UNIVERSITÀ DI PARMA

UNIVERSITÀ DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

"NEUROSCIENZE"

CICLO XXXVII

Functional properties and firing features of monkey premotor neurons during sleep

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Anni Accademici 2021/2022 – 2023/2024

TABLE OF CONTENTS

Abstract

Decades of neurophysiological research in awake, behaving primates have elucidated the role of the premotor (PM) cortex in controlling movement execution, encoding of objects and actions performed by others through a variety of neuronal cell classes, such as motor, canonical, peripersonal and mirror neurons. How do the firing properties of these neurons change during the different stages of sleep compared to wakefulness? In this study, we addressed this issue by leveraging a recently developed neurobehavioral platform for wireless intracortical recordings from the PM cortex of two *Rhesus Macaques* during both wakefulness and natural unconstrained nighttime sleep. First, we developed a novel unsupervised method to automatically classify different brain states using only intracortical biomarkers. Next, we tested single neuron properties during a reaching-grasping visuo-motor task in a traditional laboratory setting to functionally characterize neurons as self-type, other-type, or self-and-other type, and then studied their firing properties during sleep on the night following the testing. Interestingly, self-type and self-and-other type neurons characterized by narrow spikes exhibited strong suppression during NREM sleep while enhanced their activity during REM sleep as during wakefulness. In contrast, we found a set of cells apparently task-unrelated during wakefulness that mostly exhibited broad spikes and significantly increased their activity during NREM sleep relative to wakefulness and REM sleep. These findings uncover a previously unknown sleep micro-architecture within the premotor cortex, suggesting a switch between PM cortex neuronal populations involved in the orchestration of behavior during wakefulness and a subpopulation that remain silent during wakefulness but becomes active during NREM sleep, possibly contributing to sleep-related functions.

1. Introduction

The brain is central to guiding an organism's interactions with the external world, allowing it to achieve specific goals and respond to environmental challenges. Over the course of evolution, mammals have developed sophisticated neural circuits that enable them to adapt their voluntary movements to a variety of conditions, playing a crucial role in survival, such as finding food, selecting mates, and evading predators during wakefulness.

Besides coordinating actions in the outside world, the brain of all animal species needs to sleep, that is, switching periodically in an irresistible and reversible state of unconsciousness, distinct from coma or anesthesia (Eban-Rothschild et al., 2017). Sleep is just as essential as wakefulness and plays a vital role in maintaining cognitive and physical health. Since its cyclic nature, it involves the alternation between distinct phases such as non-Rapid Eye Movement (*NREM*) and Rapid Eye Movement (*REM*) sleep.

In diurnal species, like humans and non-human primates, sleep is regulated by both circadian rhythms and homeostatic processes. The circadian rhythm, controlled by the suprachiasmatic nucleus (*SCN*) in the hypothalamus, synchronizes the sleep-wake cycle with the natural light-dark cycle (Saper, 2013). Light suppresses the sleep-inducing hormone melatonin, while darkness stimulates its production, prompting the body to rest (Doghramji, 2007; Kun et al., 2019; Stone et al., 2000; Zhdanova et al., 2011; Zhdanova et al., 2001). Meanwhile, homeostatic mechanisms increase the need for sleep the longer one stays awake, with substances like adenosine building up to promote NREM sleep (Benington et al., 1995; Porkka-Heiskanen et al., 1997; Schwierin et al., 1996).

Despite sleep occupies one-third of human life - and even more in some species (Campbell & Tobler, 1984) - its function remains somewhat elusive and still under investigation. Though seemingly disadvantageous due to reduced awareness and increased vulnerability to predation, sleep is crucial for survival and well-being, and its conservation through evolution underscores its importance. Specifically, the available evidence suggest that sleep is vital for memory consolidation (Walker & Stickgold, 2006), brain plasticity (Benington & Frank, 2003), thermoregulation (Rechtschaffen, 1998), and the functioning of the endocrine (Spiegel et al., 1999) and immune systems (Imeri & Opp, 2009). Moreover, sleep is believed to support metabolic recovery by reducing energy consumption and aiding the removal of toxic metabolites (Xie et al.,

2013), though recent studies suggest that brain waste clearance also occurs during wakefulness (Miao et al., 2024). Additionally, sleep is essential for memory consolidation and synaptic homeostasis, with specific sleep stages supporting different types of memory (Bisaz et al., 2014; Stickgold et al., 2000; Tononi & Cirelli, 2006). In further support of its essential role, studies showed that insufficient sleep impairs cognitive functions, affecting vigilance, memory, and executive abilities (D'Ambrosio et al., 2019; Tucker et al., 2010), and its deprivation led to compensatory "sleep rebound" (Sallanon et al., 1983) or - in extreme cases, as demonstrated in rats (Rechtschaffen et al., 1983) and dogs (Bentivoglio & Grassi-Zucconi, 1997) - death.

1.1 Sleep and wakefulness

Recent research has expanded our understanding of the wake-sleep cycle, a natural, endogenous process comprising two alternating behavioral states that recur daily. Wakefulness can be characterized as a state of high arousal optimized for interactions with the environment (Joiner, 2016). Conversely, sleep is generally defined as a reversible state of quiescence, characterized by reduced responsiveness to external stimuli and organized into stages that vary from light to deep sleep (Eban-Rothschild et al., 2017).

The study of sleep has fascinated scientists for centuries. One of the earliest documented discussions of sleep dates back to 350 B.C., when Aristotle in his work entitled "*On Sleep and Sleeplessness*" described sleep as a restorative process essential for life (Aristotle, 350 B.C.). Despite its philosophical depth, Aristotle's view of sleep was purely speculative, lacking empirical validation.

Modern scientific exploration of sleep began in the early 20th century. A pivotal figure in this field was Henri Piéron, whose book "*Le problème physiologique du sommeil*" introduced the concept of "sleep-inducing substances" (later known as *somnogens*) (Piéron, 1913), suggesting their accumulation in the body during wakefulness and dissipation during sleep. His experiments on dogs, demonstrated that sleep was not simply the result of physical fatigue but rather governed by distinct physiological mechanisms, shaping the future of sleep research (Piéron, 1913).

One of the most groundbreaking advancements in sleep research came with the invention of the electroencephalogram (*EEG*) by Hans Berger, a German psychiatrist who, in 1929, published his findings on the electrical activity of the human brain, revealing distinct patterns during sleep and wakefulness (Berger,

1929). This discovery enabled the classification of sleep into various stages based on brainwave activity, fundamentally advancing our understanding of the neural basis of sleep.

Prior to these discoveries, sleep was widely regarded as a passive state, arising merely from the cessation of sensory inputs or brain exhaustion. However, the work of Frédéric Bremer, a Belgian neurophysiologist, challenged this idea. In the 1930s, Bremer's *"cerveau isolé"* and *"encéphale isolé"* experiments on cats provided compelling evidence that sleep and wakefulness are actively controlled by specific brain structures. In the "cerveau isolé" (Bremer, 1935), Bremer transected the brainstem at the midbrain level, isolating the forebrain from the brainstem, resulting in continuous sleep-like brain activity, as measured by EEG, with no response to external stimuli. In the "encéphale isolé" (Bremer, 1936), he performed a lower transection at the level of the medulla oblongata, separating the brain from the spinal cord, resulting in cats that exhibited a more typical wake-sleep cycle, alternating between periods of sleep and wakefulness.

Bremer's studies demonstrated that the brainstem plays a critical role in wakefulness, while the forebrain generates sleep states. His work highlighted the *active*, rather than passive, nature of sleep regulation directly influencing the work of Giuseppe Moruzzi and Horace Magoun who identified the reticular formation as a key brainstem structure that promotes wakefulness (Moruzzi & Magoun, 1949). They demonstrated that stimulating the reticular formation led to cortical activation and wakefulness, further reinforcing that wakefulness is actively maintained by specific neural circuits.

In 1953, Nathaniel Kleitman and his student Eugene Aserinsky made a groundbreaking discovery by identifying Rapid Eye Movement (*REM*) sleep, a distinctive phase characterized by fast eye movements performed under the eyelid, low muscle tone, and vivid dreaming (Aserinsky & Kleitman, 1953). This discovery was pivotal in advancing our understanding of sleep and dreams, as it revealed that REM sleep alternates with non-REM (*NREM*) sleep, which includes progressively deeper stages such as slow-wave sleep (*SWS*), known for its restorative properties. Their work provided a framework for classifying sleep into distinct phases, which are fundamental to our current understanding of sleep cycles.

The identification of REM sleep also significantly deepened our comprehension of dreams. Dreams are now recognized as a complex phenomenon influenced by neurological, psychological, and environmental factors. They are integral to the sleep cycle, crucial for processing experiences and emotions, and contribute to overall mental health and cognitive function (Maquet, 2001). The interest in dreams dates back to ancient

times. For example, Aristotle considered dreams as reflections of daily experiences or as mechanisms for processing emotions and memories (Aristotle, 330 B.C). In the 17th century, René Descartes linked dreams to brain activity during rest, suggesting a physiological basis for dreaming (Descartes, 1641). By the 19th century, Sigmund Freud made significant advancements in dream research with his seminal work, *The Interpretation of Dreams* (1900), proposing that dreams offer a window into the unconscious mind, revealing hidden desires and conflicts (Freud, 1900). The subsequent identification of REM sleep by Aserinsky and Kleitman provided further insights into the role of dreams in processing emotions and consolidating memories (Hobson et al., 2000), since modern neuroimaging techniques, such as functional magnetic resonance imaging (*fMRI*) and EEG, have revealed an involvement of these designated areas in dreaming (Nir & Tononi, 2010; Siclari et al., 2017). Despite ongoing research, dreams remain a multifaceted phenomenon with much still to be understood. They are considered a natural part of the sleep cycle, essential for processing experiences and emotions, and play a crucial role in maintaining overall mental health and cognitive function (Maquet, 2001).

In the broader context of understanding sleep, the distinctions between sleep and wakefulness have become clearer. Sleep and wakefulness are now recognized as distinct brain states, each characterized by specific patterns of neural activity, functional connectivity, and levels of consciousness. On a behavioral level, several criteria can define sleep: *1)* reduced motor activity, except for respiratory functions; *2)* diminished responsiveness to low-intensity stimuli that would typically cause a reaction during wakefulness; *3)* species-specific stereotyped postures; and *4)* easy reversibility, distinguishing sleep from coma or lethargy. Additional criteria often associated with sleep, though not universally present, include characteristic changes in brain electrical activity, behavioral quiescence, and homeostatic regulation (Campbell & Tobler, 1984; Joiner, 2016). This comprehensive understanding of sleep, including the staging and the role of REM and NREM sleep, is crucial for exploring how different sleep phases contribute to overall cognitive and emotional well-being.

1.1.1 Neural circuitry regulating sleep-wake transitions

Early insights into the brain circuitry regulating sleep-wake states were derived from von Economo's studies during World War I on encephalitis lethargica. Observations of patients with damage to the posterior hypothalamus and upper brainstem, who experienced excessive sleep often exceeding 20 hours a day, led von Economo to hypothesize that these brain regions contain crucial circuits responsible for activating the forebrain

and sustaining wakefulness (Von Economo, 1930). Conversely, studies on rats with lesions in the anterior hypothalamus revealed severe insomnia, suggesting that neurons in this region play a crucial role in inhibiting the brainstem arousal system to promote sleep (Nauta, 1946).

Lesion studies revealed an ascending arousal system in the brainstem and hypothalamus that projects to the forebrain, including noradrenergic neurons in the locus coeruleus, serotoninergic neurons in the dorsal and medial raphe, dopaminergic neurons in the midbrain, and histamine and orexin neurons in the posterior hypothalamus, all of which play a role in promoting wakefulness (Jones, 2011) (*Figure 1*). Orexin, produced in the lateral hypothalamus, is particularly important for regulating the wake-sleep cycle and food intake, and its disruption can lead to narcolepsy (Chemelli et al., 1999; Crocker et al., 2005; Lin et al., 1999). Neurons involved in these ascending pathways exhibit increased firing rates during wakefulness and can arouse animals when electrically stimulated; on the other hand, their activity decreases during sleep, indicating their role in promoting wakefulness (Lin et al., 2011).

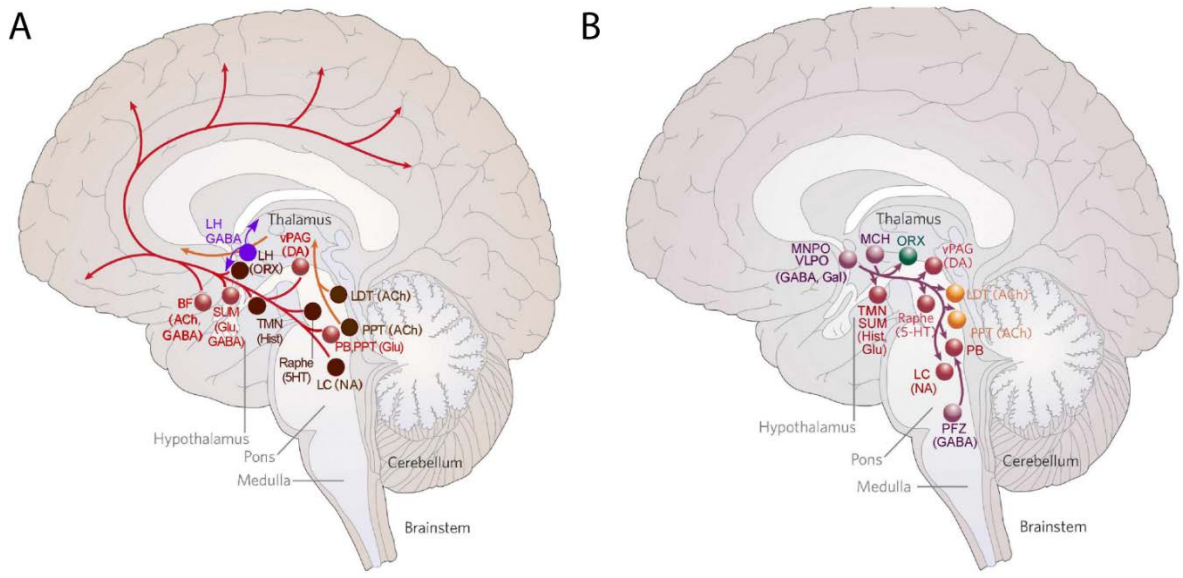

Figure 1 | **Neurotransmitter network promoting wakefulness and sleep.** A: Diagram summarizing the fast neurotransmitter systems promoting wakefulness. The backbone of the arousal system presented in this model (red), is the glutamatergic input from the parabrachial nucleus (PB) and pedunculopontine tegmental nucleus (PPT) to the basal forebrain, and the GABAergic and cholinergic neurons in the basal forebrain (BF) that diffusely innervate the cerebral cortex. Lesions at these sites cause complete loss of consciousness, whereas lesions of supramammillary (SUM) glutamatergic neurons (red) or dopaminergic (DA) neurons in the ventral periaqueductal gray matter (vPAG) near the dorsal raphe nucleus (red) cause about a 20% loss of wake time. In addition, two populations of GABAergic neurons in the lateral hypothalamus (LH) (purple), have recently been proposed to promote wakefulness by inhibiting sleep promoting neurons in the thalamus and preoptic area. The monoaminergic, cholinergic, and peptidergic neurons in the brainstem and hypothalamus (brown) play a modulatory role, but lesions in these locations have small effects on wake-sleep amounts. B: Diagram summarizing the fast neurotransmitter systems promoting sleep. Ventrolateral preoptic (VLPO) and median preoptic (MnPO) GABAergic neurons (purple) send axons to most components of the arousal system (red, yellow, and green), inhibiting them. Parafacial zone (PFZ) GABAergic neurons

in the medulla (purple) induce sleep by inhibiting the parabrachial glutamatergic arousal neurons. Melanin-concentrating hormone (MCH) neurons in the lateral hypothalamus (purple) contain both GABA and glutamate and may be able to release them at different terminal sites. Abbreviations: 5HT, serotonin; Ach, acetylcholine; Hist, histamine; LC, locus coeruleus; LDT, laterodorsal tegmental nucleus NA, noradrenaline; ORX, orexin; TMN, tuberomammillary nucleus. *Figure from Saper and Fuller (2017)*.

Later on, this theory was refined to suggest that monoaminergic and cholinergic inputs from the upper brainstem reticular formation to the thalamus and basal forebrain act as the key regulators of the sleep-wake cycle (Saper et al., 2005). However, recent evidence from lesion studies and optogenetic deactivation in the basal forebrain, pons and thalamus, has shown that the loss of these neurons doesn't affect the total amount of time spent in wakefulness or sleep, nor does it alter EEG activity during these states (Fuller et al., 2011; Saper & Fuller, 2017; Xu et al., 2015).

Recent studies have highlighted the significant role of glutamatergic, cholinergic and GABAergic neurons in maintaining the balance between wakefulness and sleep. The promotion of wakefulness seems to originate from glutamatergic and cholinergic inputs from the dorsolateral pons (parabrachial nucleus, *PB* and pedunculopontine tegmental nucleus, *PTT*) to the basal forebrain (Kroeger et al., 2017), and from the GABAergic and cholinergic neurons in the basal forebrain, projecting to the cerebral cortex (Kim et al., 2015; Kroeger et al., 2017). Moreover, GABAergic neurons in the lateral hypothalamus are thought to promote wakefulness by inhibiting sleep-promoting activities in the thalamus and preoptic area (Anaclet et al., 2015). These pathways are likely enhanced by modulatory systems involving orexin and monoamines, which are essential for maintaining full and sustained wakefulness, especially under challenging conditions.

Sleep promotion is closely linked to the inhibitory activity of GABAergic neurons in the ventrolateral and median preoptic nuclei of the anterior hypothalamus, which target the ascending arousal system of the brainstem (Sherin et al., 1998; Suntsova et al., 2002). Inhibition of these neurons leads to significant sleep loss in rats and mice, whereas their activation result in profound increases in sleep (Kroeger et al., 2018). These preoptic neurons fire at their slowest rate during wakefulness, increase their firing as animals fall asleep, and reach their highest during deep sleep (Brown et al., 2012; Hassani et al., 2009). Additionally, a population of GABAergic neurons in the parafacial zone, located near the facial nerve, inhibits the parabrachial nucleus resulting in sleep loss (Anaclet et al., 2014). Interestingly, the ventrolateral preoptic neurons receive inhibitory inputs from neurons across the arousal system, creating mutually inhibitory connections within a neural circuit

that functions similarly to an electrical *flip-flop switch*. In this circuit, each side can inhibit the other, leading to rapid and complete transitions between wakefulness and sleep (Saper et al., 2001; Saper et al., 2010; Saper et al., 2005) (*Figure 2*).

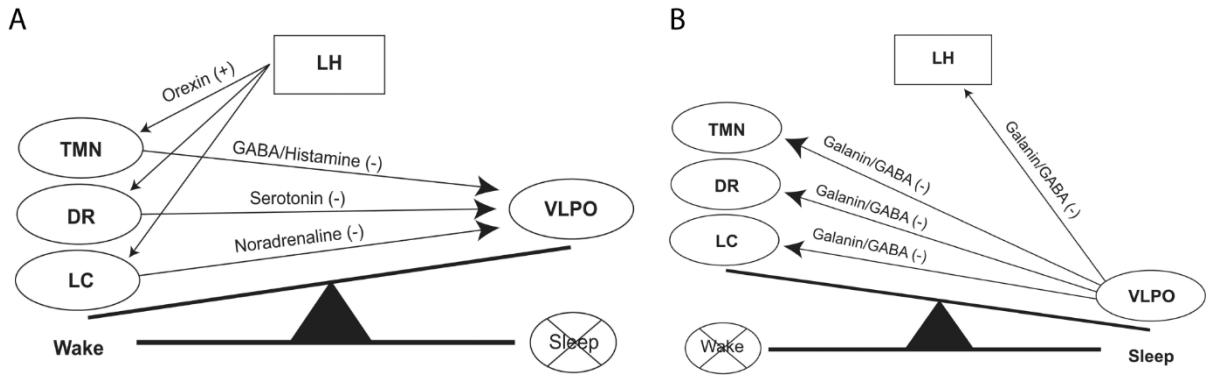

Figure 2 | **The "flip-flop" switch model of transitions between sleep and wakefulness**. A: The wake state is maintained by orexinergic input from the lateral hypothalamus (LH) to wake-promoting nuclei, such as the tuberomammillary nucleus (TMN), the dorsal raphe (DR), and the locus coeruleus (LC). B: During sleep, GABAergic and galaninergic neurons in the ventrolateral preoptic area (VLPO) inhibit these wake-promoting nuclei, including LH. *Figure from Brown et al. (2012)*.

The REM sleep state appears to be driven by glutamatergic neurons located in the subcoeruleus region of the pons (ventrally to the locus coeruleus) (Luppi et al., 2006), that presumably cause muscle atonia projecting to the ventromedial medulla and spinal cord. Here, these projections activate GABAergic and glycinergic neurons, hyperpolarizing motor neurons and exciting the nearby parabrachial nucleus and pedunculopontine tegmental nucleus, where REM-active neurons are located (Lu et al., 2006; Luppi et al., 2006). Inhibiting glutamate production in the locus coeruleus suppresses REM sleep, while promoting glutamate can strongly drive REM sleep. (Krenzer et al., 2011; Saper & Fuller, 2017). The inhibition of REM state is primarily controlled by GABAergic neurons in the ventrolateral periaqueductal grey matter (Lu et al., 2006; Luppi et al., 2006; Sastre et al., 1996), which are more active during wakefulness and NREM sleep. Lesions in these neurons result in excessive REM sleep (Lu et al., 2006; Luppi et al., 2006). GABAergic neurons in the subcoeruleus area also project back to the ventrolateral periaqueductal gray region promoting a mutual inhibition between these two neuron populations and constituting another flip-flop switch which lead to complete transitions into, and out of, REM sleep (Scammell et al., 2017) (*Figure 3*). Interestingly, the noradrenergic locus coeruleus and serotonergic dorsal raphe nucleus innervate and inhibit the subcoeruleus

region (Cassano et al., 2009), explaining why REM sleep can be reduced by antidepressants that increase serotonin or norepinephrine levels. Recent research has also highlighted the role of neurons in the ventral medullary tegmentum in REM sleep induction, with optogenetic activation of these neurons promoting paradoxical sleep (Weber et al., 2015). GABAergic neurons in this area are active during REM sleep (Sapin et al., 2009), but further studies are needed to clarify the link between their activation and the induction of REM state.

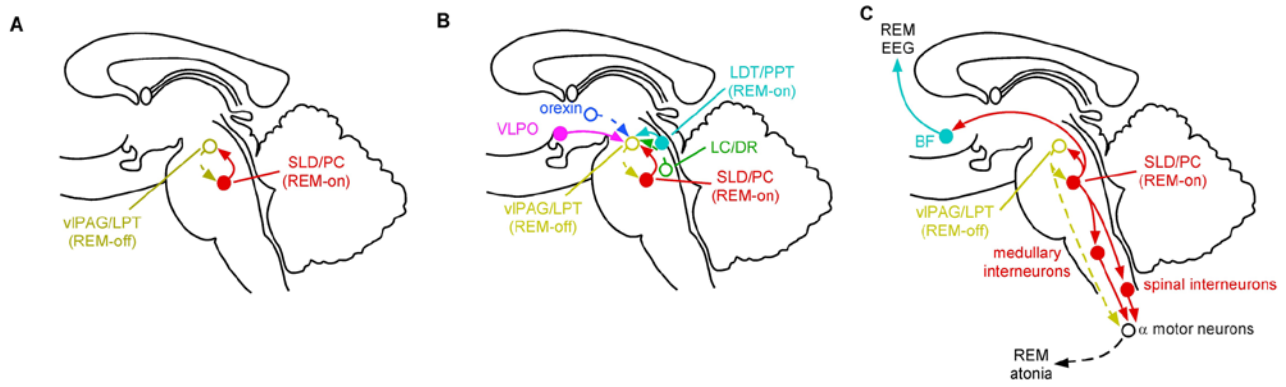

Figure 3 | **The REM-NREM sleep switch.** A: Two populations of mutually inhibitory neurons in the upper pons form a switch for controlling transitions between REM and NREM sleep. GABAergic neurons in the ventrolateral periaqueductal gray matter and the adjacent lateral pontine tegmentum (vlPAG/LPT) (dashed gold line) fire during non-REM states to inhibit entry into REM sleep. During REM sleep, they are inhibited by a population of GABAergic neurons in the sublaterodorsal region (SLD) (red). B: REM switch is modulated by neurotransmitter systems. Noradrenergic neurons in the LC and serotoninergic neurons in the DR inhibit REM-off neurons in vlPAG/LPT (gold), while during REM sleep, they are silent (dashed green line). Cholinergic neurons (aqua) promote REM sleep by having opposite actions on the same two neuronal populations. The orexin neurons (dashed blue line) inhibit entry into REM sleep by exciting neurons in the REM-off population, whereas the VLPO neurons (purple) promote the entry into REM sleep by inhibiting this same target. C: During REM sleep, a separate population of glutamatergic neurons in the SLD (red) activates inhibitory interneurons in the medulla and spinal cord, inhibiting motor neurons (dashed black line) and producing the typical atonia of REM sleep. Withdrawal of tonic excitatory input from the REM-off regions may also contribute to the loss of muscle tone. At the same time, ascending projections from glutamatergic neurons in the PB and PC activate forebrain (BF) pathways (aqua) that drive EEG desynchronization and hippocampal theta rhythms, thus producing the characteristic EEG signs of REM sleep. *Figure from Saper et al. (2010).*

Overall, these findings suggest an active role of glutamatergic and GABAergic signals in regulating sleep-wake states, suggesting the cholinergic and monoaminergic systems as modulators rather than direct promoters of these different brain states (Saper & Fuller, 2017).

1.1.2 REM and NREM sleep staging: neurobehavioral features

The groundbreaking work of Kleitman and Aserinsky in 1953 helped classify sleep into distinct phases that are now fundamental to our understanding of sleep cycles. Today, the analysis of the sleep-wake cycle, which

typically lasts about 90 minutes in adult humans (Patel et al., 2024), relies on polysomnography. This method involves three key physiological measures: scalp brain activity measured non-invasively by an electroencephalogram (*EEG*), eye movements captured by an electro-oculogram (*EOG*), and muscle tone assessed by an electromyogram (*EMG*) (*Figure 4*) (Poree et al., 2006). In clinical settings, breathing is also monitored due to its relevance in various sleep disorders. EEG, EOG and EMG are therefore the gold standard for the classification of sleep stages. Traditionally, they were classified by experts in polysomnography, but advancements have led to the development of reliable automated algorithms (Fiorillo et al., 2019).

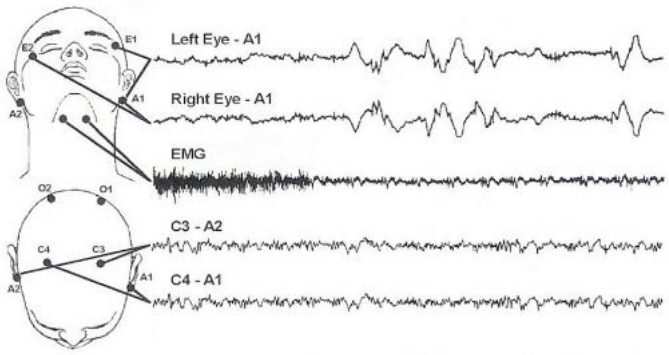

Figure 4 | **Example of EEG, EOG and EMG recording sites and signals for sleep scoring in humans**. *Figure from Poree et al. (2006)*

The identification of specific sleep states in humans follows the guidelines of the American Academy of Sleep Medicine (*AASM*) (Berry et al., 2020; Iber et al., 2007), which evaluate brain activity in 30-seconds epochs. The EEG signal is factored in frequency bands: delta waves (0-3.99 Hz), theta waves (4-7.99 Hz), alpha waves (8-13 Hz) and beta waves (> 13 Hz). The relative dominance of these frequencies, in combination with EMG and EOG allows for the classification of different stages of NREM and REM sleep.

In addition to these features, characteristic EEG patterns - such as K-complexes and spindles - are critical for identifying specific sleep stages. In particular, K-complexes and sleep spindles are hallmark features of NREM sleep, particularly during stage 2. Both play a role in sensory gating, a process that helps filtering out irrelevant external stimuli to maintain the integrity of sleep, protecting the sleeper from being easily awakened by noise or other disturbances (Dang-Vu et al., 2010). *K-complexes* are thought to originate in the cortex in response to inputs from the thalamus (Steriade et al., 1993a; Timofeev & Steriade, 1996); they are large, sudden bursts of electrical activity, visible as sharp, high-amplitude waves followed by a smooth positive

wave (Amzica & Steriade, 2002). K-complexes effectively filter sensory information, such as sounds (Halasz, 2005, 2016; Lechat et al., 2021), and it is also suggested that they may contribute to memory consolidation by coordinating with other brain oscillations during sleep (Murphy et al., 2009). *Sleep spindles* are generated in the thalamus, specifically in the reticular nucleus (Piantoni et al., 2016); they appear as short bursts of rhythmic oscillations at a frequency of 12-15 Hz, primarily associated with memory consolidation, reinforcing both declarative and procedural memories (Schabus et al., 2004). They are believed to support synaptic strengthening, critical for learning and long-term memory formation (Schabus et al., 2004), and contribute to cortical plasticity, that is essential for cognitive development and adaptation (Fogel & Smith, 2011).

The AASM manuals' scoring rules can be summarized as follows. *Wake* (W) is characterized by the presence of alpha or beta rhythm in EEG signals. Alpha waves are regular, wide, low-frequency and high-voltage, typically appearing when the subject is relaxed and not particularly stimulated. Beta rhythm, instead, are irregular waves of small amplitude and high frequency, highlighting an alert or active state of the subject. Wakefulness is also marked by rapid eye movements with open eyes and high muscular activity. *NREM1* (N1) displays low-voltage and mixed frequencies waves (2-7 Hz), including delta and theta waves. Alpha components should not exceed 50% of the total spectral band, and, during transitions into N1, it is common the occurrence of vertex sharp waves, large electronegative discharge hypothesized as a consequence of a response to an external stimulus or a mechanism to maintain sleep after a stimulus (Colrain & Campbell, 2007). Slow eye movements may be observed in the EOG, and EMG activity is lower than in the wake state. N1 can be easily disrupted leading to awakenings or arousals, and usually covers 5% of the total human sleep time. *NREM2* (N2) is characterized by the presence of K-complexes and sleep spindles, with slow eyes movement and brief body twitches both generally diminishing. N2 usually covers 50% of the total sleep time. *NREM3* (N3) exhibits predominant slow waves and delta rhythm, with high-voltage waves. Spindles and K-complexes may appear, and awakenings are rare. Usually, N3 is characterized by more than 20% of the epoch being slow waves, and it covers usually 20% of the total human sleep time. Across all stages of non-REM sleep, eye movements are basically absent, muscle tone is low, breathing is slow and regular, and body temperature falls. *REM sleep* (R) is characterized by desynchronized, low-voltage, mixed frequency brain waves (similar to wakefulness) and sawtooth waves, bursts of low frequency rhythms resembling notched triangular waves (Berger et al., 1962; Rodenbeck et al., 2006) occurring after the onset of muscle tone reduction (Geisler et al.,

1987; Pearl et al., 2002; Sato et al., 1997) and which origin is not certain yet. Key features of this stage include rapid eye movements beneath closed eyelids and a very low muscle tone, except for occasional short twitches. The low muscle tone is caused by a massive inhibition of motor neurons (except for those that support respiration and eye movements) preventing the physical enactment of dreams which are frequent, but not exclusive, of this stage (Oudiette et al., 2012; Siclari et al., 2018). Moreover, metabolic consumption increases, so as heart rate, respiration and blood pressure, and awakenings or arousals can occur. REM sleep normally covers 20–25% of the total adult human sleep time (*Table 1*, *Figure 5*).

Sleep stage	EEG features	Eye movements	Muscle tone
Wake (W)	Regular, high-voltage, low-frequency alpha waves (relaxed state) Irregular, low-amplitude, high-frequency beta waves (active state)	Rapid eye movements with open eyes	High muscle tone
NREM 1 (N1)	Low-voltage, mixed-frequencies waves (delta and theta waves) Occurrences of vertex sharp waves	Occasional slow eye movements	Low muscle tone
NREM 2 (N2)	Predominance of delta waves (more prominent than N1) Occurrences of K-complexes and sleep spindles	Occasional slow eye movements	Low muscle tone
NREM 3 (N3)	Predominance of high-voltage slow waves and delta waves Occasional occurrences of K-complexes and sleep spindles	Occasional slow eye movements	Low muscle tone
REM (R)	Desynchronized, low-voltage, mixed-frequency waves (similar to wakefulness) Occurrences of sawtooth waves	Rapid eye movements beneath closed eyelids	Muscle atonia (except for occasional twitches)

Table 1 | **Summary of key characteristics of sleep stages according to AASM manual.**

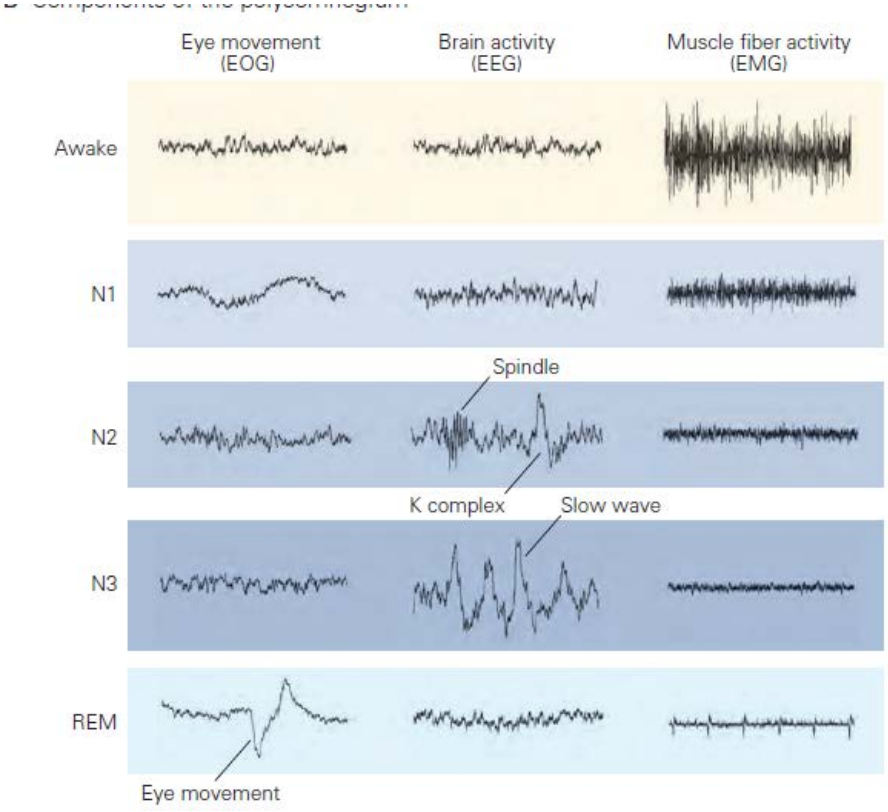

Figure 5 | **Example of sleep staging**. *Figure from Kandel et al. (2021).*

Despite the detailed criteria, there are instances in which 30-second epochs deviate from the strict classification guidelines. When this occurs, these epochs are typically reviewed and adjusted based on previous scoring. In both automated and manual scoring protocols, constraints are often applied that enforce a fixed sequence of sleep stages. For example, it is generally assumed that after REM sleep, the individual either transitions back into N3 or wakes up, and that REM sleep only follows entering N3. These assumptions are treated as standard practice, potentially introducing bias.

1.1.3 Sleep research in non-human primates

Zebrafish (Altenhofen & Bonan, 2022), rodents (Alföldi et al., 1990), cats (Ruckebusch & Gaujoux, 1976), dogs (Kaitin et al., 1986) and monkeys (Balzamo et al., 1977) are the most commonly used animals in sleep research. However, these experimental models exhibit differences in sleep-wake cycles (Lancel et al., 1991; Li et al., 2021), limiting their utility for translational studies of human sleep. Mice and rats, for instance, are the most frequently used laboratory animals but differ substantially from humans due to their nocturnal,

monophasic sleep patterns (Yasenkov & Deboer, 2012). Non-human primates (*NHPs*), on the other hand, provide a more reliable model for sleep research because of their phylogenetic closeness to humans, their neurobiology, diurnal habits, and sleep architecture, which closely mirror those of humans, thereby making them particularly valuable for investigating the neural mechanisms of sleep (Balzamo et al., 1977; Reite et al., 1965). This is especially true for species like marmosets (Bukhtiyarova et al., 2022), rhesus macaques (Hsieh et al., 2008) and cynomolgus macaques (Rachalski et al., 2014), which are considered the most reliable models for studying human sleep.

Basic research using NHPs has highlighted various phenomena occurring during the night, including memory consolidation (Takeuchi et al., 2015), neural responses to sensory stimuli (Issa & Wang, 2008) and changes in neocortex activity (Dehghani et al., 2016), but despite these, NHPs have predominantly been used in pharmacological studies (Berro et al., 2021) or research on sleep impairment in specific diseases, such as Parkinson disease (Fifel et al., 2016). These works focused more on the impacts of sleep disturbances rather than on the investigation of the neural mechanisms of sleep itself, largely due to technological limitations requiring physical restraints on the animals to perform neural recordings. Indeed, in earlier research, macaques were often placed in primate chairs with their heads fixed for extended periods of time (Benca et al., 2000; David et al., 1975), making it challenging for the subjects to sleep naturally and leading to alterations in the sleep-wake cycle (Bukhtiyarova et al., 2022). For that reason, the neural correlates of sleep were poorly explored trough only EEG-based methods, and, while informative, these studies can hardly be considered as representative of natural nighttime sleep patterns of freely behaving animals (Bert et al., 1970; Bouyer et al., 1978; Holcombe et al., 1979).

Recent technological advancements, such as the development of wireless telemetry systems, have revolutionized sleep research. These systems allow for the recording of EEG, EOG, and EMG data in unrestrained animals, providing a more accurate depiction of sleep architecture without any physical restraint (Hsieh et al., 2008; Rachalski et al., 2014), confirming remarkably similarities between macaques and humans sleep (Daley et al., 2006; Hsieh et al., 2008; Ishikawa et al., 2017), and opening new avenues to the investigation of the underlying cellular mechanisms by means of neuroethological approaches. Indeed, while telemetric EEG recordings are considered the gold standard for monitoring sleep in both humans and NHPs

(Goonawardena et al., 2019; Vuong et al., 2020), these techniques can only focus on large-scale cortical activity and not on single-neuron mechanisms.

1.2 The cortical motor system

Classical studies in neurophysiology allowed us to understand brain functioning and the relationship between neuronal activity and behaviors. Being phylogenetically close to humans, non-human primates' neural activity has been subject to a wide interest. Studies on the motor system have empirically revealed that movement is the result of extensive processes involving brain systems that continuously interact within a perception-action cycle, with crucial contributions from subcortical areas. Specifically, the direct control of movement is a function of the agranular frontal motor areas (Rizzolatti et al., 1998). Besides that, the so called *extended cortical motor system* includes a broader set of frontal as well as parietal sensorimotor regions, which in turn are connected to a variety of sensory areas (Matelli & Luppino, 2001; Rizzolatti & Matelli, 2003).

The primary motor cortex and the premotor cortex differ both anatomically and functionally. Neurons in the primary motor cortex are thought to primarily encode the parameters and details of simple movements, whereas PM cortex neurons seem to be involved in higher-order motor functions and even non-motor processes. Specifically, neurons in the premotor cortex contribute to sensory-motor transformations, converting the physical properties of objects into the appropriate sequence of actions to achieve specific goals during object-directed tasks (Gentilucci et al., 1988; Rizzolatti, 1988; Rizzolatti et al., 1988; Rizzolatti & Luppino, 2001). Moreover, these neurons may play a role in action recognition (Gallese et al., 1996; Umiltà et al., 2001) and decision-making processes (Pardo-Vazquez et al., 2008). In particular, the ventral portion of the premotor cortex, subdivided into areas F4 and F5, encodes goal-directed actions independent of the specific sequence of movements needed to achieve the goal (Umiltà et al., 2008) and is also involved in organizing defensive actions toward objects intruding into the subject's peripersonal space (Graziano et al., 2005; Graziano et al., 2002).

Premotor areas are integral to a series of parieto-frontal circuits that map a variety of sensory inputs to specific motor plans (Rizzolatti & Luppino, 2001). Classical studies have schematically organized these circuits into parallel pathways for different forelimb motor actions. For example, area F5 is heavily connected

with the anterior intraparietal area (*AIP*), forming the *"grasping circuit"* responsible for transforming the shape, size, and orientation of graspable objects into the appropriate hand and wrist postures needed to grasp and manipulate them. Area F4, connected to the ventral intraparietal area (*VIP*), underlies the *"reaching circuit"*, utilizing spatial information about objects to define the motor pattern required to reach or avoid them, often using a body-centered reference frame.

1.2.1 Neurophysiology and neuroanatomy of cortical motor system

The frontal lobe consists of two main sectors: *the rostral prefrontal cortex*, which is involved in higher cognitive functions, and *the caudal sector*, more directly involved in motor control. For centuries, it was believed that the human cerebral cortex was responsible solely for conscious and higher-order cognitive functions; however, in the mid-19th century, John Hughlings Jackson proposed that a specific region of the cerebral cortex, located rostrally to the central sulcus, played a significant role in initiating movement. This novelty was confirmed by Fritsch and Hitzig, who demonstrated that electrical stimulation of a cortical area in dogs triggered movements in the opposite side of the body, revealing a motor map crucial for voluntary control (Fritsch & Hitzig, 2009).

Advancements in anesthesia and aseptic techniques in the 1900s allowed direct experimentation on awake human subjects. Penfield and Boldrey extended these findings to humans through electrical stimulation during surgeries, confirming a motor region rostral to the central sulcus responsible for movement generation (Penfield & Boldrey, 1937). Subsequently, Woolsey identified a similar cortical map in monkeys, revealing a consistent somatotopic representation of motor functions across species (Woolsey et al., 1952).

Early 20th-century histological studies by Brodmann and Campbell categorized the primate motor cortex into the primary motor cortex (*Brodmann area 4*) and the premotor cortex (*Brodmann area 6*). Later on, the motor cortex of non-human primates has been anatomically divided into distinct areas based on their cytoarchitectonic features (Matelli et al., 1991). The primary motor cortex corresponds to area F1, while the premotor cortex is divided into three main regions: mesial (F3 and F6), dorsal (F2 and F7), and ventral (F4 and F5), each characterized by a rostral and caudal sectors (Matelli et al., 1985, 1991; Tanji et al., 1996).

The dorsal premotor cortex (*PMd*), located caudally to the superior arcuate sulcus, is subdivided into two main areas: F2 (caudal) and F7 (rostral) (*Figure 6*). Area F2 encodes arm and leg movements and integrates somatosensory feedback to guide these actions (Cisek & Kalaska, 2005), exhibiting a roughly somatotopic representation of the hand, wrist, and forearm along the rostro-caudal axis (Raos et al., 2003). In contrast, area F7 contributes to spatial coding and memory-guided motor planning, reflecting its involvement in higher-order motor processes (Pesaran et al., 2006). Unlike F2, F7 is less excitable and is hypothesized to support attention distribution, particularly through its involvement in eye movement control (Luppino et al., 1991).

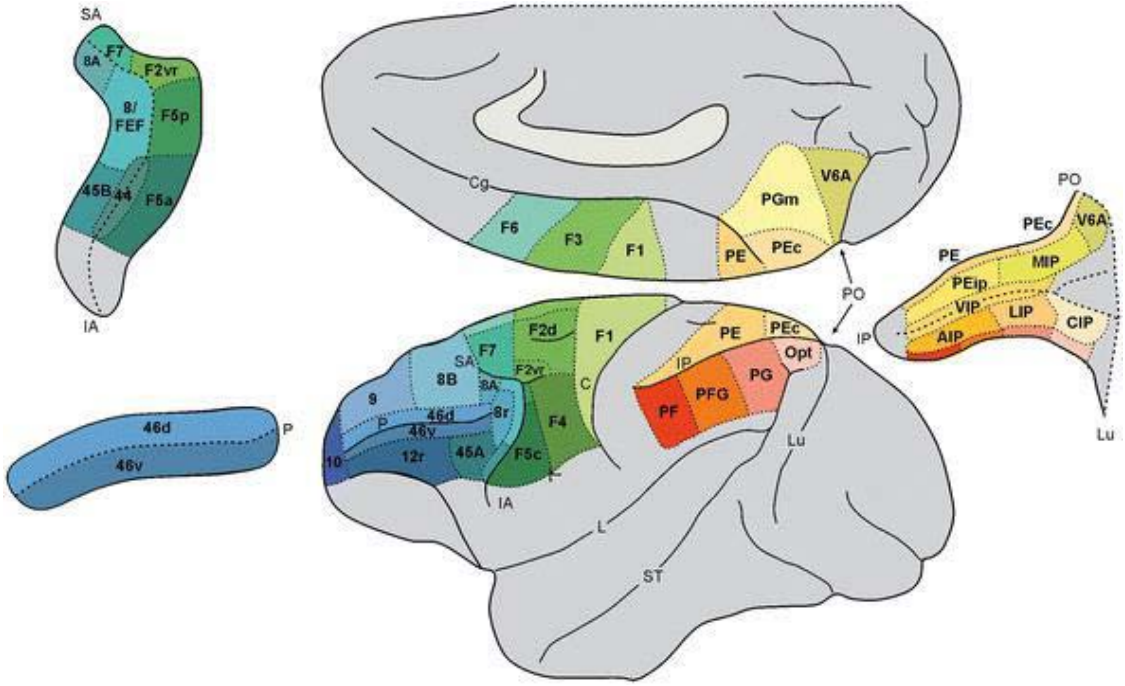

Figure 6 | **Cytoarchitectonic organization of the extended fronto-parietal motor system in the macaque.** The prefrontal cortex is subdivided according to Carmichael and Price (1994), the caudo-ventral part of prefrontal cortex according to Gerbella et al. (2007). Agranular frontal cortex is subdivided according to Matelli et al. (1991) and Belmalih et al. (2009). Parietal areas are classified according to Pandya and Seltzer (1982). *Figure modified by Gerbella et al. (2017).*

The ventral premotor cortex (*PMv*), located on the lateral cortical surface near the inferior arcuate sulcus, complements PMd by engaging in different aspects of motor control. Within PMv, areas F5 and F4 represent the rostral and caudal portions, respectively (*Figure 6*). Traditionally, distal movements were thought to be primarily represented in Broadmann Area 4 (*BA4*), or the primary motor cortex (*F1*), while proximal and axial movements were associated with BA6, located in the lateral and dorsal premotor cortex (Woolsey et al., 1952). However, further research on the PMv revealed that distal movements are also represented in BA6

(Rizzolatti et al., 1981a), and that neurons in the rostral part of BA6 respond to tactile stimulation of distal body parts (Rizzolatti et al., 1981b). In a paper published in 1988, Gentilucci and colleagues demonstrated that distal movements are represented near both the central and arcuate sulci, while proximal movements are primarily represented between these regions in both F4 and the rostral part of F1. In the ventral premotor cortex, representations of different effectors overlap, whereas they are more clearly segregated in F1 (Gentilucci et al., 1988).

Electrical stimulation with short trains of pulses delivered to the ventral premotor cortex evokes movements of the arm, mouth and face from the same cortical site (Maranesi et al., 2012), indicating a more complex organization of movement representation compared to the primary motor cortex, where stimulation more often recruits a single effector. Long-train intracortical microstimulation has been employed to better understand the cortical organization of motor maps (Graziano et al., 2005; Graziano et al., 2002). These results highlight a map of actions, such as "*reach to grasp*" or "*hand to mouth*" (Graziano & Aflalo, 2007), distributed along the ventro-dorsal extent of the premotor cortex. These maps of ethologically relevant actions in which the features of the evoked movement depend on the duration of the stimulation train, revealed a previously overlooked organizational principle of the PM cortex (*Figure 7*).

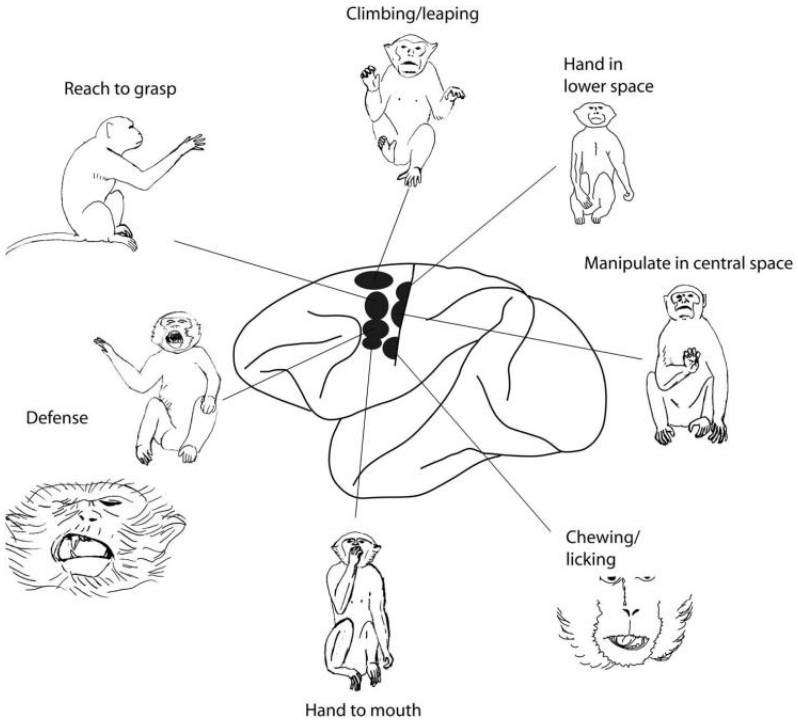

Figure 7 | **Action zones in the motor cortex of the macaque.** Thanks to intracortical stimulations, behaviorally relevant patterns were evoked. Each image shows the final posture of the monkey at the end of the stimulation-evoked movement Graziano and Aflalo (2007).

Anatomically, BA6 can be divided into two sectors based on their connectivity with different brain regions (Borra et al., 2017; Rizzolatti et al., 2014): prefronto-dependent and parieto-dependent motor areas. Prefronto-dependent motor areas are located more anteriorly, including areas F6 and F7, and mainly connected to the prefrontal cortex. They receive input from the prefrontal cortex (Caminiti et al., 2017; Gerbella et al., 2012), contribute to encoding the context and motivation underlying action selection, and determine when and whether potential motor actions should be executed (Rizzolatti & Luppino, 2001). Parieto-dependent motor areas, instead, are placed posteriorly, from F1 to F5, and connected to parietal areas. They receive sensory input from different parietal regions and are involved in sensory-motor transformations, action recognition, and peripersonal space representation (Borra et al., 2017; Gerbella et al., 2017; Schaffelhofer & Scherberger, 2016).

Areas F1, F3, and part of F2, primarily rely on somatosensory information for sensorimotor transformations, while F4, F5, F6, and the rostro-ventral part of F2 utilize both somatosensory and visual information. Area F5 is reciprocally connected with AIP, forming the core of the grasping circuit. In this circuit, areas V6A (Fattori et al., 2001) and F2vr (Raos et al., 2004) provide a more dorsal component, together with

the mesial presupplementary motor area F6 (Albertini et al., 2020; Lanzilotto et al., 2016; Livi et al., 2019). These cortical nodes use the physical properties of objects to select and activate appropriate motor plans, with different nodes exhibiting specific coding principles (Gerbella et al., 2017). Area F4 receives inputs from VIP, which transform the spatial characteristics of objects into the correct motor plan for reaching them (Matelli & Luppino, 2001; Rizzolatti & Matelli, 2003). This circuit also involves the dorsal premotor area F2 and its parietal targets in the inferior and superior parietal lobules (Gamberini et al., 2009; Rozzi et al., 2006). Similar to F4, VIP contains neurons that respond to visual stimuli, as well as bimodal and trimodal neurons (Colby et al., 1993; Duhamel et al., 1992; Schlack et al., 2005). The F4-VIP circuit is thought to be involved in defensive mechanisms and encoding peripersonal space (Cléry et al., 2015). Intracortical microstimulation of VIP has been shown to evoke defensive-like movements, as previously reported for F4 (Cooke & Graziano, 2004). When VIP is stimulated, the monkey blinks, lifts its upper lip, and makes various arm movements (Graziano et al., 2004). Specifically, VIP may play a role in constructing a head-centered representation of the environment, while F4 may be more involved in generating defensive and avoidance actions.

1.2.2 Sensorimotor transformations for actions and reactions: peripersonal, canonical, and mirror neurons

The area F4 in the ventral premotor cortex contains representations for movements of the trunk, arm, and mouth (Gentilucci et al., 1988). Intracortical microstimulation studies confirm this organization, with arm, axial, and proximal movements represented dorsally and mouth movements ventrally (Maranesi et al., 2012).

Three types of neurons have been identified in F4: somatosensory, visual, and bimodal neurons. Somatosensory neurons respond to tactile stimuli on the face, neck, arms, or hands (Rizzolatti et al., 1981b). Visual neurons respond to objects moving close to the body. Bimodal neurons respond to both tactile stimuli applied to specific body parts and to visual stimuli (Fogassi et al., 1996), moved around the subject in its peripersonal space (Gentilucci et al., 1983), typically with a clear spatial relationship between the somatosensory and the visual receptive field. Neurons with tactile receptive fields (*RF*) on the arms or hands typically have visual RFs anchored to the corresponding body part, suggesting that F4 neurons operate in a body-centered frame of reference (Graziano et al., 1994). The visual RFs remain stable even regardless of eye position, as demonstrated by Fogassi and coworkers, with only 10% of neurons exhibiting a retinocentric visual

RF (Fogassi et al., 1996). Additionally, some F4 neurons are trimodal, integrating auditory information as well. These neurons encode the auditory stimuli coming from the same visuo-tactile spatial sector, providing a multisensory representation of the nearby environment (Graziano et al., 1999), likely to direct and coordinate avoidance reactions toward external stimuli.

The rostral portion of PMv, known as area F5, is further subdivided into three distinct sectors: F5p, F5a, and F5c, each exhibiting slightly different functional properties as well as functional similarities. In general, area F5 encompasses representations of both hand and mouth movements: the hand is represented primarily in the rostral and dorsal regions, while the mouth occupies the ventral and caudal portion, with significant overlap between the two (Maranesi et al., 2012). Classical studies have identified three main types of neurons in F5: purely motor neurons, canonical neurons (mainly in F5p/a), and mirror neurons. Since the pioneering studies on this area, it emerged that neurons seem to encode the goal of the actions, not single movements (such as extension or flexion of a joint) constituting it; indeed, F5 neurons could respond when the monkey grasped food morsels regardless of the hand used (ipsilateral or contralateral) and of the direction of the reaching movement toward them (Rizzolatti, 1988). Additionally, some F5 neurons discharge independent of the effector used (e.g., hand or mouth) (Bonini et al., 2011) or the specific movement sequence employed to attain a goal (e.g., grasping objects with tools that require opposite sequence of muscle activation) (Umiltà et al., 2008). Some F5 neurons also specify the type of grip, such as whole-hand, precision grip, or finger prehension, and can be modulated by the action context in which the grip is performed (e.g. to eat the target or to place it into a container) (Bonini et al., 2012).

According to the original definition, canonical neurons exemplify the “canonical” visuomotor transformation necessary for grasping objects: they respond to the visual presentation of three-dimensional stimuli as well as during the preparation and execution of a reaching-grasping actions directed to them (Murata et al., 1997). Initially, it was believed that these neurons maintained visuomotor selectivity from the visual presentation of the target to the end of the grasping action (Murata et al., 2000), facilitating the transformation of an object’s physical properties into the necessary motor actions. Subsequent multiarea chronic recording studies emphasized the fact that the stability of the visuomotor selectivity at the single neuron level is neither an important nor a frequent property of visuomotor, so-called “canonical”, neurons in AIP and F5. Indeed, the population activity in area AIP reflects visual similarities between various objects, whereas F5 encodes the

most prominent similarities in hand shape required for grasping. These findings indicate that the visuomotor transformation occurs across a network of multiple areas, including the primary motor cortex, which finally reflect the hand posture required to perform the action (Schaffelhofer & Scherberger, 2016).

One of the most popular and intriguing classes of visuomotor cells discovered in the premotor cortex is represented by “mirror neurons”, originally discovered in area F5, and firing during the execution of an action (in the light as well as in the dark) and during the observation of an action performed by another agent. Initially, it was claimed that these cells implied the observation of the same or a similar action relative to the one motorically encoded to become active, and that they did not respond to the simple sight of a graspable object or of the motion of a non-biological effector, such as a tool. Based on these findings, it was proposed they constitute a neural mechanism for mapping observed action onto their corresponding motor representation, thereby playing a possible role in action recognition (di Pellegrino et al., 1992; Gallese et al., 1996; Rizzolatti et al., 1996). Subsequent research has expanded this idea, suggesting that mirror neurons encode the goal of both self-performed and observed actions, even when the action is partially hidden (Umiltà et al., 2001), when it is recognized only by its sound (Kohler et al., 2002), or when inferred from contextual (Bonini et al., 2010) or arbitrarily learned (Bonini et al., 2014b; Maranesi et al., 2015) information previously associated with a given motor goal.

The presence of sensory and motor responses of canonical, peripersonal and mirror type at the level of the same single neurons (Bonini et al., 2014a), suggests however a common underlying mechanism shared by all these apparently different types of cells: transforming physical or social stimuli into potential motor responses (Bonini & Ferrari, 2024; Bonini et al., 2023; Orban et al., 2021). Indeed, by recording multiple neurons simultaneously using linear probes, it has been revealed that canonical/mirror neurons exist, which respond to both observed and executed actions as well as to visually presented object depending on the space sector relative to the monkey in which they were presented (Caggiano et al., 2009; Maranesi et al., 2017), indicating that neurons once thought to be specialized for action recognition might contribute to action planning as well (Bonini et al., 2014b). In particular, the coding of pragmatic variables - such as object affordances - by these neurons aligns with the concept of “social affordances”, where observed actions afford specific motor responses during social interactions (Bonini et al., 2022).

1.2.3 The cortical motor system during sleep

Since Evarts' groundbreaking work in 1966 (Evarts, 1966), extracellular recordings of action potentials in awake, behaving primates have significantly advanced our understanding of the neural control of movement, promising the development of interventions for patients with motor deficits (Donoghue, 2002). However, despite extensive research on the motor system during wakefulness, growing interest in sleep research - particularly in studies of natural sleep in freely moving non-human primates (Bukhtiyarova et al., 2022; Hall et al., 2014; Jackson et al., 2007; Xu et al., 2019; Yun et al., 2023) - has brought a new focus to the behavior of motor-related structures during sleep, and in particular the primary motor cortex (M1).

EEG studies on unrestrained macaques have investigated phenomena such as sleep spindles, which are sometimes temporally or spatially synchronized with other cortical activities, including delta and gamma waves originating from different brain regions, such as motor areas (Takeuchi et al., 2016). These synchronized patterns across regions are believed to facilitate inter-regional information transfer and contribute to memory consolidation (Canolty & Knight, 2010). Notably, during REM sleep, muscle activity in the posterior neck muscles of macaques remains consistently inhibited due to sleep-related atonia, as confirmed by EMG recordings (Hsieh et al., 2008), despite heightened cortical activity.

Switching to intracortical recordings, sleep studies on rats have demonstrated that neurons in the motor cortex, particularly during SWS, display firing patterns that closely resemble those observed during wakeful motor tasks, highlighting the role of sleep in consolidating motor memories (Pavlides & Winson, 1989; Ramanathan et al., 2015; Ribeiro et al., 2004). Similar findings have been observed in humans, where increased correlations between firing rate patterns of repeated motor sequences from pre- to post-task rest periods support the occurrence of motor replay, sometimes at speeds 1-4 times faster than the original task execution (Eichenlaub et al., 2020; Rubin et al., 2022).

Jackson et al. (2007) conducted a landmark study, being one of the first to use a wireless neural recording system to capture intracortical activity from neurons in the macaques' primary motor cortex during sleep. The study revealed cyclical activity patterns in M1 across different sleep states, with reduced neuronal firing during SWS and bursts of activity during REM sleep - a finding recently corroborated by other researchers (Yun et al., 2023). Notably, the study also showed that the relationship between motor cortex neurons and muscle activity persisted throughout sleep, although these correlations weakened during REM.

This highlights the idea that M1 neurons maintain functional connectivity with motor outputs, even in the absence of voluntary movement during sleep (Jackson et al., 2007).

More recent research has confirmed these findings, demonstrating strong synchrony between M1 neuronal activity and slow-wave oscillations during NREM sleep in both rhesus macaques (Xu et al., 2019) and marmosets (Bukhtiyarova et al., 2022). Neurons exhibited phase-locking to low-frequency waves, reinforcing the idea that motor-related structures remain actively engaged during sleep (Xu et al., 2019). This is consistent with earlier work on cortical up/down states during SWS in other species, such as cats (Steriade et al., 1993b; Steriade et al., 2001), although the earlier studies relied on EEG methodologies.

1.3 Aims of the study

The literature so far reviewed suggest that premotor neurons may be articulated into different subpopulations, with their input and output connectivity, forming distinct but intertwined cortico-cortical and cortico-subcortical networks supporting visuomotor transformation for acting on the outside world and reacting to other subjects. An intriguing and completely unexplored issue is whether, and to what extent, the firing properties of neurons belonging to different functional classes are similarly or differently affected by different sleep stages.

To address this issue, we first developed a methodology to classify wakefulness and sleep exclusively based on intracortical recordings. Next, we aimed at leveraging this methodology to explore the firing properties of functionally classified motor and visuomotor neurons during natural, unperturbed sleep.

2. Material and methods

2.1 Experimental subjects

This study involved two purpose-bred, male adults rhesus macaques (*Macaca mulatta*, Mk1, 11 years, 13 kg; Mk2, 13 years, 14 kg), individually housed following veterinary advice in a facility with other macaques with which they always maintained visual, auditory, and olfactory contact. Recorded animals were housed in 12h/12h light/dark regimen with lights off at 7 p.m.

The animals have been first trained to interact and collaborate with researchers, and then familiarized with the process of entering and sitting in a primate chair that was later moved from the animal facility to the laboratory. Next, they were surgically implanted with a titanium head-post for subsequent head-fixed testing of neuronal properties in simple visually-guided grasping tasks and observation of similar tasks performed by the experimenter.

The training procedures were carried out by means of operant conditioning with positive reinforcement and step-by-step response shaping methods. To encourage desired behaviors that matched or resembled the target behavior, liquid rewards like fruit juice or water were provided, while undesired behavior were not rewarded and therefore gradually extinguished. At the end of each session, fresh pieces of fruit and pellet mush were given to strengthen cooperation and create as much as possible positive experience for the animals with the training. Training sessions were carried out daily to establish a predictable routine and help the animals to become accustomed to it.

Both monkeys underwent surgeries in deep anesthesia and aseptic conditions to implant on their skull a head-post, the recording chamber and the intracortical chronic electrodes. For all the surgeries, animals were prepared for the anesthesia with atropine administration (0.03 mg/kg) 15 minutes prior to the induction of anesthesia. Next, anesthesia was induced with ketamine (4.5 mg/kg) and medetomidine hydrochloride (0.05 mg/kg) and maintained with 2% isoflurane vaporized in 100% oxygen. To collect neuronal signals, each monkey was implanted with a biocompatible plastic recording chamber (4.5 × 5 × 2.5 cm) endowed with a grid of parallel grooves (width 2 mm, inter-groove distance 3 mm) for housing the Omnetics connector blocks (*Figure 8A*), subsequently sealed into a protective cap during the wireless recordings (*Figure 8B*). These

connectors interfaced the multielectrode arrays and the headstages (Deuteron Technologies Ltd) for wireless data logging (*Figure 8C*).

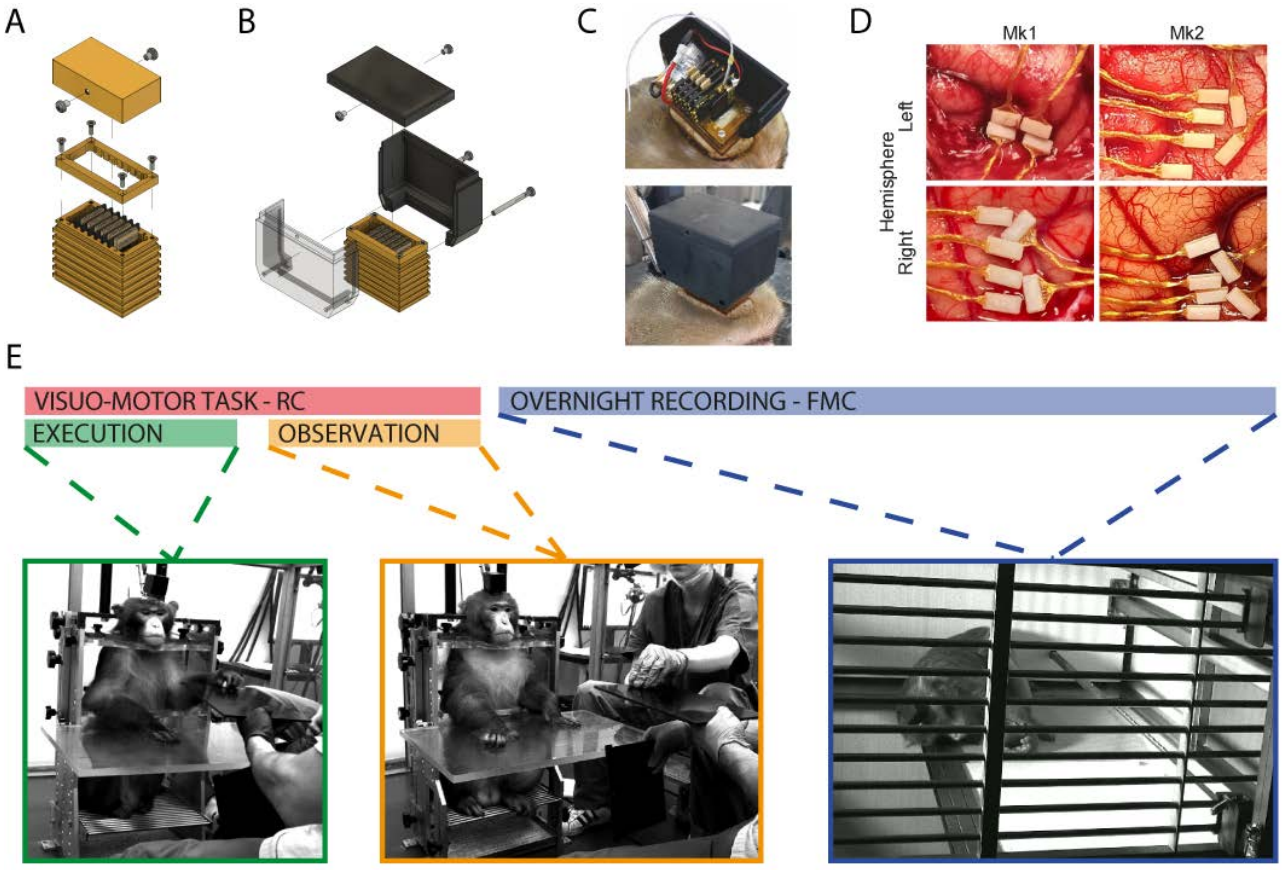

Figure 8 | **Neuro-behavioral platform enabling recording of premotor neurons.** A: recording chamber design, configured for normal monkey housing. B: Recording chamber with head-cap configuration used during neural recordings. C: Open (top) and close (bottom) head-cap before a freely-moving recording session. D: Multielectrode arrays implanted in Mk1 and Mk2 in the left and right hemispheres. E: Temporal sequence of daily (RC, restrained condition in laboratory settings) and overnight (FMC, freely moving condition in monkey's home-cage) recordings

Animals were chronically implanted with 32-channels floating multielectrode arrays (*FMA*, MicroProbes, 2.5-, 3-, 4- and 5-mm lengths, recording from the tip) placed to cover the entire cortical convexity extending from the inferior arcuate sulcus to the central sulcus (*Figure 8D*). Each hemisphere was implanted with a variable number (from four to six) of FMAs, ensuring an overall coverage of most of the ventral premotor areas F4 and F5 in both hemispheres, in addition to some sectors of M1. During the surgery, the visual identification of the main anatomical landmarks (superior and inferior arcuate sulci, the principal sulcus and the central sulcus) guided the targeting of the implantation site of the arrays in the premotor cortex. Following array implantation, animals underwent at least two weeks of recovery before the recordings started.

All experimental protocols complied with the European and Italian law on the protection of animals used for scientific purposes (2010/63/EU and Dlgs 26/2014), were approved by the Animal Welfare Body of the University of Parma and authorized by the Italian Ministry of Health.

2.2 Apparatus and behavioral paradigm

We conducted 32 overnight recordings in freely moving condition, 28 of which preceded by daytime recordings in laboratory settings, either head-free (n=9) or head-fixed (n=19), while the subject was sitting in a primate chair and performed a reaching-grasp visuo-motor task.

In the chair condition, the subject was trained to remain still while a food stimulus was presented. Upon the presentation of the food within monkey's reaching distance, it was required to execute a grasping movement with either the right or left hand and to convey the food to its mouth, or, alternatively, to remain still and observe the action performed by an experimenter (*Figure 8E*). Each action was repeated for a minimum of 11 trials with each forelimb. Recording sessions before nocturnal recordings lasted an average of 32:21 min ± 2 min per session. During the nocturnal recordings, the subject was allowed to freely move and sleep within its cage (*Figure 8E*). Recordings ended in the early morning, lasting an average of 8 hours and 21 minutes ± 10 minutes.

The experimental apparatus consisted of three laptops, a wireless neural data logger (*Deuteron Technologies*) to record neural activity, an infrared-sensitive camera with frame rate up to 100 Hz (*Basler acA2040-120um*, 2048 x1536 pixels resolution), and a dedicated video recording software (*Basler Video Recording*). Additionally, an audio interface (*Behringer UMC-404HD*) connected to a pair of stereo microphones (*Thomann tbone sc140*) recorded environmental noise. The entire system was controlled by *NIMH MonkeyLogic* (Hwang et al., 2019), a MATLAB-based package. Through a USB National Instrument DAQ board (*NI USB-6001*), the software transmitted transistor-transistor logic (*TTL*) signals to the neural activity recording software via a transceiver, and a 5V square wave at 50/100 Hz to the video recording system, enabling synchronization of neural data with audio and video recordings.

Macaques' behavior in both chair and freely moving conditions was analyzed using Behavioral Observation Research Interactive Software (*BORIS*) (Friard & Gamba, 2016), performing a frame-by-frame

scoring of the subject's behavior with a temporal resolution of 10 ms (for 100 Hz recordings) or 20 ms (for 50 Hz recordings). After reviewing many video recordings, we set an ethogram that grouped all behaviors relevant to analyze neuronal and LFP properties during the session (*Table 2-3*). To ensure accuracy and reliability of the behavioral scoring, some video recordings were independently reviewed by two observers, and the inter-rater reliability was assessed using the Cohen's kappa statistic.

Behavior	Description and Coding
Execution Task	
Food Reach – Left/Right Arm	Monkey approaches the food with its left/Right arm. Aligned with the moment when the arm begins to move towards the food.
Food Grasp – Left/Right Hand	Monkey grasps food pieces with the left/right hand. Aligned with the moment when the hand makes contact with the food.
Food to the Mouth – Left/Right Hand	Monkey actively places the food into its mouth with the left/right hand. Aligned with the moment when the food touches the mouth or, if not visible, when the hand stops advancing toward the mouth.
Observation Task	
Food Reach - Experimenter	Experimenter approaches the food with his right arm moving from the contralateral monkey's hemifield to the central position, out of monkey's reaching distance. Aligned with the moment when arm begins to move towards the food.
Food Grasp - Experimenter	Experimenter grasps food pieces with his right arm moving from the contralateral monkey's hemifield to the central position, out of monkey's reaching distance. Aligned with the moment when hand makes contact with the food.
Food to the Mouth - Experimenter	Experimenter actively places the grasped food into his mouth. Aligned with the moment when the hand stops advancing toward the mouth.

Table 2 | **Visuo-Motor task ethogram.**

Behavior	Description and Coding
Body States	
General Body Movements (GBM)	The monkey performs large movements with its body that involve two or more body parts, isolated or synergistically combined, such as changing posture, walking, climbing, yawning. The first frame where such movements are observed is marked as the onset of a state event continuing until the animal ceases movements or enters in a quiet state.
Subtle Body Movements (SBM)	The subject performs subtle movements with a single body part, such as tail, ears, mouth, arm, leg and body twitches. The first frame where such movements are observed is marked as the onset of a state event continuing until the animal ceases movements or enter in an active or immobile state.
Immobile (I)	The subject is motionless, with no part of its body in movement. The first frame where the animal is seen completely still is marked as the onset of a state event continuing until any movement is detected.
Eyes State	
Eyes Opened (EO)	The subject's eyes are visible and open. The first frame where the eyes are fully open is marked as the onset of a state event continuing until the eyes close or become not visible (undefined state).
Eyes Closed (EC)	The subject's eyes are visible and closed. The first frame where the eyes are closed is marked as the onset of a state event continuing until the eyes open or enter in a non-defined state.
Closed Eye Movements (CEM)	The subject shows eye movements visible while the eyes are closed. The first frame where eye movements are observed under closed eyelids is marked as the onset of a state event continuing until the eye movements stop, the eyes open, or an undefined state starts.
Eyes State undefined (END)	The subject's face is out of sight, or it is impossible to determine the state of its eyes. The first frame where it is not possible to determine if the eyes are opened or closed is marked as the onset of a state event continuing until the eyes are clearly opened or closed.

Table 3 | **Overnight sleep recordings ethogram.**

2.3 Wireless neural recordings

Recording sessions were conducted under two distinct conditions: awake sessions were carried out with the macaque restraint in a primate chair, while overnight recordings took place in the home-cage, allowing the animals to freely move and sleep without any constraint.

For both conditions, recordings were conducted using one, two, or four 32-channel FMAs simultaneously, utilizing a 32-, 64-, or 128-channel neural data logger synchronized with the other recording devices throughout the session by means of a transceiver communicating with the logger and connected to a PC. The neural signals were sampled simultaneously from all channels at 32 kHz and band-pass filtered (1 - 7000 Hz), capturing both Local Field Potentials (*LFPs*) and single/multi-unit signals, subsequently analyzed using MATLAB and Python. These signals were amplified, digitized, and stored locally on a microSD memory card connected to the logger, and the system was powered by a compact Li-ion battery. All components of the data logging system were housed within a cover secured on top of the chamber and could be switched on or off with a magnetic wand without physically touching the animal even when the device was sealed and the monkey was in its home cage (*Figure 8B-C*).

2.4 Local field potentials analysis

The broadband raw data obtained from the neural logger were down-sampled from 32 kHz to 1 kHz and cleaned by removing positive and negative peaks exceeding a threshold defined by the median absolute deviation (*MAD*). To assess LFP modulation, we applied a multitaper spectral analysis using the MATLAB-based *CHRONUX* toolbox (http://chronux.org/). A Fourier transform was applied to each 10-second non-overlapping window to extract spectral components across the following frequency bands: Delta – 1-4 Hz; Theta – 4-7 Hz; Alpha – 7-15 Hz; Beta – 15-31 Hz; Gamma – 31-40 Hz (Xu et al., 2019). The power for each frequency band was normalized to their respective maxima and minima. The resulting time-frequency plots over the entire recording sessions for each channel were then visually inspected by two independent expert observers to identify qualitatively invalid channels because of possible mechanical instability of the signal and/or lack of signal due to artifacts or malfunctional electrodes. All channels exhibiting no modulation or non-physiological patterns by at least one of the observers (inter-observer concordance rate 93.5%) were excluded from the analysis (on average 20% of the channels of each session).

With a data-driven approach, we identified and examined brain states from biological signals recorded throughout the night. We used *K-means* for clustering data point in the dataset corresponding to the power in each of the 5 LFP frequency bands, and the multiunit activity (*MUA*) in all 10-second bins of the overnight

recording session. Since the K-means algorithm requires user-specified parameter *k* identifying the desired number of cluster to be identified in the data (Ikotun & Ezugwu, 2022), we imposed *k*=6 assuming a correspondence between monkey and human brain states, namely: 1) active wakefulness, 2) drowsiness, 3) NREM1, 4) NREM2, 5) NREM3 and 6) REM sleep.

To gain deeper insights into the neurophysiological and behavioral fingerprints of each identified cluster and their correspondence with distinct sleep stages, we averaged the spectral power of all bins belonging to a given cluster and, for each one, we calculated the percentage of behavioral occurrences. Moreover, the temporal transitions among the 6 brain states were also analyzed by a stochastic model (Markov Chain Analysis), delineating the sequential occurrences of these states throughout the night.

To ensure a more accurate representation of the frequency spectrum for each identified cluster and to consider only periods clearly corresponding to NREM/REM sleep, drowsiness, and wakefulness, we included in the analyzed clusters associated to sleep phases only epochs consisting of at least four consecutive bins (40 seconds) that were not less than 180 seconds apart from the preceding wakefulness period, the latter determined through behavioral scoring. Additionally, to address the nuanced nature of state transitions, which can occur at any point within a 10-second bin, we excluded the final bin of each sequence to minimize the impact of state-to-state transitions. Excluding periods based on these criteria led to a stronger correspondence and more reliable alignment between neural activity and behavioral data, without altering the overall LFP fingerprint of the distinct phases performed without this refinement.

Relying on the parameters described by the AASM manual for sleep scoring (Berry et al., 2020; Iber et al., 2007) and the animal's behavior, we assigned to each cluster the correspondent brain state.

2.5 Spike sorting and data analysis

All neural data from both chair and freely moving recordings were processed together to characterize neurons across the entire experimental session based on their firing properties, and to analyze their behavior during different brain states. Signal analyses were performed offline using the fully automated software Mountain Sort (Chung et al., 2017). A threshold of -3.0 standard deviations from the signal-to-noise ratio was applied for detecting spikes from the raw signals. Spikes belonging to single units (*SUA*) were distinguished from

multi-unit activity (*MUA*) using a noise overlap parameter, with values below 0.2 assumed to correspond to single units, and the remaining detected spikes were considered as MUA. Single-unit isolation was further verified using standard criteria. On all the originally identified waveforms, first, we discarded any unit exhibiting clearly non-physiological waveform shape; second, we merged units isolated in the same channel that exhibited the same waveform shape and ISI distribution but slightly different amplitude due to progressive drift along the session; third, we verified that the inter-spike interval (ISI) distribution of the extracted single units fulfilled the criteria of a refractory period longer than ~1 ms. The waveforms that could not be clustered into a well-isolated and stable unit, together with the rejected units among those initially identified based on the above-described criteria (27.5% of the total initially isolated unit) constituted the multiunit activity. Among the excluded units, 4% consisted of well-isolated neurons that were resampled from the same channel across different sessions, as demonstrated by stable spike shape and firing properties during both VMT and overnight recordings. For each SUA and MUA detected, the absolute and normalized firing rate was computed using 10-seconds windows during the overnight recording phase.

Each SUA extracted underwent a cleaning process using 4 standard deviations from the averaged sorted waveform as threshold (2-3% of spikes discarded, on average). Next, every spike shape was sampled at 96 points centered around its negative peak, yielding a 3 ms waveform with 1 ms before and 2 ms after the peak. For each isolated unit, we confirmed its temporal stability by projecting the time stamp of each spike throughout the entire session, to evaluate the firing stability along the session in 10 second time bins. Units with highly unstable activity or suddenly lost were excluded from the analysis. Since we recorded from the same implanted probe across multiple days, although temporal separation of subsequent recording sessions from the same probes by several days/weeks generally ensured the sampling of different units, we checked for possible consistency in waveform shape, temporal firing pattern, and response properties during the visuo-motor task and the sleep phases, excluding neurons that exhibited consistent spike shapes and response profiles across sessions from the same electrode were considered only once to avoid resampling biases. The waveforms for each neuron were averaged to obtain a mean shape, and, then, every mean shape was normalized to a range between +1 and -1.

We then employed *WaveMAP*, a waveform clustering method developed to identify putative cell types based on spike shapes (Lee et al., 2021; Lee et al., 2023) leveraging both *UMAP* and *Louvain clustering*. This

method is based on three stages of analysis: *1)* the averaged normalized waveforms are processed through UMAP, a non-linear dimensionality reduction technique (McInnes et al., 2018), to generate a high-dimensional graph; *2)* the UMAP output is fed into a Louvain clustering (Blondel et al., 2008) to delineate high-dimensional clusters, maximizing intra-cluster similarity and inter-cluster differences; *3)* A reverse UMAP was applied to project the clustering result into a two-dimensional space, allowing for a classification of neurons based on their waveform shapes, from the narrowest to the broadest one.

To investigate the relationship between neuronal firing rates and wake-sleep cycle oscillations, we correlated single-unit firing rate with delta frequency time-modulations. Given that, generally, slow-wave sleep (*SWS*) is characterized by high delta power whereas REM and wakefulness exhibit reduced delta power (Berry et al., 2020), we used *Kendall's tau correlation* ($p < 0.05$) to assess the relationship between the neuronal firing rate and the average delta power within each 10-second bin across all channels of an overnight recording session. To ensure robustness, we also checked the relationship between delta power and single neuron firing rate by comparing the firing rate associated with the bins with a delta power ≥ average delta power + 1SD, and bins with a delta power ≤ average delta power -1 SD (*Wilcoxon rank-sum test*, $p < 0.05$). This dual and stringent approach enabled us to classify neurons into three distinct categories based on their relationship with delta oscillations: 1) *Delta-Positively Correlated neurons* (*DPC*), which showed significant and positive τ and z values; 2) *Delta-Negatively Correlated neurons* (*DNC*), which showed significant and negative τ and z; 3) *Delta-Uncorrelated neurons* (*DU*), when one or both the above-mentioned criteria were not satisfied.

Neuronal activity was synchronized with behavioral events recorded via a video camera and marked during frame-by-frame video analysis with BORIS software. We identified the following distinct epochs of interest relative to the main scored behavioral events: 1) *baseline epoch*, ranging from 1100 to 700 ms before the reaching onset, while the monkey was still in the inter-trial period; 2) *pre-movement epoch*, corresponding to the 400 ms interval before the reaching onset, when the target was presented; 3) *reaching epoch*, corresponding to the interval from the first frame in which the hand begins moving toward the target to the first frame in which the hand makes contact with the target; 4) *grasping-holding epoch*, ranging from the first frame in which the hand is in contact with the food to the first frame in which the food makes contact with the mouth.

We analyzed neurons' functional properties in relation to the baseline epoch while the monkey executed or observed the same reaching/grasping task. For this purpose, we performed a one-way *repeated measures ANOVA*, separately for each task context (execution or observation), examining the response across the 4 epochs (baseline, pre-movement, reaching, and grasping/holding). To ensure a restrictive classification and define neurons as “purely motor” in the absence of modulation in the visual counterpart, and conversely as “purely visual” in the absence of modulation in the motor counterpart, thereby avoiding potentially misleading inferences about mixed neurons, we employed a two-step criterion. First, a neuron was considered modulated for a given action if the ANOVA yielded a significant main effect ($p < 0.001$). Subsequently, we implemented a secondary significance threshold ($p > 0.1$), such that neurons were categorized as follows: 1) *motor neurons*, showing significant modulation relative to baseline ($p < 0.001$) during execution but not ($p > 0.1$) during observation task; 2) *visual neurons*, showing significant modulation ($p < 0.001$) in observation but not ($p > 0.1$) during execution task; 3) *visuomotor neurons*, showing significant modulation ($p < 0.001$) in both execution and observation tasks; 4) *task-unrelated neurons*, showing no modulation ($p > 0.1$) in both execution and observation; 5) *unclassified neurons*, which did not fit with the above (37% of the total neurons isolated).

2.6 Population analysis

To assess whether and how functionally defined classes of neurons discharge differently throughout the wake-sleep cycle, in a subset we investigated their firing rate changes across the previously defined brain states (*see Local field potentials analysis*).

After describing every neuron through its averaged firing pattern in each six brain state, we applied 3D t-distributed stochastic neighbor embedding (*t-SNE*) (van der Maaten & Hinton, 2008) to visualize their distribution in a 3-dimensional space, depending on the behavioral properties associated to delta oscillations and visuo-motor task. This technique reduces a set of high-dimensional points to three dimensions, such that close neighbors are attracted to each other and remain close, whereas distant points are repelled from each other and remain distant.

Then, assuming that the closest neurons in a n-dimensional space - in which *n* is the number of neurons whose temporal discharge profile - is considered have similar firing profile, we used a K-Means algorithm to

cluster the neurons based on their response similarity, using a data-driven unsupervised gap statistic to evaluate the optimal number of clusters (Tibshirani et al., 2001). The gap statistic compares the total intra-cluster variation for different values of k with their expected values under null reference distribution of the data (i.e., a distribution with no obvious clustering). A t-SNE was then used to reduce the dimensionality to 3-dimensions. Within each identified cluster, the distribution of spike shapes and functional properties of the neurons has been compared to that of the overall population using χ^2 *test* ($p < 0.05$). Additionally, the discharge profile of each cluster identified was computed, and significant differences in brain states' firing rate was evaluated using a *Kruskal-Wallis* non-parametric test (Kruskal & Wallis, 1953), followed by a *Dunn* post hoc test.

3. Results

We collected behavioral data and neural signals from 128 (n=9), 64 (n=5) or 32 (n=18) simultaneously recorded channels across 32 recording sessions (totaling 1645 recorded channels). From these sessions, we extracted LFPs, MUA, and single-unit activity, resulting in 398 well-isolated and temporally stable neurons (see *Spike sorting and data analysis*). Furthermore, prior to most of the overnight recording sessions (28 out of 32), we carried out formal testing of single neuron responses during the execution and observation of a visuomotor task (VMT) with monkey in a classical chair condition (RC, *Figure 8E*), typically used for neurophysiological experiments.

During the VMT, the execution and observation trials were administered in a randomized order, and the behavioral events of interest were subsequently scored offline with dedicated software (see *Apparatus and behavioral paradigm*). Next, we could monitor the activity of the same neurons (303 out of 398) while the monkey naturally slept in its home cage in a freely moving context (FMC, *Figure 8E*).

3.1 Intracortical signatures and classification of sleep stages

3.1.1 Dynamic changes of LFPs and MUA throughout wakefulness and sleep

We utilized intracortical neural signals recorded throughout the night, specifically local field potentials (LFPs) and multi-unit activity (MUA), to identify various brain states, including wakefulness and putative slow-wave (SWS/NREM) and REM sleep.

Consistent with previous findings in humans (Hayat et al., 2022), we observed recurrent and synchronized patterns within the 1–40 Hz range in the LFP time-frequency spectrogram, across channels and throughout the night (*Figure 9A*). These changes are evident in the normalized power spectrum across key frequency bands (delta/δ: 1-4 Hz, theta/θ: 4-7 Hz, alpha/α: 7-14.9 Hz, beta/β: 15-31 Hz, gamma/γ: 31-40 Hz), identifying repetitive fluctuations in both low and high-frequency rhythms which are persistent in all channels (*Figure 9B*).

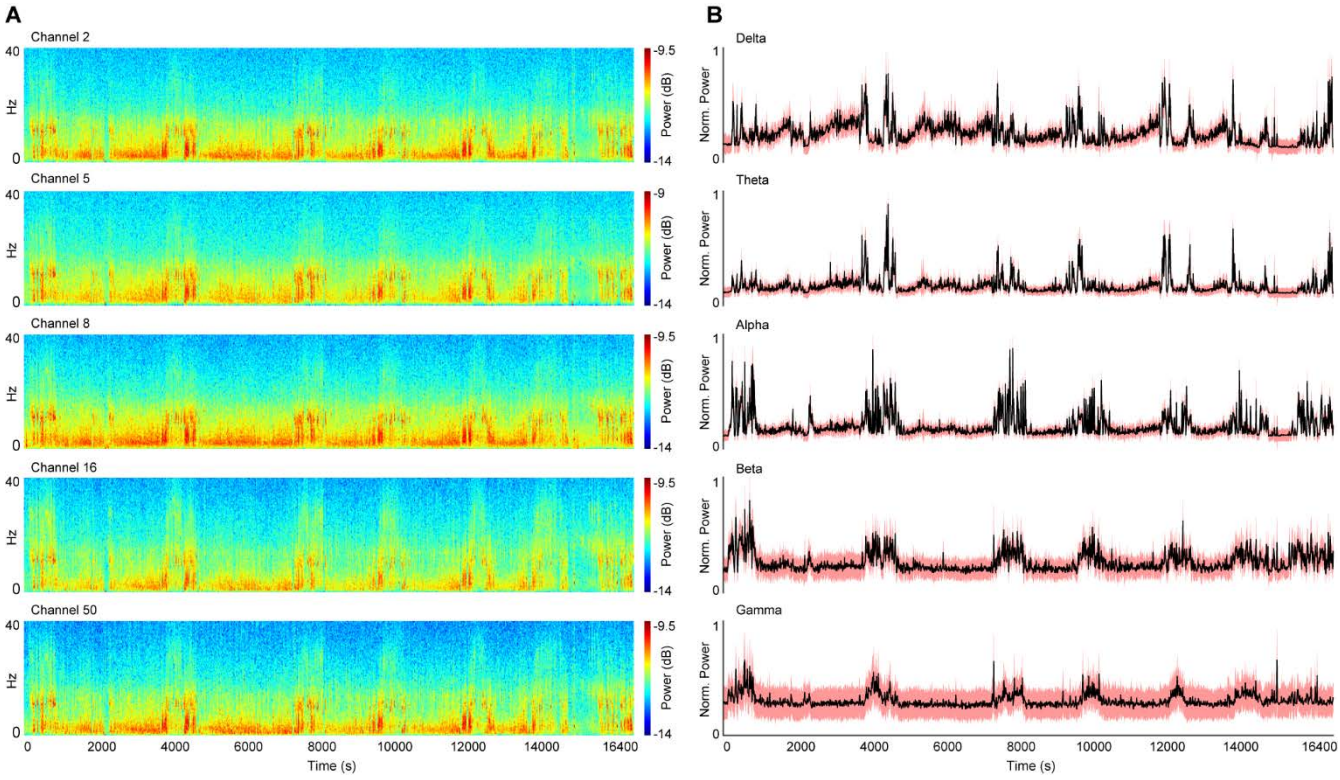

Figure 9 | **Common LFPs patterns during an overnight recording session**. A: Example of randomly-selected LFP time-frequency spectrograms from five channels during a single nocturnal recording session. B: Averaged LFP power spectrum across channels for different frequency bands; black lines represent the average normalized power across channels, while red shadings indicate the standard deviation from the mean.

On these bases, we averaged the signals from all the analyzed channels within each overnight session, resulting in a time-frequency spectrogram and a power spectrum across frequency bands as shown in *Figure 10A-B.* Similar modulations in high- and low-frequency rhythms were also found in the normalized MUA activity pattern (*Figure 10C*).

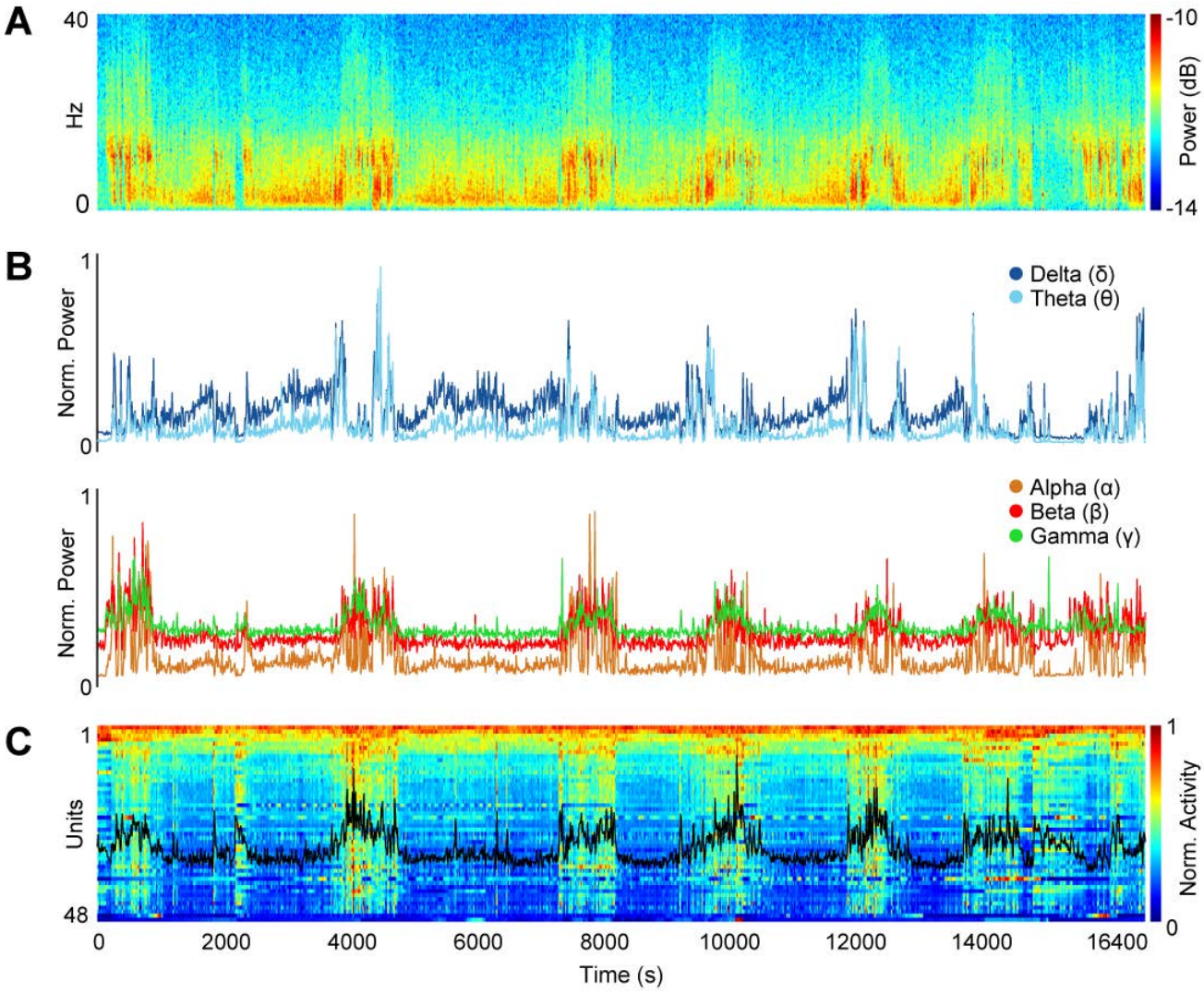

Figure 10 | **LFP and MUA modulations during an overnight recording session**. A: Averaged LFP spectrogram across channels from a single nocturnal recording session. B: Averaged LFP power spectrum across channels of different frequency bands. C: Multi-unit activity of each channel in descending order, along with their averaged activity (black line).

In line with previous EEG human literature (Berry et al., 2020), we observed extended periods of sleep dominated by low-frequency rhythms, particularly delta power, which were interrupted by shorter episodes of wakefulness characterized by elevated high-frequency rhythms, especially beta and gamma bands (*Figure 10B*). These data support the feasibility of developing a methodology to classify commonly recognized wake and sleep states in an unsupervised manner, based solely on intracortical signals verified through behavioral observation. Additionally, this approach demonstrates the potential for obtaining reliable results across multiple sessions.

3.1.2 Automated brain state classification and behavioral monitoring

To characterize sleep stages using solely intracortical neuronal signals, we developed a multi-dimensional framework leveraging the changes in power within different frequency bands and multiunit activity, to extract a number of clusters (k=6) corresponding to the assumed number of wake and sleep phases in an overnight recording (see *Local field potential analysis*). Indeed, based on previous literature (Hsieh et al., 2008), we assumed that the sleep architecture of rhesus macaques consists of six distinct stages: active wakefulness, drowsiness, NREM (N1–N3), and REM sleep. The results of the k-means clustering were visualized over time and aligned to the behavioral scoring in *Figure 11A-B*.

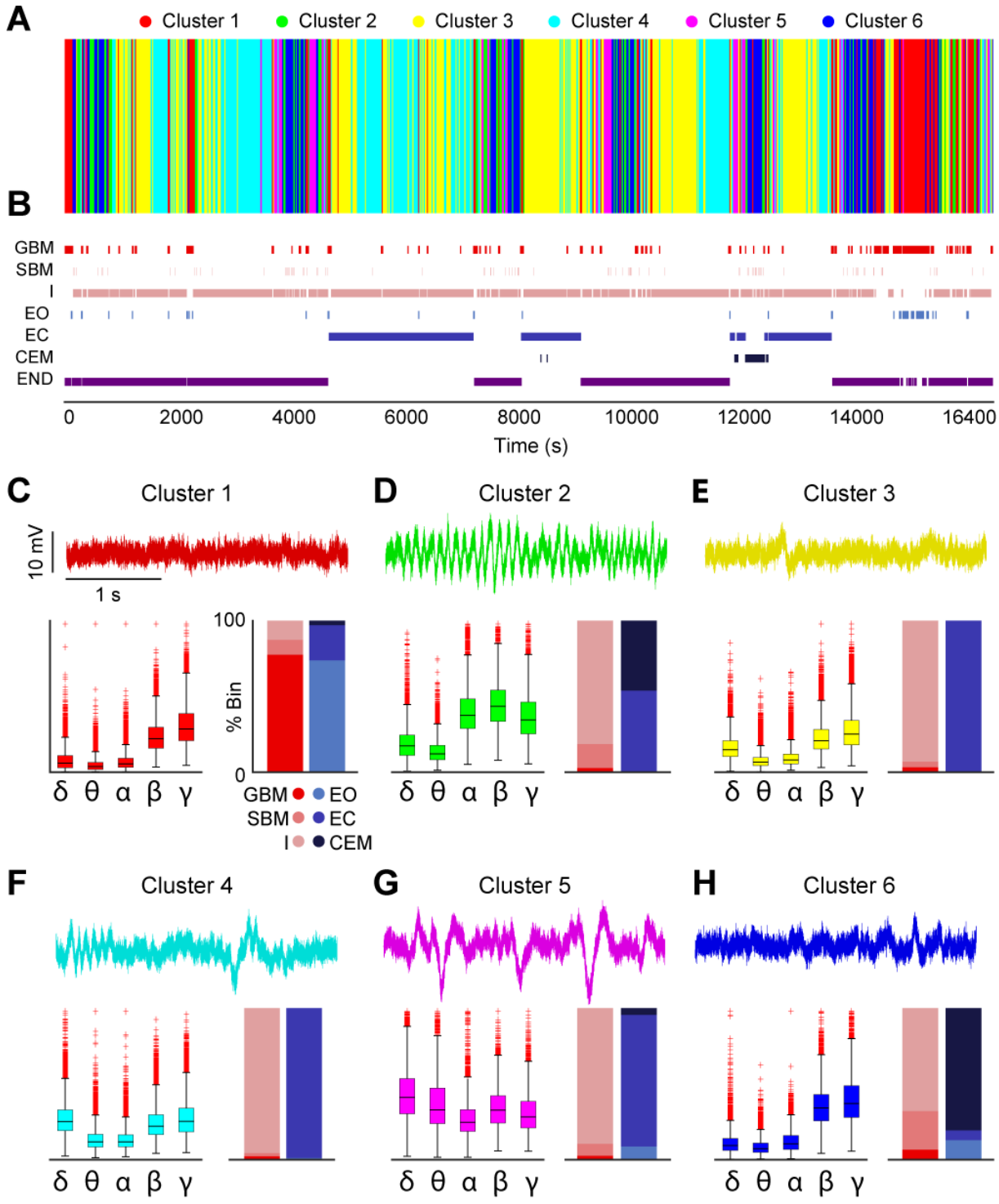

Figure 11 | **Neural and behavioral fingerprints of k-means clusters during an overnight recording session.** A: K-means algorithm results identifying 6 brain states which alternate throughout the overnight recording session. B: Behavioral scoring throughout the entire night. C-H: Frequency band contributions, body/eye state occurrences, and raw signals zoomed into 3 seconds for the putative phases of Wakefulness (C), Drowsiness (D), NREM1 (E), NREM2 (F), NREM3 (G), and REM sleep (H). Bar plots showing the percentage of occurrences of eye states only consider eye-defined behaviors, excluding those labeled as eye-non-defined. GBM = General Body Movements; SBM = Subtle Body Movements; I = Immobile; EO = Eyes Opened; EC = Eyes Closed; CEM = Closed Eyes Movements; END = Eyes state Non-Defined.

To gain deeper insights into the neurophysiological and behavioral signatures of each identified cluster and their correspondence with distinct sleep stages, we averaged the spectral power of all bins within a given cluster and calculated the percentage of associated behavioral occurrences (*Figure 11C-F*). We found that cluster 1 (*Figure 11C*) was characterized by a dominance of low-voltage, high-frequency activity (beta and gamma, 15-40 Hz) accompanied by several general body movements (*GBM*) and eyes opened (*EO*), clearly indicating a wakefulness state. The second cluster suggest a wake-sleep transitions (*Figure 11D*), showing an increase in alpha waves and low-frequency rhythms, including theta, driven by increased frequency of bin in which monkey is immobile (*I*) and its eyes are closed or are slowly moving under the eyelid (closed eyes movement, *CEM*) – as typically happening during drowsiness (Ogilvie et al., 1988). Notably, the alpha range may also include the high-amplitude rhythmic activity in the sigma range (9-16 Hz) resembling the mu-rhythm described in both human (Niedermeyer, 1997) and marmoset (Bukhtiyarova et al., 2022). Cluster 3, 4 and 5 (*Figure 11E-G*) exhibited a gradual increase in high-voltage, low-frequency activity - which is a main feature of NREM sleep (N1-N3) - with cluster 4 (*Figure 11F*) and 5 (*Figure 11G*) displaying over 20% and 40% delta power, respectively, alongside quiet, restful periods. These stages, in addition to being generally characterized by a state of continuous immobility (*I*) and eyes closed (*EC*) (*Figure 11E-G*, bottom right), were further marked by the presence of spindles followed by K-complexes or high-voltage delta waves, usually occurring during NREM sleep (Iber et al., 2007). Lastly, the sixth cluster (*Figure 11H)* mirrored the low-voltage, fast, desynchronized rhythms that distinguishes wakefulness from sleep states (*Figure 11H*, top and bottom left), though with the absence of movements - except for subtle ear movements and short spasm, categorized as subtle body movements (*SBM*) - and eye movements detected through the eyelids (*CEM*) (*Figure 11H*, bottom right), which are typical features of REM sleep stage (Iber et al., 2007).

To further verify the reliability of the proposed classification of the identified clusters, we analyzed the temporal transitions among the clusters using a stochastic model (*Markov Chain Analysis*, see *Local Field Potential analysis*), revealing a distinction between the putative NREM states (cluster 3, 4 and 5), following each other, and the other clusters (*Figure 12*).

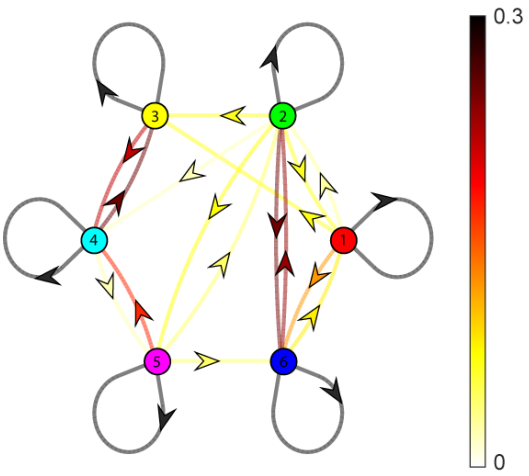

Figure 12 | **Probability of transitions among k-means clusters during an overnight recording session.** Colored dots represent a previously identified cluster; colored lines and arrows indicate the probability of transitioning from one cluster to another during the night.

To ensure that each time bin assigned to a cluster accurately reflected a specific sleep stage - both in terms of frequency spectrum and observed behavior - and to clearly distinguish NREM and REM sleep during quiet and restful periods, we selected a subset of bins based on the previously described criteria (*Figure 13A*; see *Local Field Potential analysis*). Next, we aligned them with the behavioral scoring (*Figure 13B*), and we extracted their neural and behavioral fingerprint (*Figure 13C-H*). This approach offers a faithful and more robust representation of the different sleep stages, supporting the assumption of a correspondence between the subsets of clusters and brain states.

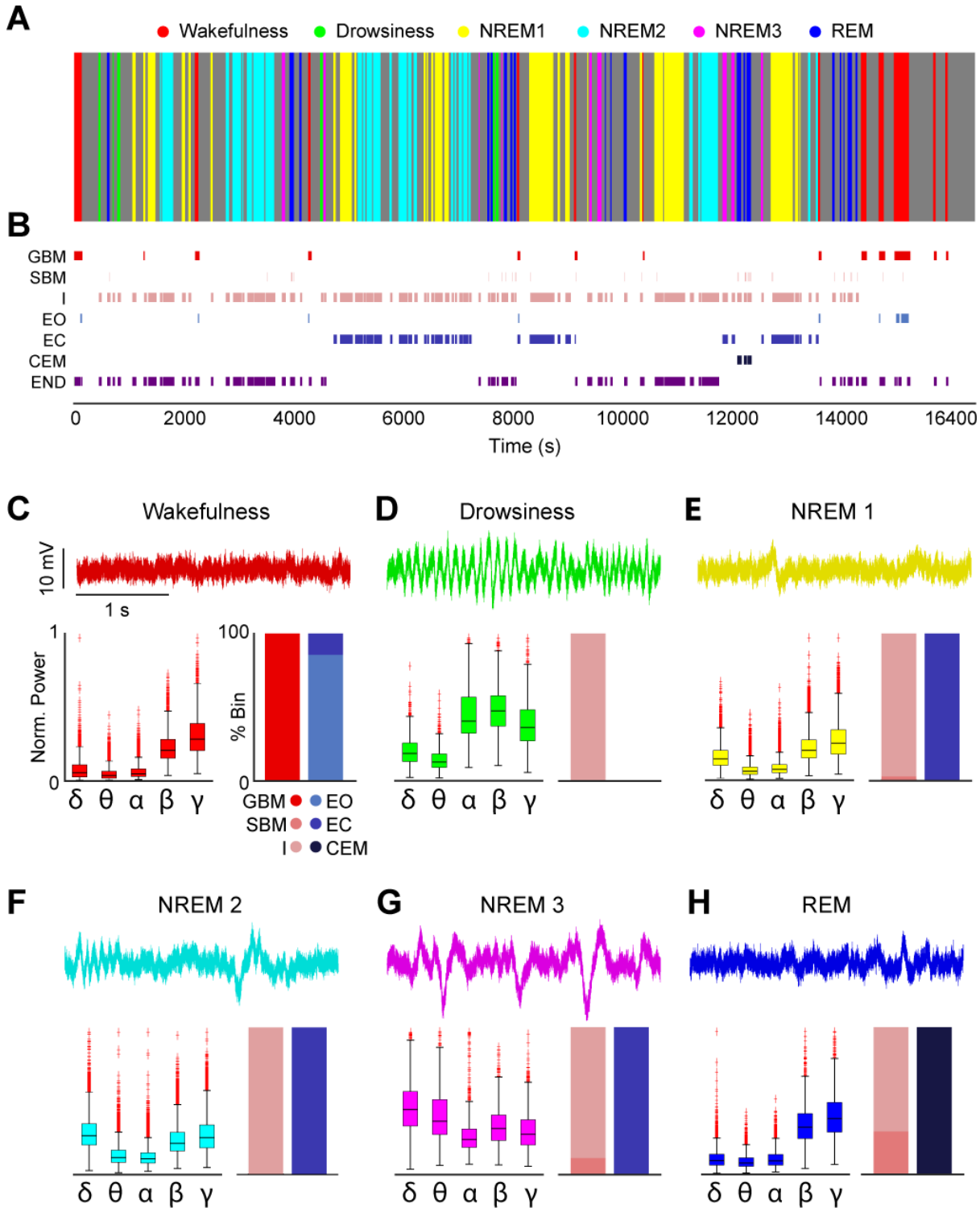

Figure 13 | **Neural and behavioral fingerprints of identified brain states during an overnight recording session.** A: K-means algorithm results after applying the cleaning criteria. Grey areas represent bins that do not meet the described criteria. B: Behavioral scoring in the subset of selected bins throughout the night. C-H: Frequency band contributions in the selected bins, body/eye state occurrences, and raw signals zoomed into 3 seconds for the putative phases of Wakefulness (C), Drowsiness (D), NREM1 (E), NREM2 (F), NREM3 (G), and REM sleep (H). Bar plots showing the percentage of occurrences of eye states only consider eye-defined behaviors, excluding those labeled as eye-non-defined. In panel D, the bar plot representing the eye state occurrences is missing because, in all bins considered in the analysis, the animal's eye state was classified as non-defined. GBM = General Body Movements; SBM = Subtle Body Movements; I = Immobile; EO = Eyes Opened; EC = Eyes Closed; CEM = Closed Eyes Movements; END = Eyes state Non-Defined.

3.2 Delta-related firing dynamics and cell-type classification

3.2.1 Firing rate patterns and delta activity

Based on the sleep stages identified as described so far, we investigated whether the firing rate of PM neurons reflect changes across these various sleep stages.

Initially, we correlated the firing rate of each well-isolated neuron (n=398) from each monkey (n=200 from Mk1; n=198 from Mk2) with the averaged normalized delta power oscillations (see *Local field potential analysis*). In this way, we found *Delta-Positively Correlated* (DPC, n=130) and *Delta-Negatively Correlated* neurons (DNC, n=210), whose firing rate during sleep phases characterized by high power of delta increased or decreased, respectively; furthermore, we found a set of *Delta-Uncorrelated* (DU, n=58) neurons, which did not show a significant and consistent relationship between their firing and the delta power (*Figure 14*).

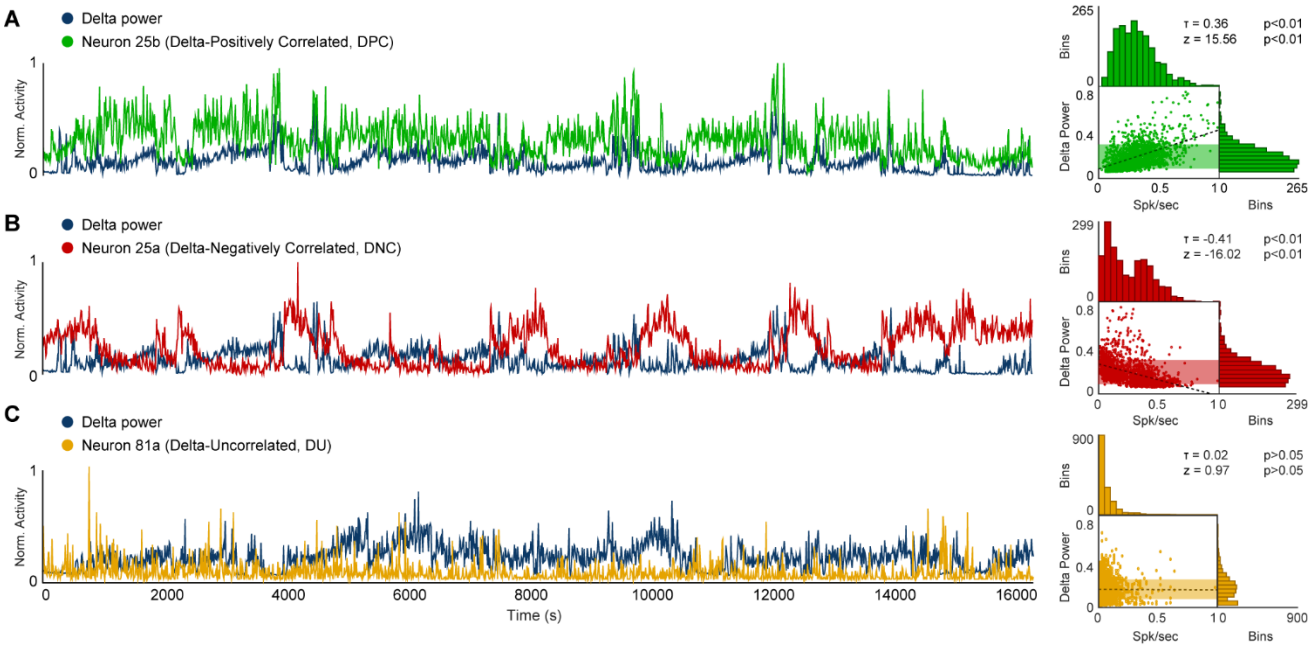

Figure 14 | **Correlation between averaged delta power and neuronal firing rate identify three distinct classes of neurons.** Example of a positive (A, DPC neuron, green), negative (B, DNC neuron, red) and none (C, DU neuron, yellow) correlation between firing rate and averaged delta power of the corresponding session. In the plots on the right, each data point represents the firing rate within a 10-second bin characterized by different delta power.

The majority of PM neurons (53%) exhibited a significant suppression of their firing rate during periods of high delta power (DNC neurons, chi-square test, χ^2=86.90, $p < 0.0001$). However, a sizeable fraction (33%) showed increased firing when delta power is low (DPC neurons) (*Figure 15A*). Next, to assess the

activity fingerprint of the three identified neuronal classes during the six brain states identified, including wakefulness and REM sleep, we averaged their normalized firing rates during each of the six previously identified cluster.

DPC neurons exhibited significant differences between wakefulness and NREM sleep (*Figure 15B*; Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.05$). No significant differences were observed when comparing the power spectrum between NREM stages (N1-N3) (Kruskal-Wallis test followed by Dunn post hoc test, $p > 0.05$; *Figure 15B*) or between phases with higher frequency rhythms (Wakefulness vs. Drowsiness; Wakefulness vs. REM). This indicates a specific enhancement of these neuronal classes exclusively during NREM sleep, with a return to baseline activity during REM phase, using wakefulness as the reference. DNC neurons showed significant suppression between wakefulness and all other brain states (*Figure 15C*; Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.05$), most pronounced during NREM sleep and less so during wake-like phases, where their firing rate begins to rise significantly compared to NREM (*Figure 15C*; Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.001$). DU neurons exhibited no significant modulation across different sleep stages (Kruskal-Wallis test followed by Dunn post hoc test, $p > 0.05$; *Figure 15D*), suggesting that they maintain a stable firing rate throughout the entire sleep-wake cycle.

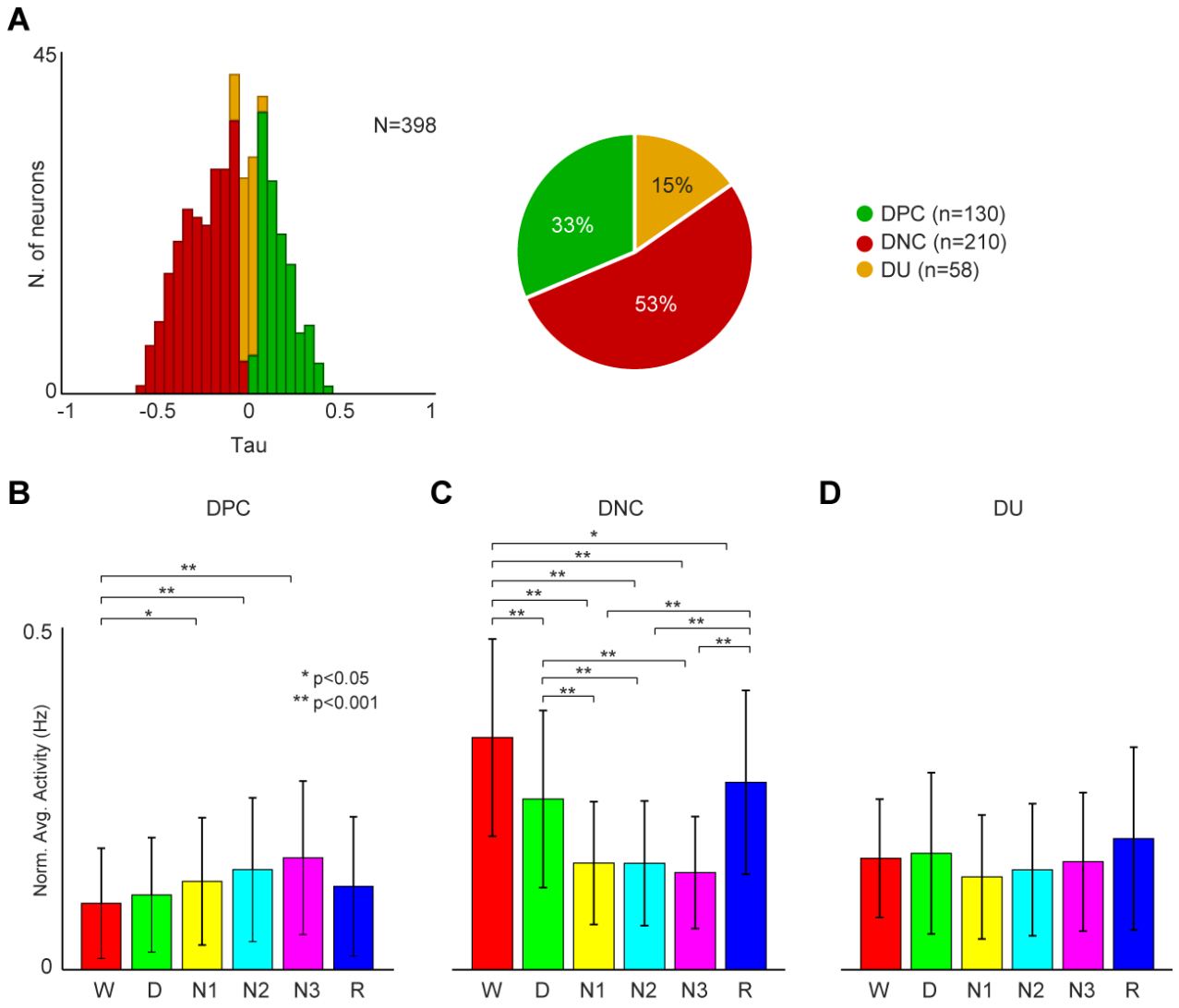

Figure 15 | **Classification and firing rate patterns of different delta-classified neurons in premotor cortex.** A: On the left, a bar plot displaying the distribution of Tau values across the dataset. On the right, a pie chart showing the percentage of Delta-Positively Correlated (DPC), Delta-Negatively Correlated (DNC), and Delta-Uncorrelated (DU) units. B-C-D: Bar plots showing the average firing rate and standard deviation (black lines) of DPC, DNC and DU neurons during wakefulness (W), drowsiness (D), NREM1 (N1), NREM2 (N2), NREM3 (N3), and REM (R). Asterisks denote significance according to the chi-square test ($p < 0.05$).

3.2.2 Neuronal cell-types and their delta-related firing properties

To gain insight into the internal organization of the PM cortex during natural sleep, we examined whether isolated neurons with specific patterns of activity during periods of different delta power also exhibit different spike shapes, suggesting possible insight regarding their relative functional role during wakefulness and sleep.

To address these issues, first we extracted the extracellular waveforms of the isolated neurons. Traditionally, cell types have been classified using one or two dimensions, such as action potential width and repolarization time, to distinguish between putative inhibitory neurons, characterized by narrow spikes and

higher firing rate, and putative excitatory pyramidal cells, characterized by broad spikes and lower baseline firing (Ferroni et al., 2021; Hussar & Pasternak, 2009; Mitchell et al., 2007; Trainito et al., 2019). Utilizing a novel unsupervised multi-dimensional protocol that leverages the entire shape of spike waveforms (Lee et al., 2021), we could identify five distinct waveform clusters (*Figure 16A*). These clusters occupied different regions within the multi-dimensional space (*Figure 16B*).

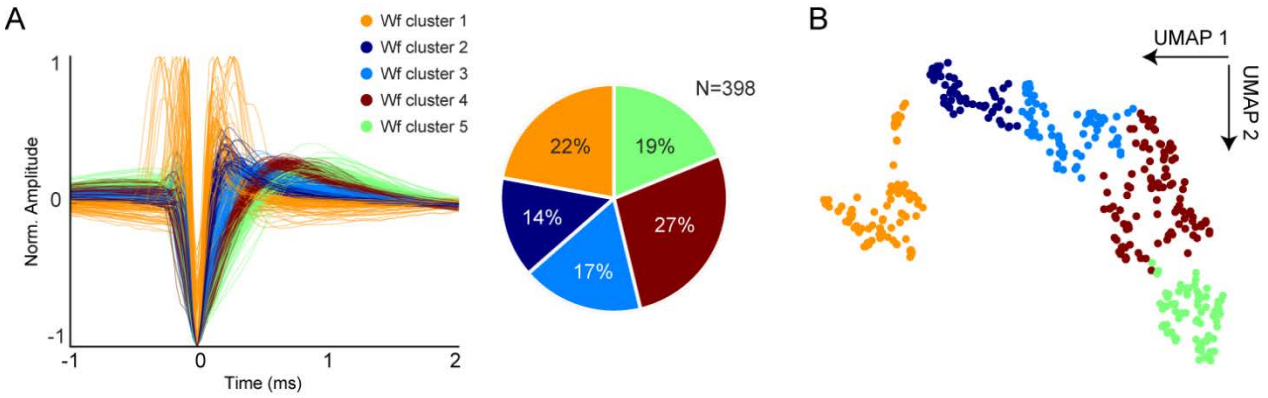

Figure 16 | **Structural properties of PM neurons.** A: On the left, normalized average single-unit waveforms, colored according to their cluster membership. On the right, a pie chart displays the percentage of units belonging to different shape clusters. B: Distribution of normalized average single-unit waveforms; units are colored by their cluster membership.

When integrating information about spike shapes with their activity patterns towards delta power (*Figure 17*), we observed that cell types in clusters with a through-to-peak time of less than 0.5 ms (Clusters 1, 2, and 3) displayed a significant increase in the proportion of DNC neurons (60%) and a decrease in DPC neurons (26%) compared to the overall population (*Figure 16A*, χ^2=6.25, p=0.044). In contrast, for cell types with a through-to-peak time greater than 0.6 ms (Clusters 4 and 5), there was a notable increase in the proportion of DPC neurons (41%) and a decrease in DNC neurons (43%) compared to the entire population (*Figure 16A*, χ^2=7.26, p=0.026). Since, according to the existing evidence (Lemon et al., 2021), the first group should include inhibitory interneurons whereas the second group should be mostly made of medium-to-small size pyramidal cells (Vigneswaran et al., 2011), these findings suggest that during periods of high delta power inhibitory interneurons may be mostly suppressed, whereas excitatory medium-to-small size pyramidal neurons, deemed to contribute to local and cortico-cortical processes (Ghosh & Porter, 1988), increase their activity.

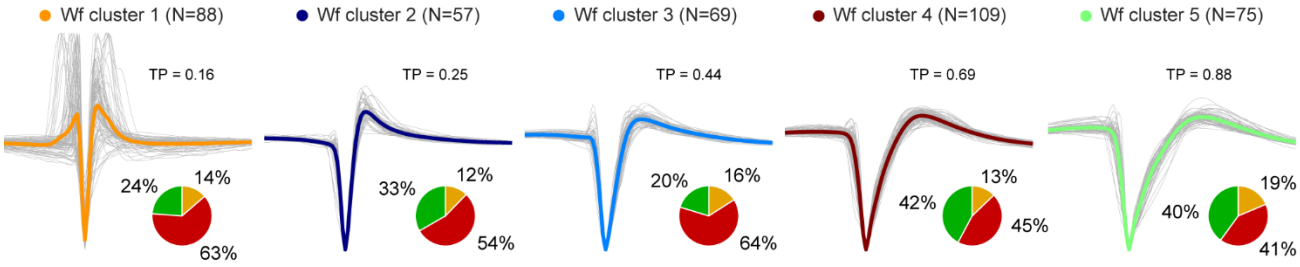

Figure 17 | **PM cell-types identified and percentage of Delta-classified neurons belonging to each shape's group.** The colored shapes represent the averaged waveform of each identified cluster, while the grey shaded shapes represent individual waveforms within each cluster. The pie chart illustrates the percentage of DPC (green), DNC (red), and DU (yellow) neurons belonging to each cluster.

3.3 Exploring PM cortex features across brain states

3.3.1 Single neuron's functional properties during VMT

To further evaluate the functional characteristics of PM neurons previously classified based on their spike shape and discharge pattern during sleep, we leveraged the testing in head-restrained conditions carried out prior to most of the overnight recording sessions, with which we could characterize 303 neurons during both the execution and observation of the VMT.

We applied a one-way repeated measures ANOVA using epoch as factor (baseline, pre-movement, reaching, grasping, see *Spike sorting and data analysis*) to the execution and observation response, separately. We identified 122 task-related neurons out of 303 (40%) modulated significantly ($p < 0.001$) during at least one of the three epoch of the VMT relative to baseline. Among these, 79 neurons (65%) were modulated only during the execution of manual actions (self-type neurons, *Figure 18A, B*), 35 neurons (29%) responded during both the execution and the observation of the same action (self-other type neurons, *Figure 18A, C*), while a few (n=8, 6%) responded only during the observation task (other-type neurons, *Figure 18A, D*). Moreover, 68 neurons out of 303 (22%) were not significantly modulated during either the execution or observation of the VMT and were thus classified as task-unrelated neurons (*Figure 18A, E*). Additionally, 113 out of 303 neurons (37%) were categorized as "unclassified neurons" because they did not meet the criteria adopted for the classification of neuronal categories. This choice was aimed at providing more robust neuronal categories accepting the increase rate of false negative results (*see Spike sorting and data analysis*).

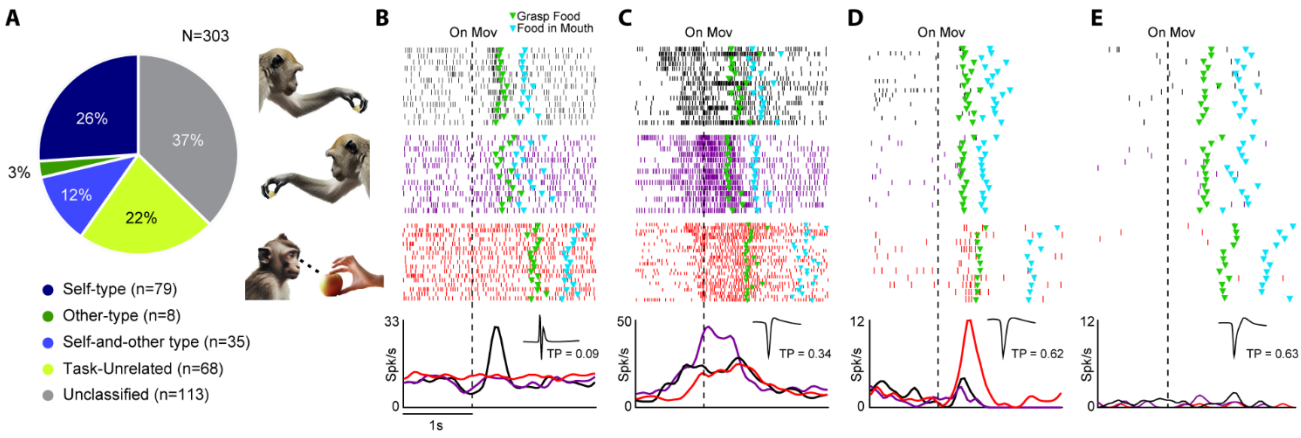

Figure 18 | **Classification of PM neurons based on their firing properties.** A: Pie chart showing the percentage of units classified by distinct firing properties. B-C-D-E: Peri event raster plots representing one example of a self-type neuron (black line and bars: contralateral hand; violet line and bars: ipsilateral hand), self-and-other type neuron, other-type neuron (red line and bars), and task-unrelated neuron during the visuo-motor task, aligned to the onset of the reach-to-grasp movement (black dashed line). The shape and trough-to-peak time for each neuron are depicted alongside each example.

3.3.2 Single neuron's functional properties during sleep stages

After classifying neurons based on their firing properties during VMT, we examined their correlation with delta activity and explored the potential relationship between these patterns and their spike shape.

We found that the number of self-and-other type neurons belonging to the DNC group was significantly higher than in the general population (*Figure 19A;* chi-square test, $\chi^2=7.29$, $p < 0.05$). Conversely, task-unrelated neurons included a significantly higher proportion of DPC neurons compared to the overall population (*Figure 19A;* chi-square test, $\chi^2=12.87$, $p < 0.01$). Additionally, self-type neurons followed the general population trend (*Figure 19A;* chi-square test, $\chi^2=2.3$, $p > 0.05$) with more DNC than DPC neurons. Other-type neurons showed a greater number of DPC neurons, though this was not statistically significant (*Figure 19A*; chi-square test, $\chi^2=2.51$, $p > 0.05$). Furthermore, no significant differences were found in the distribution of self-type, self-and-other type, other-type and task-unrelated neurons across spike shape group (*Figure 19B;* chi-square test, $p > 0.05$).

Taken together these findings suggest that, regardless of spike shape, task-unrelated neurons are less active during wakefulness but increase their activity during periods of high delta power, in contrast to self-and-other and most self-neurons, which exhibit the opposite pattern.

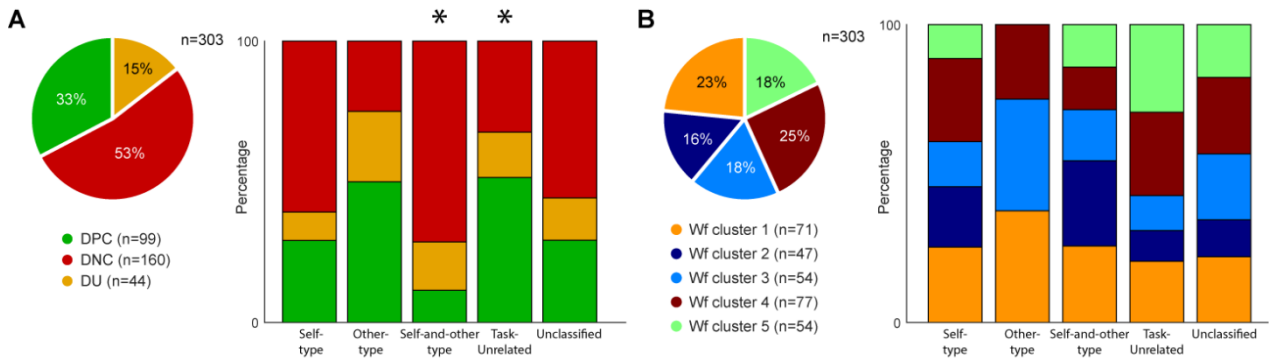

Figure 19 | **VMT-classified neurons and their distribution across spike shape clusters and Delta-classified groups.** A: On the left, a pie chart displaying the percentage of DPC, DNC, and DU units among the population of neurons isolated during VMT. On the right, bar plots showing the percentage of VMT-classified neurons belonging to each Delta-classified group. B: On the left, a pie chart displaying the percentage of neurons classified into spike shape clusters among the population of neurons isolated during VMT. On the right, bar plots showing the percentage of VMT-classified neurons within each spike shape group.

Next, to investigate PM neurons' firing profile across all sleep phases, we calculated their average normalized firing rate across the six brain states and used these values as dimensions in a multiple-dimensional space, subsequently reduced to three by applying *t-SNE* (*Figure 20-21*).

First, to assess whether neurons classified based on their discharge properties during VMT or Delta correlations exhibited similar behavior across sleep stages, we tagged neurons according to their functional classifications - self-type, self-and-other type, other-type, task-unrelated, and unclassified neurons for VMT (*Figure 20A*, left); DNC, DPC, and DU for Delta correlations (*Figure 20A*, right). Interestingly, in addition to revealing a spatial separation of neurons based on facilitation or suppression patterns following delta correlations (*Figure 20A*, left), task-related neurons - namely self-only and self-and-other neurons - segregated in distinct regions of the multi-dimensional space compared to task-unrelated neurons, suggesting different firing behavior between these two groups across brain states (*Figure 20A*, right).

Next, since not all neurons within the same functional group - whether Delta or VMT - necessarily exhibit a uniform sleep behavior or consistently differ from neurons in other functional classes, we evaluated the firing fingerprint of PM neurons independently from their belonging classes. Using gap statistics to determine the optimal number of clusters, we applied a K-means clustering algorithm to group neurons in an unsupervised way (see *Population analysis*). This approach identified nine distinct clusters of neurons (*Figure 20B*), each with a unique firing fingerprint across the six sleep stages (*Figures 20B, 21*).

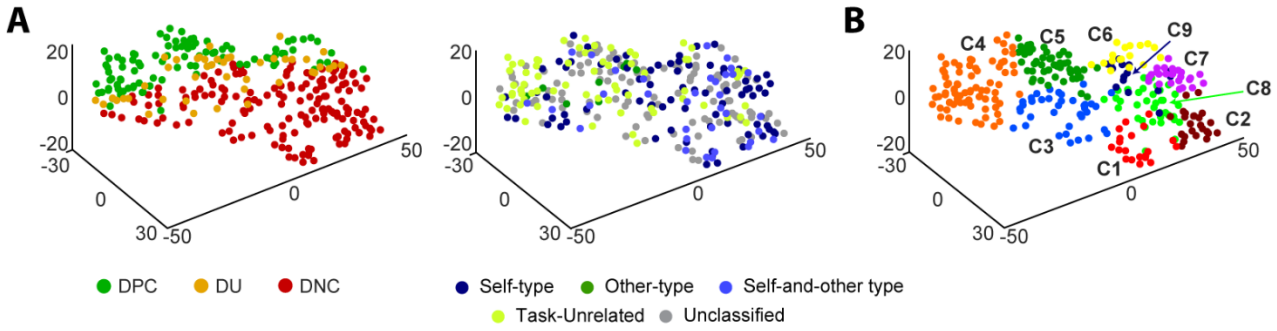

Figure 20 | **Population coding of averaged firing rate across sleep stages.** A: 3D visualizations showing the distribution of neurons, tagged based on their firing properties during delta activity (A, left) and VMT (A, right). On the left, DPC neurons (green dots) are segregated in the upper part of the graph, DNC neurons (red dots) in the lower part, and DU neurons (yellow dots) positioned between the DPC and DNC groups. On the right, task-unrelated neurons (yellow dots) are principally grouped on the left, while task-related neurons (blue and light blue dots) are on the right. Unclassified neurons (grey dots) are mixed across both sides of the graph. Each dot in the graphs represents a neuron, described by its normalized average firing rate across the six brain states. B: 3D visualizations showing the distribution of neurons grouped by the unbiased K-Means algorithm and gap statistic classification. Each dot in the graphs represents a neuron, described by its normalized average firing rate across the six brain states.

The identified clusters exhibited differences not only in their neural fingerprint but also in their percentage distribution across various functional groups (Delta and VMT) and structural classifications. These results are presented in *Figure 21*.

Cluster 1 grouped DNC neurons (significantly different from the reference population shown in *Figure 19A*; chi-square test, χ^2=17.879, $p < 0.001$), primarily task-related (self- and self-and-other neurons, significantly different from the reference population in *Figure 18A*; chi-square test, χ^2=11.558, $p < 0.05$), and narrow-spiking (i.e., waveform clusters with trough-peak < 0.5 ms, significantly different from the reference population in *Figure 19B*; chi-square test, χ^2=11.991, $p < 0.05$). This cluster was characterized by a fingerprint showing complete suppression during NREM sleep, with increased firing rates during REM, similar to wakefulness (Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.001$).

Cluster 2, also consisting exclusively of DNC neurons (chi-square test, χ^2=21.462, $p < 0.001$) and task-related neurons (mainly self-type neurons; chi-square test, χ^2=10.05, $p < 0.05$), had a balanced spike shape distribution that was not significantly different from the reference population (*Figure 19B*), with a firing pattern similar to Cluster 1 in terms of NREM/REM sleep and wakefulness (Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.001$).

In contrast, Cluster 4 showed a significantly higher number of DPC neurons (chi-square test, χ^2=42.25, $p < 0.001$), a large proportion of task-unrelated neurons (30 out of 68, 44%; chi-square test, χ^2=14.92, p <

0.01), and broad-spiking cells (i.e., waveform clusters with trough-peak > 0.6 ms, 49 out of 131, 37%; chi-square test, χ^2=14.47, $p < 0.01$) compared to the reference populations (respectively *Figures 19A, 18A, 19B*). This cluster exhibited increased activity during NREM sleep, followed by suppression during the REM phase, although this suppression was not significantly different from wakefulness (Kruskal-Wallis test followed by Dunn post hoc test, $p > 0.05$).

Similarly, Cluster 5 showed a higher proportion of DPC neurons (chi-square test, χ^2=22.92, $p < 0.001$) and task-unrelated neurons (chi-square test, χ^2=9.84, $p < 0.05$), but with a balanced spike shape distribution that was not significantly different from the population (*Figure 19B*). Its neural fingerprint showed a significant increase in activity during N3 and REM phases compared to wakefulness (Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.05$).

Interestingly, Cluster 6 - like Clusters 4 and 5 - had a greater proportion of DPC neurons (chi-square test, χ^2=17.21, $p < 0.001$) and narrow-spiking cells (chi-square test, χ^2=13.35, $p < 0.01$) compared to the reference populations (*Figures 19A, 19B*), but no significant differences in the number of neurons classified based on VMT. However, most of these neurons (65%) were self- or self-and-other type. The firing pattern of this cluster showed a significant increase in activity during NREM compared to REM (Kruskal-Wallis test followed by Dunn post hoc test, $p < 0.01$), but not when compared to wakefulness (Kruskal-Wallis test followed by Dunn post hoc test, $p > 0.05$).

The remaining clusters, although each exhibiting distinct firing patterns, did not show significant differences across spike shape, Delta-correlated, or VMT discharge properties - except for Clusters 3 and 8, which had a higher proportion of DNC neurons compared to the reference population (Cluster 3, chi-square test, χ^2=22.92, $p < 0.001$; Cluster 8, chi-square test, χ^2=28.631, $p < 0.001$).

These findings suggest the existence of distinct and mixed neuronal groups that behave differently throughout the night, highlighting a dualism between subgroups of self- and self-and-other putative interneurons, which are suppressed during NREM sleep, and those consisting mainly of task-unrelated putative pyramidal neurons, which are excited during these sleep phases.

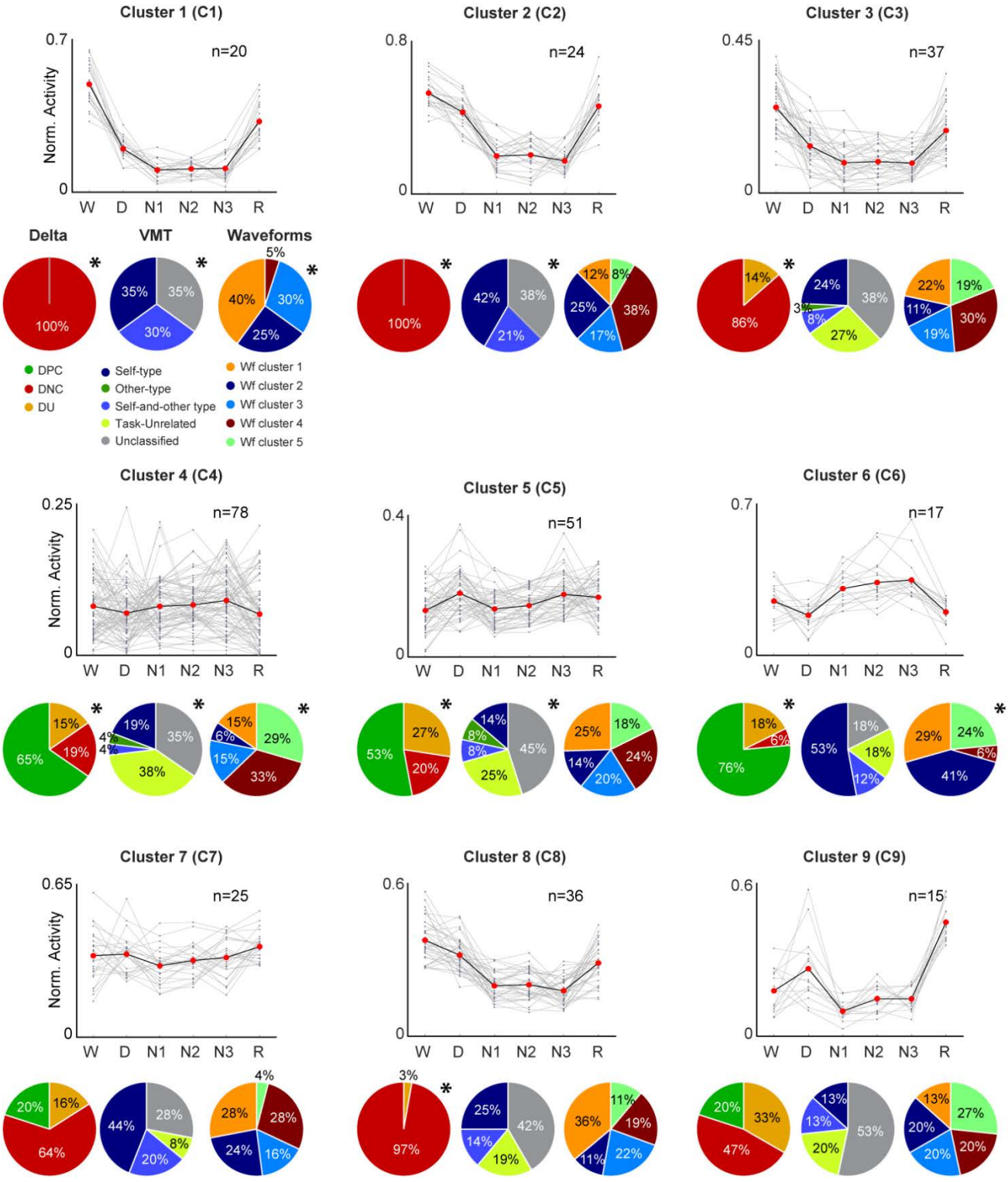

Figure 21 | **Population fingerprint of K-Means identified groups.** Normalized activity for each group identified by the K-Means algorithm, based on firing properties across brain states. The percentage of neurons belonging to Delta-, VMT-, and waveform groups is represented under each group's fingerprint. Black lines indicate the averaged signal, grey shaded lines represent individual neurons' firing rate within each cluster, red dots mark the mean firing rate in each brain state, and asterisks denote significance compared with the reference population (see *Figure 19A*, *Figure 18A* and *Figure 19B*) according to the chi-square test ($p < 0.05$).

4. Discussion

The neurophysiology of motor cortices during wakefulness has been extensively studied for over 50 years (Bonini et al., 2012; Fogassi et al., 1996; Gentilucci et al., 1988; Rizzolatti et al., 1981a). However, animals - and primates, including humans - spend a substantial portion of their lives sleeping. Since sleep in a laboratory setting often requires physical restraints, such as head fixation in primate chairs, this can deeply affect the quality of sleep and the duration of its cycles (Benca et al., 2000; Bukhtiyarova et al., 2022). For those reasons, the motor system remains understudied during sleep, especially regarding the functional properties that neurons exhibit during various sleep stages and their possible contribution to the processes occurring during this state.

Here, we classified brain states solely through intracortical neuronal signals and examined PM neuron activity during both a visuomotor task (VMT) in chair condition and natural sleep in their familiar home-cage, identifying 1) neuronal classes in the PM cortex that increase or decrease their activity following delta correlations, 2) neuronal classes exhibiting different firing patterns across various brain states, and 3) a specific subsets of self-type, self-and-other-type, and task-unrelated neurons showing distinct and complementary activity patterns during different sleep phases.

4.1 From intracortical signals to sleep staging

Most of the existing sleep literature relies on the gold-standard EEG, EOG, and EMG measures for sleep staging (Berry et al., 2020; Goonawardena et al., 2019; Yasenkov & Deboer, 2012), using tethered or - more recently - telemetric systems. Given the similarity between EEG and LFP signals - with the former considered a spatiotemporally smoothed version of the LFP integrated over a wider area (Buzsaki et al., 2012) - some studies have attempted to use LFP for identifying sleep stages (Bukhtiyarova et al., 2022), although this is typically supplemented by EOG or EMG signals to accurately identify REM sleep (Xu et al., 2019).

For the first time, our study utilized only intracortical signals with high temporal resolution (10-second bins) to identify sleep stages in rhesus macaques, a species considered to have a sleep architecture most similar to humans (Hsieh et al., 2008). The synchronized and repetitive low- and high-frequency rhythms - particularly delta rhythms during slow-wave sleep (SWS), and beta and gamma during wakefulness – are consistent with

those observed in in mice and humans (Franken et al., 2001). By leveraging LFP and multi-unit activity patterns, we identified six putative brain states. Their overnight behavioral scoring, LFP rhythms fingerprints, and the stochastic model of state transitions – all consistent across both experimental monkeys - largely reflected the guidelines used in human sleep scoring (Berry et al., 2020). This allowed us to define groups of bins as corresponding to specific sleep phases.

Our approach is consistent with recent evidence in non-human primates (Bukhtiyarova et al., 2022; Xu et al., 2019) using LFP signals to classify sleep stages and delta power to identify NREM depth.

4.2 Sleep-driven dynamics in the PM cortex

By studying neuronal firing rates during overnight recordings in freely moving macaques, we discovered rhythmic and repetitive patterns throughout the night. By detecting sleep stages we identified functional classes whose firing rates followed delta rhythm oscillations, a typical feature of NREM sleep (Berry et al., 2020) and a biomarker of homeostatic sleep drive - since its enhancement after prolonged wakefulness, and its decline as sleep deepens (Achermann & Borbély, 1997; Long et al., 2021). The positive or negative correlation between these variables revealed the already known existence of neurons that are suppressed during NREM phase and facilitated during wakefulness and REM sleep (Xu et al., 2019) – here named as “Delta-Negatively Correlated neurons”. Moreover, this study demonstrated the presence of neurons that follow delta rhythm variations and become excited during NREM phases - referred to here as “Delta-Positively Correlated” neurons: to our knowledge, these cells have not been previously documented in the cortex sleep literature of non-human primates. This evidence points to a dynamic modulation of PM neuron activity throughout the night, indicating that transitions from wakefulness to NREM and REM sleep are marked by a cyclical reorganization of the PM cortex activity. This reorganization is likely mediated by a specific balance between excitatory and inhibitory inputs in the brain - a recurring theme in sleep-related processes such as memory consolidation (Pereira & Lewis, 2020), energy and synaptic recovery (Niethard et al., 2017; Rybalchenko & Greene, 2022), and molecular mechanisms involving the interplay between GABA and Glutamate (Siegel, 2004).

To gain deeper insights into these classes of neurons, we performed a structural classification of neurons based on their spike shape (Lee et al., 2021; Lee et al., 2023). The differentiation of cell types based

on waveform shape arises from transcriptomic differences in ion channel expression (Bakken et al., 2021; Bomkamp et al., 2019) and other related electrophysiological characteristics, such as inter-spike interval (ISI) distribution as seen in fast-spiking interneurons (Latuske et al., 2015; Schneider et al., 2023). Together with neuronal circuitry and connectivity influencing the input a cell receives (Zaitsev et al., 2009), these features shape firing rate patterns in response to external stimuli and task events (Pinto & Dan, 2015; Yu et al., 2019). However, spike width alone is not always a reliable indicator of cell type (Vigneswaran et al., 2011), as not all interneurons have "thin" spikes, and not all pyramidal neurons exhibit broad spikes (Lemon et al., 2021). While further validation through optogenetic or viral labeling is needed to confirm spike duration as a classification tool, our approach aligns with that of Trainito et al. (2019), who classified waveform groups that transcend the traditional pyramidal vs. interneuron distinction (Trainito et al., 2019). In our study we identified five neuronal classes with different excitatory/inhibitory properties correlated with delta power. Two of them, (cluster 4 and 5 with trough-to-peak time > 0.5 ms), are likely composed of pyramidal neurons, whereas other two, waveforms clusters 1 and 2 (trough-to-peak time $\leq$ 0.25 ms), most likely include putative inhibitory interneurons (Connors & Gutnick, 1990; Gold et al., 2006; McCormick et al., 1985; Tahvildari et al., 2012). The classification of waveforms class 3 (mean trough-to-peak time = 0.44) remains uncertain. However, our results indicate that neurons belonging to classes with an average trough-to-peak time < 0.5 ms share a similar excitatory/inhibitory balance, showing a higher proportion of suppression during periods of high delta power (NREM sleep). In contrast, neurons with a trough-to-peak time > 0.5 ms - traditionally classified as broad-spiking, putative excitatory pyramidal cells (Lemon et al., 2021) - showed a higher proportion of neurons facilitated during the same periods.

The shift in excitatory/inhibitory balance observed in our study during NREM sleep aligns with what is seen in ketamine-induced unconsciousness, which already shares notable similarities with NREM sleep (Lydic & Baghdoyan, 2005; Radovanovic et al., 2023). Ketamine, known for inhibiting GABAergic interneurons (Gerhard et al., 2020), not only leads to thalamic deactivation (Höflich et al., 2015) and induces delta waves and sleep spindles (Nelson et al., 2002) - both hallmarks of NREM sleep - but also heightens the activity of cortical glutamatergic excitatory neurons (Brown et al., 2010), resulting in unconsciousness. The cortical excitatory increase seen in both ketamine-induced states and NREM sleep suggests a common mechanism driving unconsciousness.

Our findings highlight notable differences in the activity patterns of different PM neurons across brain states, with a particular focus on VMT task-unrelated neurons. Specifically, we found groups of neurons predominantly composed of task-unrelated, putative excitatory pyramidal cells that show facilitation when the delta power is high (NREM sleep). In contrast, other groups, primarily consisting of self- and self-and-other neurons, exhibit suppression during the same periods (NREM sleep), with one group notably characterized by putative inhibitory interneurons. Each group displays unique activity fingerprints, reflecting their putative involvement in different phenomena during sleep. The complementary nature of these groups suggests a potential interaction, supporting the hypothesis of a dual network in PM cortex where task-related neurons rest during sleep while task-unrelated neurons become more active, taking on sleep-specific functions. Although some regions in the rat brain show energy consumption during sleep that is similar or equal to wakefulness (Dworak et al., 2010), the general reduction in metabolic activity during NREM sleep (~ 85% of wakefulness metabolic consumption) (DiNuzzo & Nedergaard, 2017) could be here likely explained by the decreased activity of a putative wake-dedicated network, replaced by a sleep-specific one, allowing the former to rest.

The duality of this hypothetical double network may be supported by interneurons, which play an inhibitory role on excitatory neurons during wakefulness. Evidence from rodents demonstrates that interneurons, particularly somatostatin-expressing and parvalbumin-positive subtypes, play a critical role in inhibiting pyramidal neurons during wakefulness(Fino et al., 2013; Liguz-Lecznar et al., 2022; Murayama et al., 2009; Yu et al., 2019), underlying adult cortical plasticity (Fu et al., 2015). This inhibition could be essential for maintaining proper neural circuit function and preventing excessive excitation during wakefulness, contributing to the initiation and execution of correct motor actions (Giordano et al., 2023).

On the other hand, cortical excitability during NREM sleep is known to impact neural processes crucial for cortical plasticity (Tononi & Cirelli, 2014), energy restoration (Tononi & Cirelli, 2014; Vyazovskiy & Harris, 2013), memory consolidation (Diekelmann & Born, 2010) and offline behavioral improvements trough the reactivation - or replay - of motor memories (Eisenstein et al., 2024). Although we did not directly test memory consolidation or neuronal reactivation, our results are in line with the increased cortical excitability observed during NREM sleep. This fits well with the growing evidence that offline memory reactivation occurs not only in the hippocampus in relation to declarative memories but also at the level of the motor cortex, both in animals (Ramanathan et al., 2015) and humans (Buch et al., 2021), particularly following motor learning.

Recent discoveries in the human motor cortex (Rubin et al., 2022) have raised the question of whether motor replay might be the result of decreased inhibition of pyramidal neurons, a reduction that aligns with our findings. Several lines of evidence suggest a causal relationship between slow-wave oscillations and memory consolidation mechanisms (Lemke et al., 2021; Petzka et al., 2022; Schreiner et al., 2021), with motor replay playing a central role (Genzel & Robertson, 2015) to re-activate the connections most utilized during wakefulness, improving memory performance and leading subsequent changes in memory circuits. Underlying these processes may be mechanisms of short- (STP) and long-term potentiation (LTP) (Lynch, 2004) occurring in motor cortices linked to sleep spindles and slow-wave oscillations (Rosanova & Ulrich, 2005) and at the basis of the synaptic downscaling occurring during SWS (Tononi & Cirelli, 2006). Most research has focused on neurons that are active during task performance and undergo replay during sleep. However, it should be noted that there is currently no evidence indicating that other neurons, which are not directly involved in the task – such as those observed in our study -, do not participate in this process, suggesting the possibility of a broader and more intricate neural involvement during sleep. Further research is necessary to validate this hypothesis.

4.3 Conclusions

In conclusion, our study, though still in its preliminary stages, provides novel insights into the firing properties of PM neurons during different brain states in rhesus macaques. These findings contribute significantly to our understanding of the PM cortex, especially regarding its activity during sleep - a domain that has remained still underexplored in neurophysiological research.

One of the main limitations of this study lies in the lack of telemetric control for the validated identification of sleep stages. Although our analysis provides a robust characterization of PM neurons' activity during sleep stages, as inferred from LFP and MUA signals, it remains essential to validate these findings against a gold-standard classification based on telemetric EEG, EOG, and EMG recordings. This will be addressed in future studies, where upcoming sessions will incorporate a telemetric system to allow more precise and validated measurement of brain states.

Nevertheless, the present findings represent the first empirical demonstration of the functional diversity of PM neurons during sleep and their property discharge during the night. Specifically, this study sheds light on the presence of complementary neuronal activity, with a particular focus on task-unrelated neurons, which were previously underexplored, speculating - in PM cortex - a dual network where task-related neurons rest during sleep, while task-unrelated neurons become more active and play a role in sleep-related functions.

It would be beneficial to further explore these findings with cross-correlation analyses between neurons - already part of our upcoming analysis pipeline, to hypothesize whether specific patterns of firing are temporally linked and whether these patterns are responsible for the cortical reorganization that occurs during sleep - as well as with optogenetic studies to further elucidate the neuronal circuitry underlying PM cortex sleep-related functions. These potential findings may provide important clues about the inner working of the premotor cortex and how its neural networks - specifically active during different brain states - supports different brain functions.

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