM).
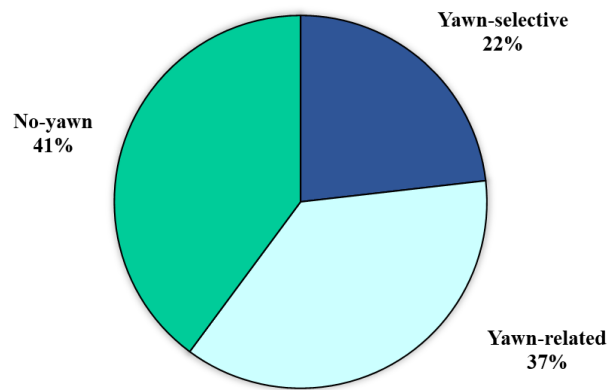
Figure 12. Pie chart representing the distribution of neurons across the three identified classes based on their response (N = 147).

YAWN-RELATED		NO-YAWN	
Behaviors	**N of neurons**	**Behaviors**	**N of neurons**
Y+S	21	S	13
Y+S+BM	10	S+BM	10
Y+S+B	6	BM	9
Y+S+B+BM	6	B	7
Y+T+S+B+BM	4	S+B	5
Y+BM	3	T+S+B+BM	5
Y+B	1	S+B+BM	4
Y+T+S	1	B+BM	3
Y+T+S+BM	1	T	1
Y+T+S+B	1	T+S	1
		T+BM	1
		T+S+BM	1
		T+S+B	1

Table 3. Yawn-related neurons are listed on the left, while no-yawn neurons are on the right. The table shows the number of units displaying significant modulation for each recorded combination of behaviors. Behaviors are listed in descending order, starting from the combinations with the highest number of responsive neurons and ending with those with the fewest.

4.2 Yawn's kinematic analysis

In order to determine if cortical responses to yawning could be accounted for some low-level kinematics parameters, for a subset of the sessions (4 out of 8), we analyzed yawning kinematics by tracking both the mouth opening and head elevation components (Figure 13). Due to visibility issues arising from the freely moving conditions, it was possible to track head elevation for all 4 sessions, while mouth opening only for 3 of them. Then, we could analyze the possible correlation between the yawn kinematics aspects and the neurons firing rate on 67 yawn-responsive neurons for head elevation and 64 for mouth opening (out of 87) and testing the possible correlation between the kinematics aspects of yawning and the firing rate of the neuron.
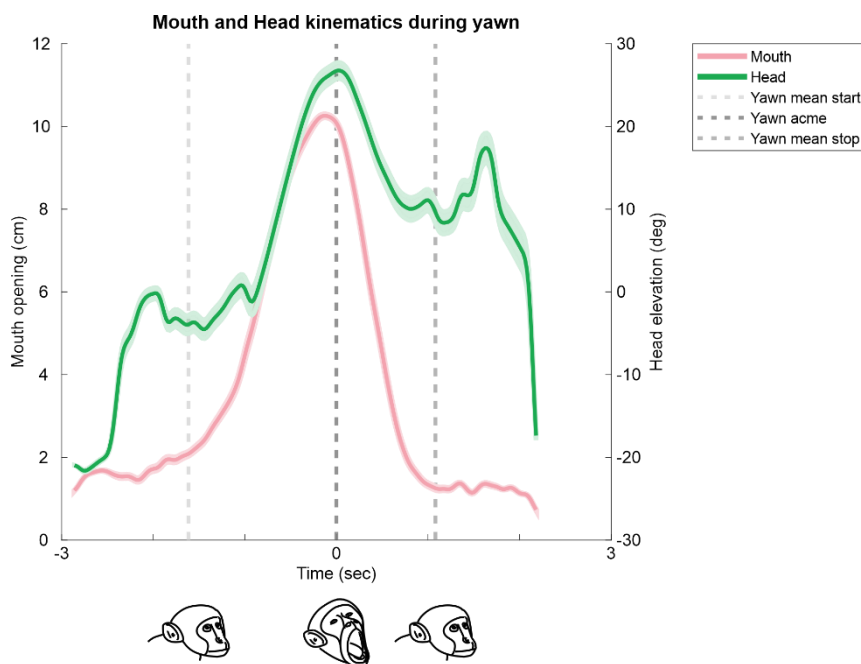

Figure 13. Graph showing both head and mouth kinematics during yawning. The peak of both mouth opening and head elevation represents the central moment of the yawning behavior, indicated by the 0 on the x-axis. On the right y-axis the degrees of head elevation are reported: 0 degrees correspond to the straight position of the head, positive values indicate the lifting of the head, negative values its lowering.

To statistically determine if there was a correlation between the peak firing rate and both head and mouth kinematics, we used the Kendall rank correlation coefficient. Specifically, we used the critical values for Kendall's rank correlation coefficient tau according to our α level and sample size ($\alpha = 0.05$; N = 5-20) (Kendall, 1938). We identified 5 neurons whose firing rate correlated with

the degree of head elevation (3 yawn-selective neurons and 2 yawn-related neurons), and 3 other whose firing rate correlated with the extent of mouth opening (2 yawn-selective neurons and 1 yawn-related neuron). Since the analysis did not consider the temporal relationship between the maximal firing rate and the maximal mouth opening and head elevation, we also investigated the possible correlation between these two variables and found that only one yawn-selective neuron exhibited a significant correlation between the timing of peak activity and the timing of head elevation, and another neuron whose firing rate correlated with the timing of mouth opening. The p value of these few correlated pairs of variables (both positively and negatively correlated), however, did not passed the Bonferroni-Holm method to correct for the family-wise error rate probably supporting the idea that they appeared randomly (examples of neurons in Figure 14). This indicates that the magnitude and timing of mouth aperture or head elevation could not explain the observed modulations.
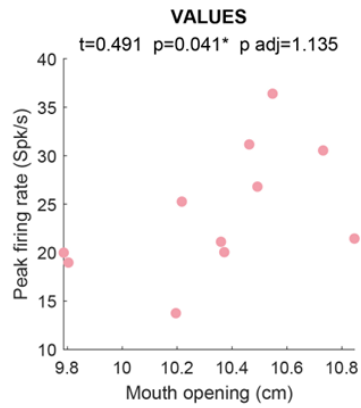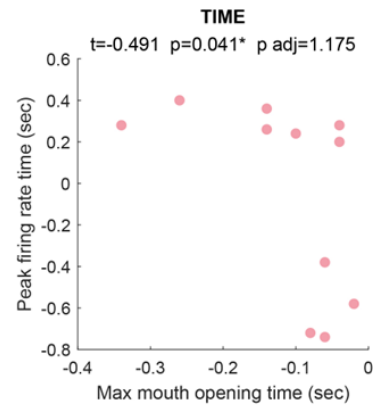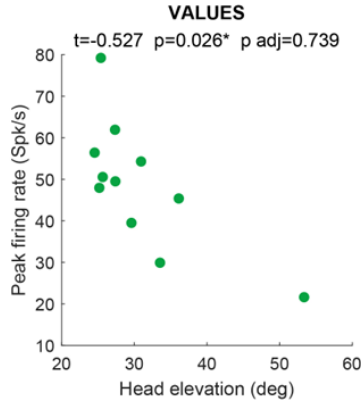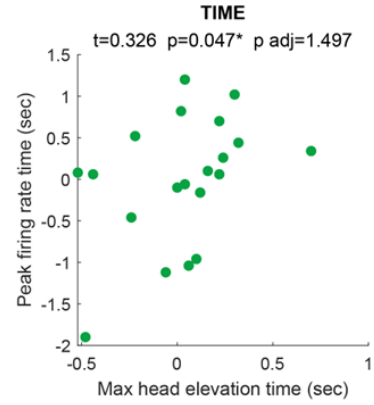
Figure 14. Scatter plots representing examples of neurons that showed a correlation between the peak firing rate and mouth/head value and time correlations. In all four cases the correlations disappear considering the adjusted pvalues.

4.3 Contextual modulation of neuronal responses to yawning

Having demonstrated the presence of neurons modulated, sometimes even selectively, during yawning in the ventral premotor cortex, next we wanted to investigate if neurons can exhibit different modulations to yawning in different contexts. Indeed, in 3 sessions from which we could isolate a total of 48 neurons, neural activity was recorded when monkeys were both in the NER and in their home-cage and we obtained a total of 61 yaws (25 occurred in the NER and 36 in the home-cage). Among the 48 neurons, 17 yawn-responsive neurons were identified: 10 showed a response to yawning exclusively in one context (example in Figure 15A) and 7 displayed similar responses in both contexts (example in Figure 15B).

A)

B)

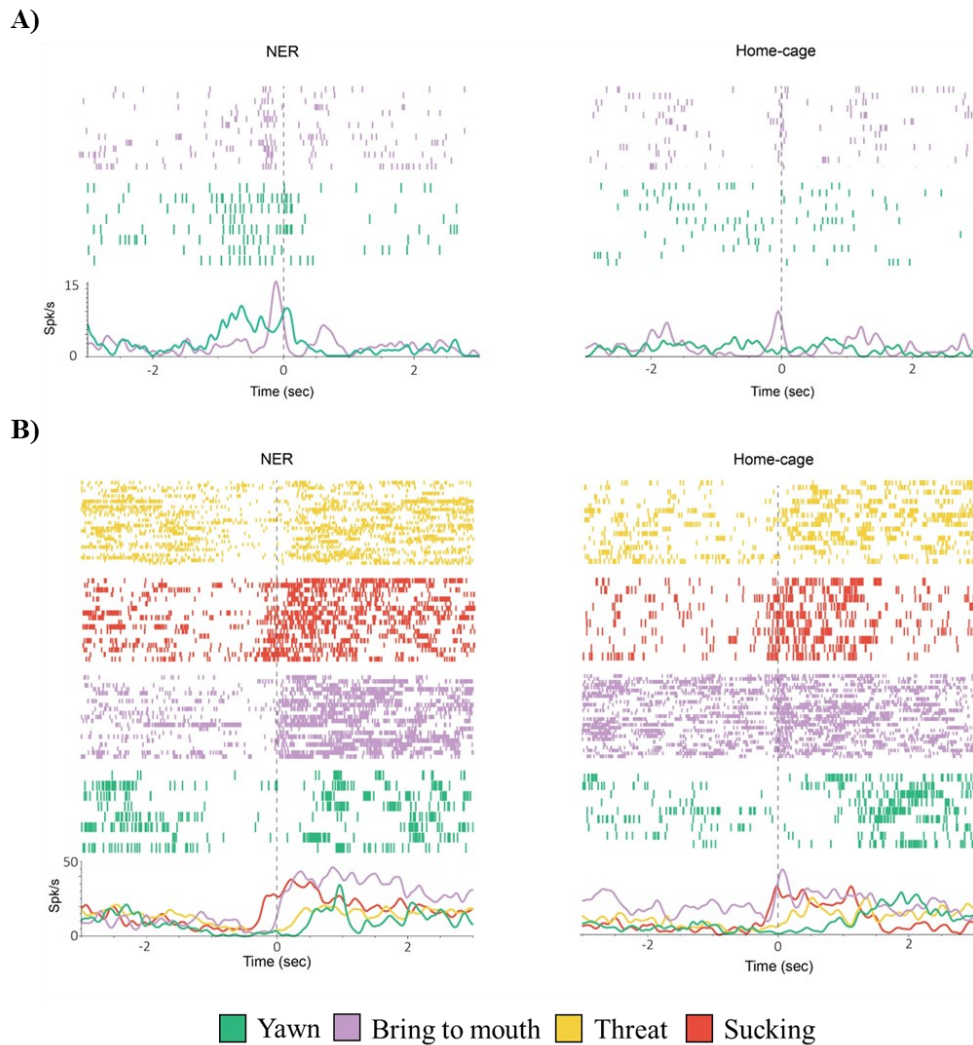🟩 Yawn ⬜ Bring to mouth 🟨 Threat 🟥 Sucking

Figure 15. Examples of neurons recorded in both the NER and the home-cage. Rasters and polyline of significant responses to behaviors are plotted. The lines represent the mean of the firing rate in all trials of the behavior, while rasters represent the neuron's spikes in each trial of the behavior.
A) Example of a neuron differently modulated in the two contexts: in the NER, there is a significative response to both yawning and bringing to mouth, while in the home-cage the neuron does not respond to yawning but still responds to bringing to mouth.
B) Example of neuron with similar response across the two contexts: the response appeared suppressed during yawning, while excited during the other behaviors in both contexts.

   The NER could be a more stressful environments than the home-cage, thereby making plausible that yawning may be displayed differently (e.g. displaying canines) in the two contexts, subserving different functions and resulting from different triggering mechanisms. Thus, we investigated possible differences in the yawning display that could explain the different modulation of some neurons depending on the context. Specifically, we first analyzed the display of canines and we found that in the NER, all yawning occurrences (except two) were accompanied by a display of the canines. In contrast, in the home-cage, the majority of the yawns occurred without a display of the canines (teeth = 36%; no teeth = 64%) and this differential distribution across the two contexts

was significant ($\chi2 = 19,05$; $p < 0.001$). Another possible variable is the posture assumed during yawning: all yawns in the NER were performed in a sitting position, whereas in the home-cage during night there was more variability (sitting = 39%; laying = 61%) and this different distribution across contexts was significant ($\chi2 = 23,90$; $p < .001$) (Figure 16).
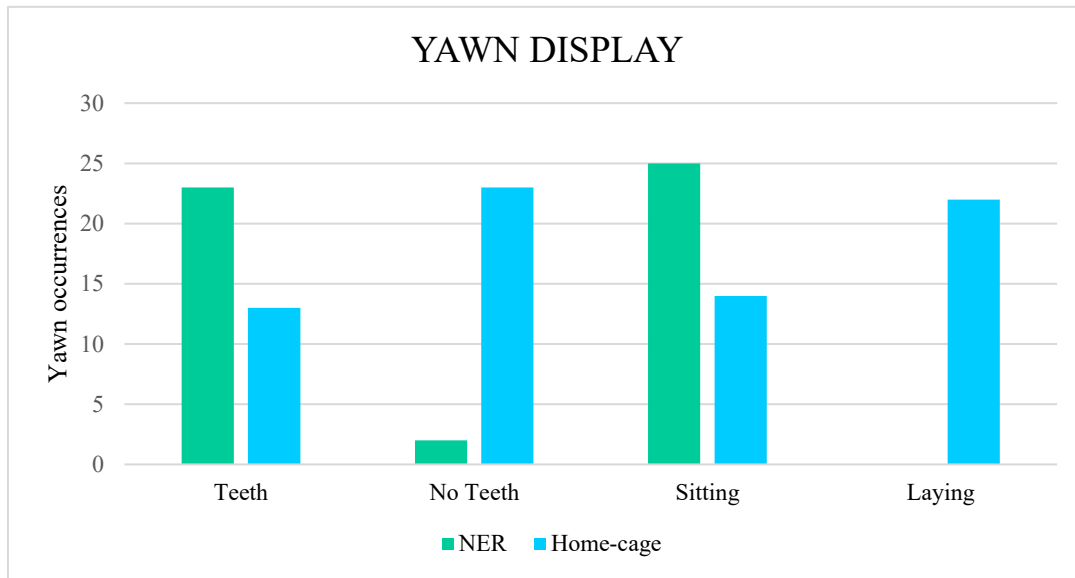

Figure 16. Histogram representing the yawn display in the two conditions: NER and home-cage. A yawn could be performed with or without a display of the canines and in a sitting or laying position.

	NER		HOME-CAGE	
	Mean (sec)	**sd**	**Mean (sec)**	**sd**
Teeth	2,04	0,55	2,39	0,50
No teeth	1,61	0,18	1,68	0,55

Table 4. Table presenting the average durations and standard deviations of yawns categorized by the context and teeth exposure.

As differences in yawning behaviors (teeth/no teeth, sitting/laying) might reflect potential variations in the communicative purposes of yawning, it is essential to consider not only the manner of display, but also the duration of yawning (see paragraph 1.2 and 1.4). Therefore, we have examined the duration of various types of yawning in different contexts to try to link display variances with potential differences in communicative intent, possibly explaining the differential neuronal responses we found. Table 4 and Figure 17 show the yawn's mean durations based on teeth display and on the context in which they were performed.
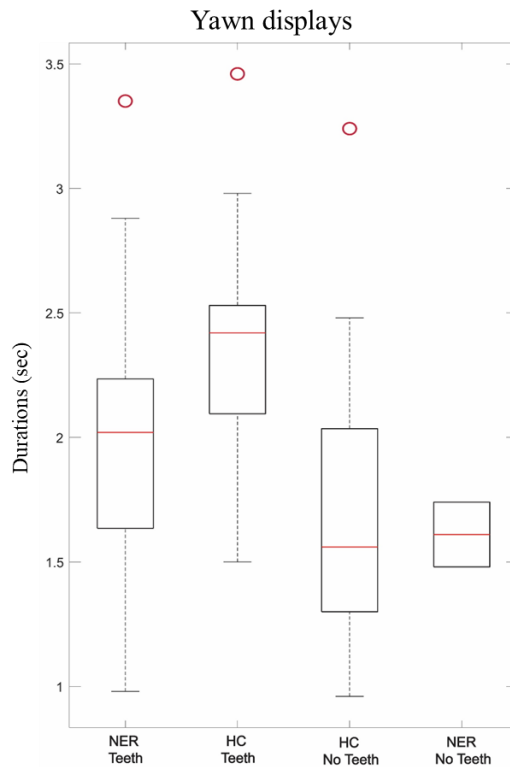
Figure 17. Boxplot representing durations of yawns divided into four groups: two performed in the NER and two in the home-cage context. Each context includes a category with teeth exposure and another without teeth exposure.

We then conducted a two-way ANOVA to investigate whether yawning durations are significantly affected by the context, teeth exposure, or a combination of both factors according to Palagi's classification (Zannella et al., 2021). Interestingly, the analysis (F = 6,79; p = 0,0117) reveals that the presence or absence of teeth exposure is the only significant factor impacting on yawning duration.

Since neurons that respond only in a specific context mainly do so in the NER where yawns are consistently performed with teeth exposure, we addressed whether the different neural responses observed in the two contexts could be linked to this difference in yawn display. Thus, in one session, where we had a reasonable number of yawn occurrences with teeth exposure in both contexts, we directly compared neuronal responses in these trials in both contexts. Out of a total of 19 neurons, we identified 5 yawn-responsive neurons: 4 exhibited a significant response to yawning only in the NER (example in Figure 18A), while one neuron responded to yawning in both contexts

(example in Figure 18B). Among the 4 neurons showing differential responses, we investigated whether the disparity was due to the teeth exposure. Interestingly, apart from one neuron, there was no significant response to trials involving teeth display in the home-cage.

We also examined whether neurons exhibited a different response during yawning displayed in different postures: sitting position in both the NER and the home-cage and laying posture only in the home-cage. Our analysis revealed no significant response during yawning to the sitting position in the home-cage (although there was a significant response to this posture in the NER, where every yawning trial was performed while sitting) (example in Figure 18C).
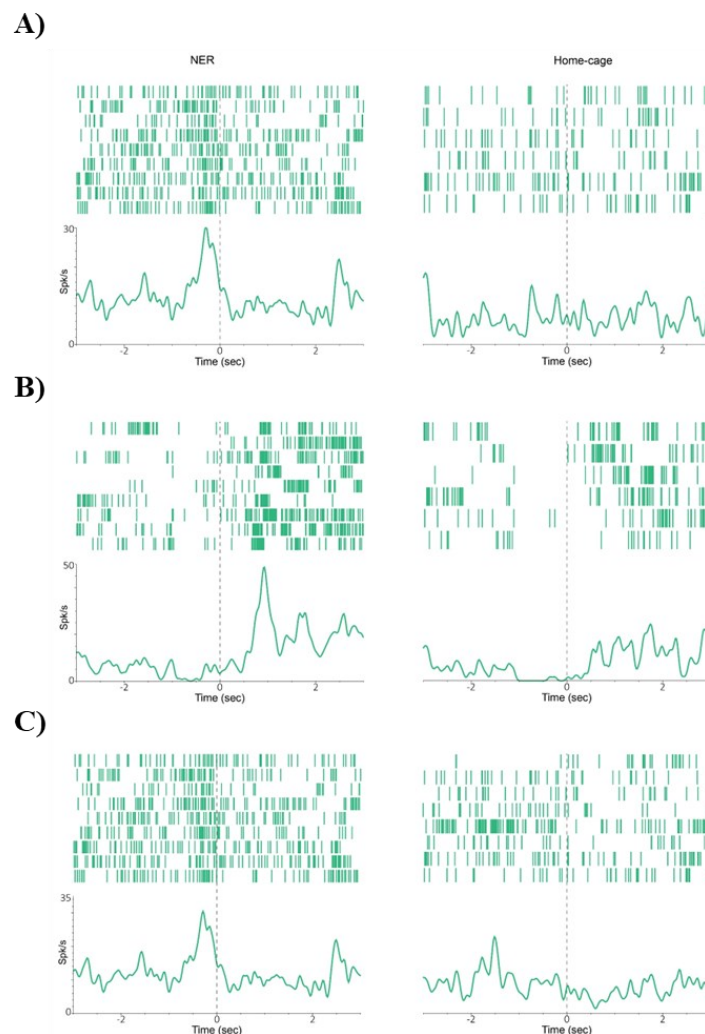

Figure 18. Examples of neurons: A) Neuron exhibiting a different response to yawn conducted with teeth exposure across the two contexts; B) Neuron responding during yawn with teeth exposure in both contexts; C) Neuron discharging only in the NER during yawns done in the sitting position.

4.4 Movements evoked by Intracortical Microstimulation (ICMS)

After single neuron recording sessions, we performed ICMS experiments in one monkey (Mk1). Out of the 67 stimulation sites, movements of at least one effector were evoked in 48 sites (72%) through current intensity of 100 µA, and in 58 sites (87%) through current intensity of 150 µA. In the remaining sites, no movements were observed. Figure 19 shows the cortical location of excitable sites in terms of the effector activated by ICMS matched with the presence of neurons with different functional properties (i.e. yawn-responsive and no-yawn neurons). To test for a possible association between neurons functional properties and electrical excitability, we carried out a $\chi 2$-test that firstly revealed us that the proportion of yawn-responsive and no-yawn neurons among the excitable and non-excitable channels is not statistically different at both current intensities. Considering only the excitable sites at 100µA, we found a significantly higher proportion of sites with yawn-responsive neurons where ICMS evoked mouth movements ($\chi 2$ = 11,77; p = 0,0006), and a higher proportion of no-yawn neurons where ICMS evoked head movements ($\chi 2$ = 12,3; p = 0,0005). See Table 5 for all the comparisons.

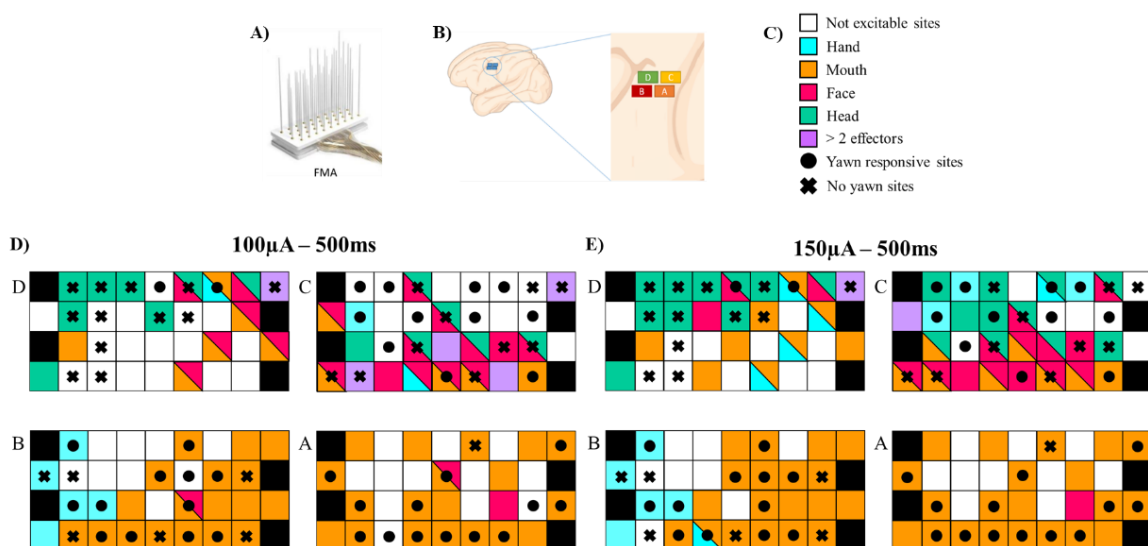*Figure 19. A) Reproduction of the FMAs; B) Schematic representation of the four probes of the implant along with their cortical locations; C) Legend; D) Schematic representation of the four probes that underwent stimulation during the ICMS procedure with the parameters of 100 µA for 500ms; E) Schematic representation of the four probes that underwent stimulation during the ICMS procedure with the parameters of 150 µA for 500ms.*

100µA – 500ms	Excitable		Head		Face		Hand		Mouth	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Yawn sites	26	12	0	26	3	23	5	21	22	4
No yawn sites	22	7	10	12	8	14	1	21	7	15
χ²	0,45 (p = 0,5)		12,3 * (p = 0,0005)		2,87 * (p = 0,09)		1,20 * (p = 0,27)		11,77 (p = 0,0006)	

Table 5. Results of the chi-square analysis for the stimulation parameters of 100 µA for 500ms. Significant results are highlighted in red. An asterisk indicates the results where the Yates correction was applied.

We obtained similar results considering the excitable sites at 150 µA (Table 6), with a significantly higher proportion of sites with yawn-responsive neurons where ICMS evoked mouth movements ($\chi 2 = 6{,}41$; $p = 0{,}02$), and a higher proportion of no-yawn neurons in those sites where ICMS evoked head ($\chi 2 = 9{,}59$; $p = 0{,}002$) and face ($\chi 2 = 4{,}72$; $p = 0{,}03$) movements.

150µA – 500ms	Excitable		Head		Face		Hand		Mouth	
	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Yawn sites	35	3	4	31	2	33	9	26	24	11
No yawn sites	23	6	12	11	7	16	1	22	8	15
χ²	1,35 * (p = 0,25)		9,59 * (p = 0,002)		4,72 * (p = 0,03)		3,07 * (p = 0,08)		6,41 (p = 0,02)	

Table 6. Results of the chi-square analysis for the stimulation parameters of 150 µA for 500ms. Significant results are highlighted in red. An asterisk indicates the results where the Yates correction was applied (when there less than 5 observations in at least one of the cells).

Remarkably, we observed that in the sites where we have recorded yawn-responsive neurons, ICMS predominantly triggered mouth movements at both current intensities employed, suggesting a certain level of cortical control over yawning behavior.

5. Discussion

In the present neuroethological investigation, we explore the neural activity of neurons within the ventral premotor cortex (PMv) of macaques during yawning behavior. Yawning, characterized by the sequential opening and closing of the mouth, is often perceived as a stereotypical and reflex-like behavior, leading to limit the investigation of its underlying mechanisms to subcortical areas (especially the brainstem and the hypothalamic paraventricular nucleus) ignoring a possible cortical modulation. Given that yawning involves complex movements of both mouth and head, and that PMv is involved in facial and oral motor control (Maranesi et al., 2012), it is plausible that this region may host neurons encoding some aspects of yawning. Moreover, PMv contains neurons responsive to communicative mouth actions, both observed and performed (Ferrari et al., 2003), and yawning has been hypothesized to convey a communicative signal, particularly among NHPs. By conducting such studies, we aim to deepen our understanding of the neural mechanisms underlying yawning behavior in primates, especially considering its potential top-down regulation, potentially shedding light on its evolutionary significance and potential communicative functions.

The main novel finding of this work consists in the demonstration of PMv neurons modulated during yawning, either exclusively or concurrently with other mouth-related behaviors, such as sucking, biting, bringing food to mouth and threatening acts. Indeed, within our overall neuronal pool, the majority of modulated neurons showed response to yawning and to at least one other mouth behaviors (yawn-related neurons). Although a minority of the total recorded neurons, we also found a subset of neurons (defined as yawn-selective), which exclusively responded during yawning, supporting the hypothesis that PMv could play a role in the yawning display.

It is crucial to discern both the functional roles and the properties of these neurons within PMv, especially when considering that subcortical areas seem to predominantly control yawning. Therefore, we examined whether yawn-responding neurons could encode yawning's kinematics parameters, such as mouth opening and head elevation, as these are prominent motor components of

the behavior. This exploration aimed to deepen our understanding of if and how these neurons contribute to the overall motor control of yawning in the macaque's brain. In our analysis we wanted to identify any possible correlation between the peak firing rates of yawn-responding neurons and both the values and timing of mouth and head kinematic peaks. Surprisingly, we discovered that no neurons exhibited a correlation with the kinematics. This finding is intriguing as it suggests that while these cortical neurons are involved in the yawning response, they do not encode for low-level kinematics parameters, but likely exert a different form of control over this behavior.

If the primary property of these neurons is not to encode the kinematic aspects of yawning, what function do they serve in PMv? Drawing from existing literature it has been suggested the existence of different types of yawns: a physiological yawn, which occurs during the transition between wakefulness and sleep, and the emotional yawn, triggered by stressful situations, that appears to convey a subtle form of threat, less direct and severe compared to the actual threat (Deputte, 1994; Leone et al., 2014; Scopa & Palagi, 2016; Zannella et al., 2021; Zannella et al., 2017). To explore potential variations in neuronal responses to yawning based on the conveying message and contextual factors, we analyzed neuronal activity during yawning in two distinct contexts: the laboratory setting (NER), that is supposed to induce stress in the monkey, and the home-cage during nighttime, presumed to be a relaxing context. Surprisingly, we observed that yawn-responsive neurons exhibited different patterns of response depending on the context: indeed, some neurons only activated in response to yawning in the NER, but not when yawning was performed in the home-cage, while others displayed consistent responses across both contexts. Since variations in the yawning display could explain the observed differences in neuronal responses, we focus our analyses on canine display and yawn duration, based upon a previous classification of three types of yawns depending on teeth exposure and duration (Zannella et al., 2021). We noted that yawns performed in the NER were predominantly accompanied by canine display and were always performed while sitting, whereas in the home-cage yawns occurred both

with and without canine exposure and were executed in both sitting and laying positions. Our analysis revealed that the duration of yawns with teeth display did not significantly differ between the NER and the home-cage contexts. In fact, we couldn't replicate the findings of Palagi's group (Zannella et al, 2021) since, in our study, the duration of yawns alone did not identify different yawn displays, suggesting that it did not account for the differential neuronal responses across contexts. This discrepancy in findings could potentially be explained by the difference in species used in the two studies (*Macaca tonkeana* in Palagi's group study, *Macaca mulatta* in the present study). These species differences may introduce variations in yawning behavior and accompanying displays, suggesting that further exploration into species-specific yawning patterns and their neural correlates could provide valuable insights about yawning.

Notably, the only significant difference in duration was observed between yawns with and without teeth exposure. Then, since only yawns with teeth exposure were performed in both contexts, we compared neuronal responses between the two contexts focusing exclusively on this type of yawn display. Interestingly, we found that, although the similarity of yawn, neurons responded exclusively in the NER and not in the home-cage. Therefore, contextual differences, particularly in the display of teeth during yawning, may not fully explain the divergent neuronal response observed across contexts suggesting that the triggering factors (e.g. stress) underlying this behaviors have an impact on its neural representation in the ventral premotor cortex.

To deepen the understanding of cortical mechanism controlling yawning, we compared the movements evoked through Intracortical Microsctimulation (ICMS) among the sites where we recorded yawn-responding neurons (both yawn-selective and yawn-related) and the sites where we didn't extract any yawn-modulated neurons. Remarkably, we found a significant positive association between the sites where yawn-responsive neurons were recorded and those sites where mouth movements were elicited, in contrast to the sites where yawn-responsive neurons were absent. Regarding the sites in which ICMS evoked head and face movements, we found a significant association between the sites in which no-yawn units were recorded, suggesting that in

PMv yawn-responsive neurons may exert greater control over the mouth-related aspects of yawning rather than the head/face-related ones. This finding provides valuable insights into the potential roles of these neurons in orchestrating the intricate motor sequence involved in yawning.

In summary, our findings suggest that neurons in the PMv may indeed regulate yawning as a communicative behavior, with its expression influenced by contextual factors. Our data revealed a mouth motor control in sites where yawn-responding neurons were identified. Despite this association, our kinematic analysis revealed that this control operates beyond basic motor parameters, as we did not observe a significant correlation between the peak firing rate and mouth/head kinematics. Moreover, when considering neuronal response in different contexts, it becomes evident that these neurons likely play a role in orchestrating yawning display. This suggests potential involvement in modulating the downstream network of yawning for social and communicative purposes.

This study provides a valuable foundation for further investigations, particularly regarding the communicative and social aspects of yawning, thereby enhancing our understanding of its possible functions.

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