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Dopamine neurons in the ventral tegmental area modulate REM sleep

Jimmy J. Fraigne¹, Pierre H. Luppi², Carrie E. Mahoney³, Roberto De Luca³, Priyattam J. Shiromani⁴, Franz Weber⁵, Antoine Adamantidis^{6*}, John Peever^{7*}

COMMENTARY ON:

Rapid eye movement sleep is initiated by basolateral amygdala dopamine signaling in mice (Hasegawa *et al. Science*, 375, 994–1000, 2022)

¹Department of Cell and Systems Biology, University of Toronto, Toronto, Canada.

²Centre de Recherche en Neurosciences de Lyon (CRNL), INSERM, and Université Claude Bernard Lyon 1, Lyon, France.

³Department of Neurology, Beth Israel Deaconess Medical Center and Division of Sleep Medicine, Harvard Medical School, Boston, MA 02215, USA.

⁴Laboratory of Sleep Medicine and Chronobiology, Department of Psychiatry, Medical University of South Carolina, Charleston, SC 29425, USA.

⁵Department of Neuroscience, Perelman School of Medicine, Chronobiology and Sleep Institute, University of Pennsylvania, 3400 Civic Center Boulevard, Philadelphia, PA 19104, USA.

⁶Centre for Experimental Neurology, Departments of Neurology and Biomedical Research, Inselspital University Hospital Bern, University of Bern, Bern, Switzerland.

⁷Department of Cell and Systems Biology, University of Toronto, Toronto, Canada.

*Corresponding Authors.

Commentary Overview (Antoine Adamantidis and John Peever).

The discovery of REM sleep is attributed to the landmark observations of Eugene Aserinsky, William Dement, Michel Jouvet and Nathaniel Kleitman. In 1953, Aserinsky and Kleitman identified periods of ‘active sleep’ that are marked by rapid-eye-movements that alternate with ‘quiescent sleep’ periods in human infants. Several years later Dement and Kleitman showed that rapid-eye-movements are correlated with specific patterns of brain-wave activity and that vivid dreaming occurs during periods of rapid-eye-movements in human adults. Shortly thereafter, Jouvet identified a similar behavioural state in cats, showing that cats also experience periods of rapid-eye-movements that occur during periods of muscle atonia and wake-like cortical activity. REM sleep, or REM sleep-like states, have subsequently been identified in a variety of animals, including marsupials, birds, fish, insects, octopi, and lizards. These observations suggest that REM sleep is conserved across the animal kingdom and imply that REM sleep plays a role in normal biology and physiology.

Although REM sleep was initially characterized by rapid-eye-movements, we now know that it is also characterized by a range of physiological features, including reduced amplitude and faster frequency cortical electroencephalogram (EEG) that is reminiscent of waking, high-amplitude theta waves in the hippocampus, active suppression of skeletal muscle activity (i.e., REM atonia), intermittent muscle twitches, autonomic and respiratory activation, fluctuations in

brain/body temperature, and an elevated arousal threshold. Because REM sleep is marked by a waking-like EEG pattern coupled with skeletal motor atonia, some scientists use the terms 'active sleep' or 'paradoxical sleep' when referring to REM sleep. Research over the past several decades has shed light on the biological functions of REM sleep and has provided scientists/clinicians with considerable insights into the anatomical bases by which it is regulated.

Although Jouvet found that REM sleep is generated by structures in the pons, considerable advances in our understanding of REM sleep mechanisms have emerged over the past two decades. Many of these advances are attributable to the advent of novel neuroscience tools (e.g., optogenetics and genetic sensors) that have enabled high-precision interrogation of the brain circuits and neurochemicals that control REM sleep. For example, we now know that circuits beyond the pons (e.g., medulla, midbrain and hypothalamus) also influence the timing, duration and hallmark features of REM sleep.

This commentary was sparked by recent findings that a new, but unexpected, cluster of midbrain neurons appear to play a role in REM sleep control. In a recent edition of *Science Journal*, Hasegawa and colleagues, in their paper entitled *Rapid eye movement sleep is initiated by basolateral amygdala dopamine signaling in mice*, found that dopamine neurons in the ventral tegmental area and dopamine receptor-expressing neurons in the amygdala influence the timing of REM sleep. Hasegawa *et al.*'s findings are not only novel but also provocative because dopamine neurons are typically thought, and have previously been shown, to promote arousal. Dopamine neuronal control of REM sleep therefore represents a new frontier in the search for the circuits that modulate REM sleep.

Based on the novel and provocative nature of Hasegawa *et al.*'s findings, members of SLEEP's editorial board felt that it would be useful to engage the opinions and feedback of experts who study REM sleep mechanisms. Below are the commentaries from Drs. Fraigne, Luppi, Mahoney, De Luca, Shiromani, and Weber wherein they discuss the strengths, limitations and new questions that Hasegawa *et al.*'s study provides to the field of sleep science.

Dopamine neurons in the ventral tegmental area modulate REM sleep (Jimmy J. Fraigne).

The role of dopamine neurons in regulating sleep-wake states has, until recently, been mostly overlooked^{1,2}. This is despite the fact that the most efficient wake-promoting drugs (e.g., modafinil) enhance arousal via dopamine-dependent mechanisms^{3,4}, and that pharmacological manipulation of dopamine receptors influences sleep⁵ (both D1 and D2 dopamine receptor agonists cause arousal), and that some dopamine neurons change their activity across the sleep-wake cycle⁶. A recent paper by Hasegawa *et al.* proposes that dopamine cells in the ventral tegmental area (VTA^{DA}) and neurons expressing dopamine receptor D2 in the basolateral amygdala (BLA^{D2R}) gate the onset of REM sleep (**Fig. 1a**), and that VTA^{DA} neurons might play a role in the pathophysiology of cataplexy - a core disease symptom in narcolepsy⁷. My commentary not only aims to highlight Hasegawa *et al.*'s findings but also aims to raise questions that were not resolved/answered in Hasegawa *et al.*'s paper.

In 2016, *Eban-Rothschild et al.* used the genetically encoded calcium indicator (GcAMP6) to show that VTA^{DA} neurons, a structure historically linked with motivation, reward and addiction, are predominantly active in both wakefulness and REM sleep, and increase their activity at the transition from NREM to REM sleep¹. Based on this observation, *Hasegawa et al.* aimed to elucidate which projections from VTA^{DA} neurons are responsible for the dopamine action on REM sleep⁷. VTA^{DA} neurons project to the cerebral cortex, the lateral hypothalamus (LH), thalamus, the amygdala, the dorsal raphe (DR), pontine tegmentum, cerebellum, and has the strongest projections synapsing onto neurons in the nucleus accumbens (NAcc)⁸. To identify which of these projections might modulate REM sleep, they used a genetically encoded dopamine sensor based on a modified Gi-coupled dopamine receptor D2 (GRAB_{DA})⁹. This sensor allows for temporally precise measurement of relative dopamine release in the post-synaptic field of VTA^{DA} neurons, including the BLA, while mice go through states of wakefulness, NREM and REM sleep. They found that dopamine release increased transiently in both the BLA and NAcc a few seconds before mice transition into REM sleep (**Fig. 1b**), while it decreased in the LH and medial prefrontal cortex (mPFC).

To establish a functional role of VTA^{DA} circuits, *Hasegawa et al.* optically activated VTA^{DA} terminals using a stabilized step-function opsin (SSFO), and revealed that activation of VTA^{DA} terminals in the BLA induced transitions from NREM to REM sleep⁷. Then, using a vertebrate low-wavelength opsin (vLWO) that hyperpolarizes cells through a Gi-mediated mechanism, they optically silenced D2 receptor-expressing neurons in the BLA, and found that inhibiting them also induced transitions from NREM to REM sleep⁷. They concluded that increased dopamine release from the VTA in the BLA inhibits D2-expressing neurons which cause downstream disinhibition and eventually activate brainstem structures that initiate REM sleep. These downstream circuits, however, remain unidentified.

Cataplexy, the involuntary loss of waking muscle tone, is a characteristic feature of narcolepsy. We have previously shown that this occurs through the recruitment of the brainstem circuit that causes REM sleep muscle atonia¹⁰. These events are often triggered by positive emotions elicited by laughter in humans¹¹ and chocolate in mice¹², and we and others have shown that this is mediated through activation of GABA cells in the central bed of the amygdala (CeA)^{13,14}. Finally, we have also shown that pharmacological activation of dopamine D2 receptors in narcoleptic mice increases the occurrence of cataplexy¹⁵. Accordingly, the hypothesis by *Hasegawa et al.* of whether the VTA^{DA}→BLA^{D2R} circuit could also trigger cataplexy was timely and their finding that dopamine release in the BLA precedes episodes of cataplexy in narcoleptic mice⁷ extended the importance of this circuit in the pathophysiological mechanisms of cataplexy. Importantly, *Hasegawa et al.* found that stimulation of VTA^{DA} during wakefulness produces transient dopamine release in the BLA and after some delay, triggers behavioral arrests that resemble cataplexy. This observation is striking as this was produced in wild-type animals, which never exhibit cataplexy. They further found that silencing BLA^{DR2} cells in non-narcoleptic mice with an intact orexin system (the lack of which underlies narcolepsy) produce similar cataplexy-like events. We and others have previously identified similar behavioral arrests in wild-type animals^{10,16-18}. This was obtained through manipulation of either norepinephrine neurons of the locus coeruleus (LC^{NE})¹⁶, GABA cells of the pontine reticular formation (PRF^{GABA})¹⁷, serotonin signaling¹⁸ or activation of sublaterodorsal tegmental (SLD) neurons¹⁰.

Distinction between behavioral arrest, cataplexy and even REM sleep can be difficult, and needs to be carefully assessed by analyzing both cortical and motor activity as well as a detailed behavioral analysis. For example, cataplexy is characterized by a sudden loss of muscle tone that is preceded and followed by heightened motor activity, a specific cortical activity that differs from what is observed in REM sleep, and an inability to be terminated by sensory stimuli (e.g., toe pinch, gentle handling)^{10,16,19}. The cataplexy-like events triggered by Hasegawa et al. will need to be carefully studied to further understand the mechanisms that underlies these events.

The work by *Hasegawa et al.* revealed that VTA^{DA} neurons modulate the timing of REM sleep through their connection with BLA^{D2R} neurons⁷; however, some of their conclusions contrasted with previous evidence and even their own findings. First, the authors claimed that VTA^{DA} dopamine release in the BLA initiates REM sleep. Even though it is clear that dopamine release in the BLA happens a few seconds before the onset of each REM sleep episode, the rather long delay (~100s) from the onset of stimulation of VTA^{DA} terminals, or inhibition of BLA^{D2R} cells, until the onset of REM sleep is somewhat puzzling. How can such a physiologic event as the transition into REM sleep which occurs only few seconds after the release of dopamine in the BLA during spontaneous sleep transitions (**Fig. 1b**) take ~100s when artificially triggered? One possible explanation is that the $VTA^{DA} - BLA^{D2R}$ connection does not produce sufficient dopamine to rapidly (i.e., in less than 10s) trigger the transition into REM sleep. Accordingly, it is possible that the dopamine detected in the BLA prior to REM sleep onset comes from multiple sources of dopamine neurons to sufficiently affect cells in the BLA (both the substantia nigra²⁰ and hypothalamic dopamine nuclei (e.g., $A11^{DA}$)²¹ also project to the amygdala). It is also conceivable that the parameters of optogenetic manipulation (i.e., SSFO) used by *Hasegawa et al.* did not reveal the full scope of this dopamine-mediated mechanism. Like most dopamine neurons, VTA^{DA} neurons fire in a bimodal pattern, either tonically at low frequencies (0.5-5Hz) with low release of dopamine that predominantly activate inhibitory ($G_{\alpha i}$) dopamine D2 receptors or phasically by bursting at high frequencies (10-30Hz), causing transient high concentration of dopamine release activating post-synaptic excitatory ($G_{\alpha s}$) dopamine D1 receptors (**Fig. 1c**)⁶. Importantly, VTA^{DA} neurons fire phasically during REM sleep similar to what is observed under locomotion, feeding and reward⁶. The opsin used in the *Hasegawa et al.* study (i.e., SSFO) increased the firing pattern of VTA^{DA} from 0.5Hz to ~2.5Hz (tonic), preferentially targeting D2-expressing BLA neurons. In this context, it would be interesting to manipulate the same $VTA^{DA} \rightarrow BLA$ circuit with tonic or phasic frequencies by using opsins that can produce both slow and rapid stimulation (e.g., ChETA)¹ to observe the effect on REM sleep and the involvement of other dopamine-sensitive neurons (i.e., D1R-expressing) in the region.

It also remains unclear how BLA^{D2R} neurons are functionally connected with the brainstem circuits that generate REM sleep because these neurons do not appear to project outside the amygdala complex (**Fig. 1a**). *Hasegawa et al.* suggest that silencing of BLA^{D2R} might release the inhibition to other BLA as well as CeA neurons. Consistent with this idea, we¹⁴ and others¹³ have previously shown that chemogenetic activation of GABA-releasing CeA neurons (CeA^{GABA}) promotes emotionally-induced cataplexy, and that these neurons directly project to GABA ventrolateral peri-aqueductal grey ($vIPAG^{GABA}$) and lateral pontine tegmentum (LPT^{GABA}) neurons, that in turn, connect directly with SLD cells (**Fig. 1a**)¹⁴. Presumably, activation of CeA^{GABA} cells inhibits $vIPAG^{GABA}$ neurons and disinhibits SLD neurons, which ultimately cause

muscle atonia during both REM sleep and cataplexy. However, during our manipulations, activation of CeA^{GABA} neurons only triggered cataplexy but did not affect REM sleep, suggesting that other circuits are required to trigger episodes of REM sleep.

Previous work by us and others has shown that the state of cataplexy is objectively different from the state of REM sleep^{10,11}. For example, we showed that the power spectrum profile during cataplexy is different than the typical high theta (θ) activity seen during REM sleep, and that muscle twitches seen in REM sleep are absent during episodes of cataplexy¹⁰. Importantly, narcoleptic subjects experiencing episodes of cataplexy remain fully conscious and aware of their environment, which is strikingly different to what happens during REM sleep¹¹. It would be of interest to investigate how a similar dopamine transient in the BLA can either trigger REM sleep from NREM sleep or cause a cataplexy-like episode when initiated during wakefulness. Along this line, it remains to be shown whether the circuits involved in generating both states are different.

Many nuclei play a role in modulating REM sleep²²⁻²⁵, and it is clear that this newly discovered VTA^{DA}→BLA^{D2R} circuit has to be integrated in our understanding of the widespread network that regulates REM sleep. Considering the network as a whole will help us understand why activation of this specific pathway promotes REM sleep albeit with a significant delay⁷, and why inhibition of REM-promoting nuclei are unable to abolish REM sleep by themselves²²⁻²⁵. In addition, *Hasegawa et al.* reported that peak dopamine release in the BLA does not correlate with any measurement of REM sleep homeostasis (e.g., REM sleep or inter-REM duration). Yet, it will be crucial to identify how these nuclei (including the BLA) contribute to REM sleep homeostasis, a process that shapes the timing of REM sleep. REM sleep homeostasis is characterized by the fact that REM sleep pressure is dissipated during longer REM sleep periods and causes an increased duration of inter-REM intervals to initiate the next REM sleep episode²³.

The D2-mediated circuit described by *Hasegawa et al.* is supported by our finding that systemic injection of a D2-agonist increased cataplexy in narcoleptic mice; however, this pharmacological treatment had no effect on REM sleep amount or transitions¹⁵. The use of D2-agonists in narcoleptic dogs also increased cataplexy, but in this case the agonist was injected either in the VTA^{DA} or A11^{DA} nuclei²⁶. These injections specifically targeted D2-autoreceptors and ultimately decrease the activity of dopamine neurons (**Fig. 1c**). This suggests that the increase in cataplexy observed in these studies was due to a decrease in dopamine tone rather than the activation of dopamine release.

Finally, the findings described by *Hasegawa et al.* need to be reconciled with data showing that increasing dopaminergic tone, either pharmacologically^{4,5} or through direct stimulation of dopamine neurons¹, causes sustained arousal. The most widely used wake-promoting drugs (e.g., amphetamines, modafinil) stimulate arousal by directly blocking dopamine transporter (DAT) to prevent dopamine reuptake, thereby increasing the amount of dopamine in the synapse which will then saturate post-synaptic dopamine receptors (**Fig. 1c**)^{4,5}. Activation of the D1 receptor pathway with systemic D1 agonists leads to increased arousal whereas high doses of D2 agonists activate postsynaptic D2 receptors, causing arousal and decreases in both NREM

and REM sleep⁵. Finally, the study by *Eban-Rothschild et al.*, which demonstrated that VTA^{DA} neurons are active during both wake and REM sleep, also showed that optogenetic activation of VTA^{DA} neurons (~20Hz) induced arousal from sleep and promoted wakefulness even after 4-hours of sleep deprivation¹. Arousal was observed not only when VTA dopamine cell bodies were activated, but also when optogenetic stimuli activated the release of dopamine from VTA fibers in the NAcc¹. This was most likely mediated by activation of D1-expressing NAcc (NAcc^{D1R}) neurons, which are also active during both wake and REM sleep (**Fig. 1c**)²⁷. Hence, it is surprising that light-activation of VTA^{DA}→NAcc by *Hasegawa et al.* had minimal effects on wakefulness. One possible explanation is the use of different firing frequencies and the modulatory effect of dopamine through either D1 or D2 receptor-mediated mechanisms. This could be similar to how a bidirectional D1 to D2 shift in the VTA^{DA}→DR^{5HT} inputs can either prevent or promote feeding²⁸. In this recent study *Cai et al.* showed that a stronger stimulation (i.e., phasic, 20Hz) of the VTA^{DA}→DR^{5HT} circuit activated D1 receptors and prevented feeding while a weaker stimulation (i.e., tonic, 2Hz) activated D2 receptor and promoted feeding²⁸. Hence, in the context of the VTA^{DA} role in sleep-wake regulation, a fine control of VTA^{DA} firing and dopamine release in either BLA or NAcc might explain the balance through which either wake or REM sleep are promoted (**Fig. 1c**).

In conclusion, this study offers new insight into our understanding of REM sleep control and highlights the importance of the dopamine system in sleep-wake regulation⁷. This study also raises important questions. For example, How do VTA^{DA} neurons cause both arousal and REM sleep?; 2) How is the VTA^{DA}→BLA^{D2R} circuit connected to the brainstem structures that generate REM sleep, and what is the role of D1 and D2 receptors in this process?; and, 3) How can the same dopamine release trigger two different behavioral states – i.e., REM sleep or cataplexy? There is no doubt that future work will eventually answer these questions, which could lead to new therapeutic approaches for treating narcolepsy and other sleep disorders (e.g., insomnia).

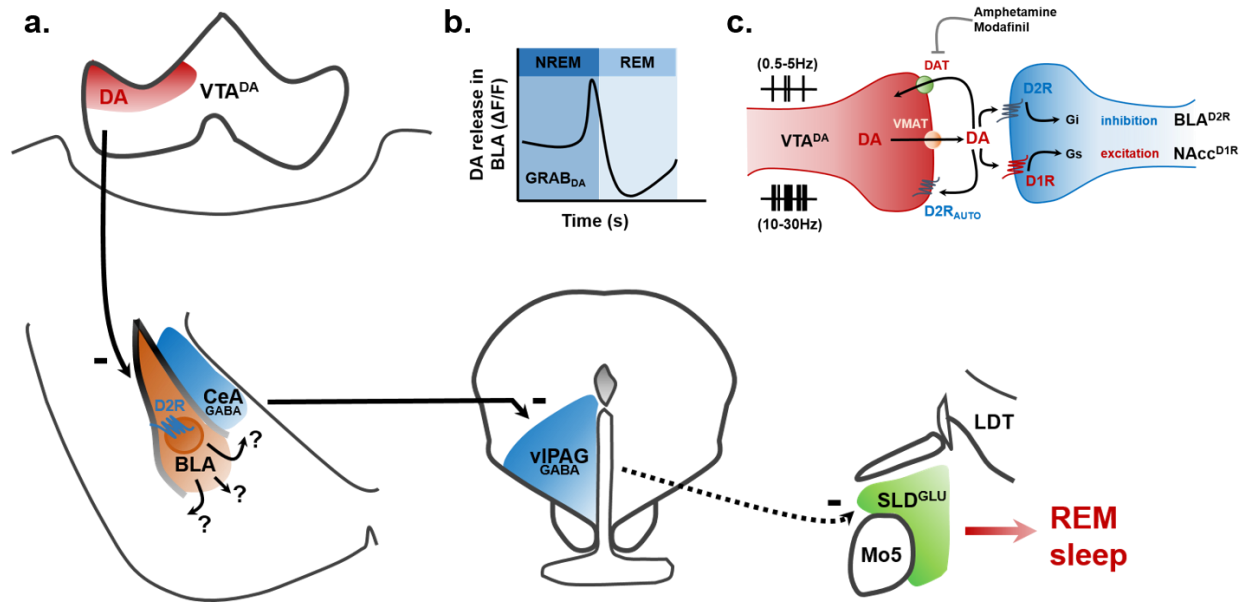


Figure 1. Dopamine control of REM sleep. *a.*, Hasegawa *et al.* proposed that dopamine (DA) cells in the ventral tegmental area (VTA^{DA}) silence neurons expressing dopamine receptor D2 in the basolateral amygdala (BLA^{D2R}) to engage REM sleep. However, it remains unclear through which pathway they connect with REM-generating neurons of the mesopontine junction. This could be done through activation of GABA releasing neurons of the central bed of the amygdala (CeA^{GABA}) cells that inhibit GABA ventrolateral peri-aqueductal grey (vlPAG^{GABA}) neurons and induce the disinhibition of sublaterodorsal tegmentum (SLD^{GLU}) generating REM sleep. *b.*, Hasegawa *et al.* showed that transient DA release ($\Delta F/F$) in the BLA (and in the nucleus accumbens, NAcc) occurs a few seconds before the transition into REM sleep from NREM sleep by using a genetically-encoded DA sensor (GRAB_{DA}). *c.*, VTA^{DA} neurons fire in a bimodal pattern, either tonically at low frequencies (0.5-5Hz) producing low level of dopamine release that predominantly activate inhibitory (G_{ai}) dopamine D2 receptors (both pre- and post-synaptically) or phasically by bursting at high frequencies (10-30Hz), causing transient high concentration of dopamine release activating post-synaptic excitatory (G_s) dopamine D1 receptors. The action of dopamine is tightly regulated by the ability of presynaptic terminals of dopamine-containing neurons to reuptake neurotransmitters through dopamine transporters (DAT) which can be inhibited by wake-promoting drugs (e.g., Amphetamine and Modafinil). Inhibitory D2 receptors are also located on the pre-synaptic side as autoreceptors and regulate the activity of dopamine neurons. Both BLA and NAcc neurons express both D1 and D2 receptors. It remains to be investigated whether a shift in D1 to D2-receptor mechanism could regulate both wake and REM sleep.

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Circuit mechanisms controlling paradoxical (REM) sleep (Pierre-Hervé Luppi)

In their report, Hasegawa et al. (1) propose a completely unexpected new hypothesis on the genesis of paradoxical (REM) sleep. Indeed, they propose that a phasic release of dopamine specifically in the basolateral amygdala (BLA) and not in the lateral hypothalamic area (HLA) nor in the nucleus accumbens (NAC) or the prefrontal cortex (mPFC) plays a crucial role in inducing REM sleep. Their proposal is completely new since until their publication it was admitted that the state of REM sleep was generated by brainstem structures under the control of the lateral hypothalamic area (2). Even more puzzlingly is the demonstration that after the occurrence of a phasic peak of dopamine at the NREM-REM transition, there is decrease of DA level during REM sleep in the BLA and in the other structures. It suggests that a peak of dopamine release at the NREM-REM transition limited to the BLA induces REM sleep whereas the decrease of dopamine release is not playing a key role.

In the following experiments, they excited dopamine terminals in the BLA during NREM for 1 sec per 30 minutes for three hours using optogenetics. They found that each stimulation specifically of the BLA caused a transition to REM sleep after 142s. Amazingly, with such very sparse stimulation, they also increased REM sleep quantities from 10% to more than 30%. They further obtained a switch to REM from NREM after a delay of 89s when inhibiting BLA neurons expressing the D2 dopamine inhibitory receptors, suggesting that dopamine release induced by the stimulation of the DA terminals in the BLA inhibit these neurons. It means that in fact the

neurons they target in the BLA are REM-off and not REM-on neurons and therefore inhibit the onset of REM sleep.

The increase of REM sleep they obtained is similar to what is seen during REM sleep rebound after 48h of total REM sleep deprivation (3, 4). The long delay they report suggests that their stimulation is not directly inducing REM sleep. It suggests that the release of dopamine in the BLA is not acting directly on a generator of REM sleep. Indeed, when you stimulate a structure inducing REM sleep such as the MCH neurons the induction of REM sleep is nearly immediate (5). Conversely, when you stimulate neurons inhibiting REM sleep like the GABAergic neurons of the ventrolateral periaqueductal gray, the cessation of REM sleep is also nearly immediate (6). In addition, Hasegawa et al.(1) did not only induce REM sleep but induce a REM sleep hypersomnia of similar magnitude (30% of total time) to that obtained after a long-lasting REM sleep deprivation. In a way, it looks like their stimulation induced a need for REM sleep.

In a second part of their demonstration, they inhibited DA axons in the BLA again using sparse inhibition for 5 minutes per 25 minutes during three hours. They amazingly observed a quasi-absence of REM sleep during the first hour but with a disappearance of the effect during the third hour of stimulation. The fact that the decrease vanished over time is puzzling and indicates that some mechanisms of adaptation occur.

Then, they report that following the inhibition of the dopamine D2-expressing neurons in the BLA, a number of surrounding cells express cFos and they further showed using anterograde tracing that these neurons project to the brainstem structures generating REM sleep. In fact, they also found cFos neurons in the neighbouring central amygdala and it is more likely than these neurons are those projecting to the brainstem since BLA neurons do not project there. They rather strongly project to the ventral hippocampus, nucleus accumbens and central amygdala (7). It would have been particularly helpful if the authors had analysed cFos staining across the entire brain to determine whether the classical REM inducing structures in the pons are activated and whether additional ones than those seen during REM sleep rebound are also labelled.

In the second part of their article, the authors found that there was also a transient increase in DA in the BLA when narcoleptic mice were eating chocolate, which was followed by cataplectic episodes. Then, they stimulated the DA terminals in the BLA and induced after a delay of around 80s an increase in cataplectic episodes. Similar results were obtained when inhibiting the BLA neurons expressing D2 receptors. Finally, optogenetic inhibition of DA terminals in the BLA almost completely inhibited the occurrence of cataplectic episodes. They finally demonstrated that VTA dopaminergic neurons projecting to the BLA are different than those projecting to the NAC and HLA.

Overall, and as stated above, Hasegawa et al.(1) results are quite surprising since if the amygdala has been previously involved in the induction of cataplectic attacks, it is not the case for REM sleep. Indeed, the amygdala has been classically involved in emotional regulation (8) and its lesion reduced emotionally triggered cataplexy in mice but had no effect on REM sleep

(9). Further, amygdala lesions in monkeys did not induce a decrease in REM sleep (10). Finally, although it has been reported that VTA dopaminergic neurons have similar levels of activity during wakefulness and REM sleep (11), their chemogenetic inhibition inhibits wakefulness and induces sleep with remarkable increase in REM sleep quantities (12). The increase of REM was due to an increase in the number of bouts not of their duration. Conversely, excitation of DA VTA neurons by optogenetics induced an immediate transition to wakefulness. These results indicate that dopamine release from VTA DA neurons is rather inhibitory than excitatory to REM sleep and are against a role of DA in REM sleep induction (12).

In summary, the results provided by Hasegawa et al. (1) are very convincing in demonstrating that the inhibition by dopamine of BLA neurons via D2 inhibitory receptors disinhibit surrounding neurons and neurons localized in the neighbouring central amygdala. Their results indicate that such neurons play a role in REM sleep and cataplexy. However, there are a number of results indicating that DA and the central amygdala do not play a direct role in REM sleep genesis. In addition, the fact that the induction of cataplexy and REM sleep occurred after very long latencies and that they obtained a REM sleep hypersomnia similar to that obtained after a 48h of REM sleep deprivation clearly indicates that BLA neurons are not a core element of the REM sleep generating network but are rather acting upstream to it. The latency to obtain REM sleep further suggests that the effect obtained is not due to a disinhibition of central amygdala neurons projecting to the core REM generating systems in the brainstem. Indeed, in such case, the effect would have been nearly immediate. The fact that the BLA is a core system for controlling emotion rather suggest that the effect obtained is linked with the function of REM sleep. Indeed, a large body of data indicate that REM sleep plays a key role in emotional regulation (2, 13). It is therefore tempting to propose that by acting on a key structure involved in emotional regulation, the authors touched upon neurons that are key for regulating such a function during REM sleep. One can speculate that emotional regulation occurred during REM sleep and is regulated by BLA neurons and their projections to the ventral hippocampus. By stimulating or inhibiting these neurons, it might be that a signal is send to the REM sleep generator that more or less REM sleep is needed to do the job. Additional experiments are necessary to explore such hypotheses.

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Why do we have REM sleep? (Roberto De Luca and Carrie E. Mahoney)

This question may be addressed from a mechanistic standpoint, as to which brain regions or neurotransmitters cause the transition into the state of REM sleep, or a functional view as to what are the advantages of REM sleep for the organism in terms of development or maintenance of physiology and/or brain plasticity. Here, we discuss the recent findings from Hasegawa and colleagues on the role of VTA dopamine (VTA^{DA}) neurons in REM sleep initiation and the research perspectives on the evolutionary origin of mammalian sleep.

Previously, VTA^{DA} neurons have been reported to display higher activity during wakefulness, REM sleep and before NREM-REM sleep transitions and lower during NREM sleep [1]. Additionally, other neurochemically-distinct excitatory (glutamatergic/nitroergic) and inhibitory (GABAergic) but non-dopaminergic neuronal populations of the VTA, have been described to have a role in wake and sleep regulation [2].

In this study, Hasegawa and colleagues described an important pathway by which the release of DA within the basolateral amygdala (BLA) from VTA neurons that express the DA transporter (VTA^{DAT}) anticipates the onset of REM sleep. The DA accumulation in the BLA showed a transient peak that precedes the NREM-REM sleep transition. More importantly, photo-stimulation of $VTA^{DAT} \rightarrow$ BLA pathway caused a rapid transition into REM sleep. The peculiarity of this finding resides in 1) describing that DA release from VTA^{DAT} neurons to BLA anticipates REM sleep and that 2) DA release from VTA^{DAT} neurons into the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and lateral hypothalamus (LH), is excluded from REM sleep participation (Fig.1). There are multiple dopaminergic cell groups throughout the central nervous system (CNS) of mammals. Many have been demonstrated to increase their activity during wake, often in response to salient stimuli [3-5]. Whether these populations act in concert to also facilitate REM sleep entry, is not known. Is the pattern of neuronal firing a trigger for REM sleep, such as burst firing from the VTA^{DA} neurons required? Burst firing of DA neurons leads to more synaptic DA accumulation than single spike firing [6]. Is the cooperation of the VTA^{DAT} , VTA^{GABA} and VTA^{Glut} neurons that contribute to REM regulation recruited by the same or different upstream regions? Are the VTA neurons further subdivided by their receptor profile and this sensitivity allows for regulation of salient stimuli processing and facilitation of REM sleep?

The fact that the GABAergic neurons of the BLA expressing the DA receptor 2 (DRD2) may serve as a switch to dis-inhibit the amygdala outputs – those involved to indirectly activate the REM-on pontine and medullary structures – is a novel and highly valuable finding. Reluctantly, the authors did not provide evidence of circuit connection from the amygdala to REM regulatory regions to support their finding of BLA's direct involvement in REM initiation by the dis-inhibition of REM-on structures. The optogenetic silencing of BLA DRD2 neurons increased the time spent in REM sleep as well as NREM-REM sleep transitions. In a mouse model of narcolepsy, Hasegawa et al., reported that chemogenetic silencing of D2R-expressing neurons in the BLA increased cataplexy. This is consistent with the observations that administration of a D2R agonist increased, and D2R antagonist reduced, cataplexy in mice [7]. These neurons may represent a potential and selective pharmacological target to treat many of the REM sleep-related disorders, such as narcolepsy (type 1 and 2) and other sleep-related pathologies where DA signaling is involved.

However, how these local GABAergic neurons of the BLA that express the DRD2 dis-inhibit the other neurons in the amygdala complex remains unclear. The fact that their activation can reduce the glutamatergic inputs onto the non-DRD2 neurons nearby – and therefore that their inhibition could dis-inhibit the other amygdala neurons – does not fully clarify the circuit mechanism responsible for REM sleep onset. Indeed, there is no indication on where this excitatory input may come from or how the dis-inhibited neurons in the amygdala could promote REM sleep and/or cataplexy. The pontine REM circuitry, specifically the periaqueductal gray (PAG), sends both glutamatergic and GABAergic projections to VTA^{GABA} neurons. This extended loop from the PAG to the VTA may facilitate REM sleep entry [8]. Additional candidate regions recruiting the VTA^{GABA} neurons to promote REM sleep include the hippocampus (Hip), lateral dorsal tegmentum (LDT) and pedunculo-pontine nucleus (PPT). The

last two are highly active during REM sleep and project to and regulate VTA^{DA} firing [9,10]. (Fig.2).

Moreover, the circuit experiment performed by using the *Drd2-cre::DAT-cre* mice with the aim to record from identified BLA neurons expressing the DRD2 while stimulating the input from VTA^{DAT} neurons (VTA^{DAT}→BLA^{DRD2}), could accidentally “enlarge” the source of DA coming from the “true” DA neurons from VTA, into the BLA (VTA^{DAT/DRD2}→BLA^{DRD2}) since the DRD2-expressing neurons in the VTA, co-express TH+/DAT+ and are inhibited by the DRD2 agonist quinpirole [14]. These results could be misleading and not completely in line with the work done in the previous experiments from Hasegawa and colleagues that focused on the manipulation of the VTA^{DAT}→BLA input, *in vivo*.

As shown in several studies, DA tone is high during wake [11] and intra-nuclear administration of DA into REM sleep regulatory regions of the tegmentum, reduced REM sleep duration and increased REM sleep latency [12,13]. Additional research is needed to clarify the role of subpopulations of DA neurons and their respective roles in sleep. Some concern emerges from the selection of the DA neurons in the Hasegawa paper done solely through the expression of the DAT in the VTA. It has been described that not every DAT-expressing neuron in the VTA expresses tyrosine hydroxylase (TH) [14]. Indeed, DAT-expressing neurons in this region comprise a population of “truly” DA neurons (TH+/DAT+, 70%) and neurons that express DAT but not TH enzyme (that could be considered as “conditionally” dopaminergic and may turn into a dopaminergic phenotype if L-DOPA is available) [14]. Therefore, these neurons are able to potentially release GABA or glutamate [15]. It has also been reported that the VTA^{Glut} neurons are both wake and REM sleep active [2]. So, in addition to DA, other neurotransmitters could be released from the VTA^{DAT} neurons in the BLA and modulate the activity of BLA neurons and the amygdala output itself. The release of GABA and/or glutamate from the VTA^{DAT} neurons into the BLA was not investigated and may represent a new experimental avenue raised by the study.

The findings of Hasegawa et al., do bring to light potential mechanisms underlying the proposed evolution of mammalian sleep. There is much investigation and thought on whether sleep evolved to conserve energy and/or if sleep provides a state to allow reinforcement and elimination of memories. As described in [16,17], mammalian NREM-REM cycling may have evolved from reptilian basking in the sun (resting) and arousal to complete goal directed behaviors (feeding, fighting, etc.). Observations placing the VTA as a triggering signal to enter REM sleep open an avenue to test the interface of temperature regulation and brain plasticity (Fig. 2).

Warm-sensing neurons in the preoptic area (POA) that modulate the autonomic nervous system and thermoregulatory brain regions can promote sleep by inhibiting wake-promoting centers in the brain [18]. In humans, thermoregulation around the time of sleep onset, particularly the increase in distal skin temperature (Tsk) and the decrease in core body temperature (CTB), are important determinants of sleep latency, sleep consolidation, and overall sleep quality [19]. During REM sleep, CTB is more influenced by ambient temperature as thermo-protective responses are irregular [20]. CTB influences the propensity to enter REM

sleep [21] and the coordination of body temperature and sleep cycles is necessary for optimal patterns and quality of sleep.

Do thermoregulatory neurons of the preoptic and median preoptic area project to the VTA and gate those neurons of the VTA involved with induction of REM sleep? Or do the thermosensing neurons project directly to the VTA? Is prior experience a factor in whether REM sleep is attained under thermal stress conditions?

Disrupted sleep and suppression of REM sleep does impact memory and brain plasticity [22]. Although the various methods of sleep disruption, stress models, etc. have resulted in inconsistent findings around sleep and brain plasticity. Further studying the role of the VTA in REM sleep may clarify the function of REM sleep. It has been proposed that one function of REM sleep is to permit replay of newly acquired memories during sleep resulting in improved or strengthened synaptic connections [23]. If we focus on aspects of behavior the VTA is known to process, i.e. motivated behaviors, there is sufficient evidence to support the VTA^{DA} neurons in promotion of neuronal plasticity. The VTA projects to numerous regions involved with processing novel and rewarding stimuli, e.g. nucleus accumbens, cortex, and hippocampus [24]. Many studies have demonstrated that DA promotes plasticity (Long-Term Potentiation) in the hippocampus [25-27]. It is plausible that the VTA facilitates memory linked to rewarding experiences by way of promoting REM sleep and also modulating brain plasticity in multiple but related brain regions (Fig.1).

A direct test of whether the VTA receives input from thermoregulatory and REM regulatory regions to facilitate entry in REM sleep under different conditions is warranted. If the VTA^{DA} neurons are fundamental to REM sleep, we would predict that the heating of cooled rodents back to thermoneutrality while in NREM sleep would result in the increase in activation of VTA^{DA} neurons [28]. Additionally, inhibition of VTA^{DA} neurons may reduce the likelihood of transition to REM sleep following cooling of rodents that are held at a temperature above thermoneutrality. Ultimately, can memory consolidation disrupted by thermal stress be rescued by activation of the VTA to BLA circuit?

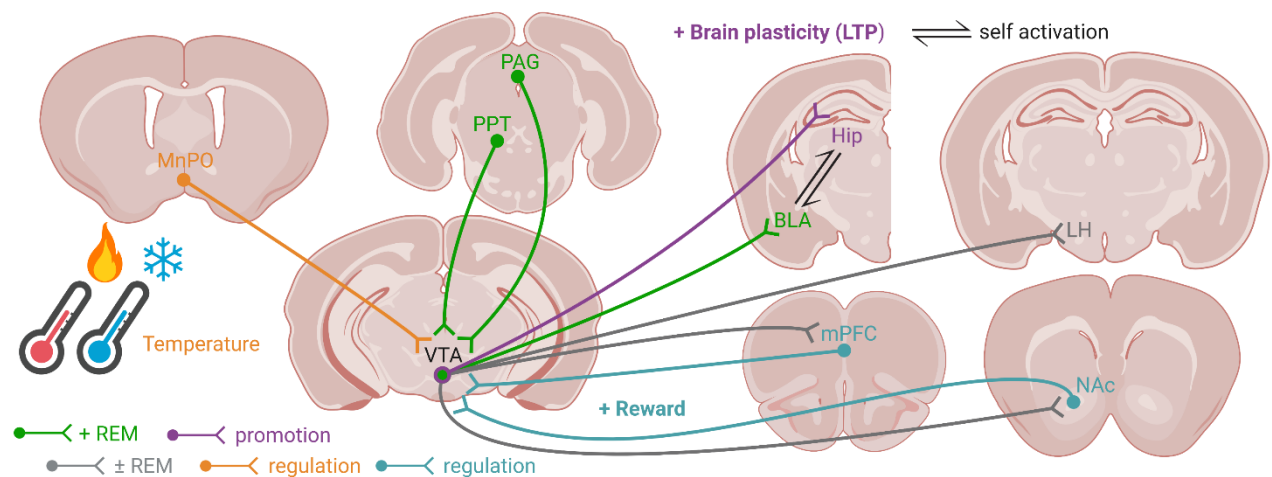


Figure 2. VTA^{DA} neurons gate REM sleep and brain plasticity by receiving inputs from REM sleep-promoting, thermoregulatory and reward processing brain regions. Median preoptic area (MnPO); Periaqueductal gray (PAG), Pedunculopontine nucleus (PPT); Hippocampus (Hip); medial prefrontal cortex (mPFC); nucleus accumbens (NAc) and lateral hypothalamus (LH). Long term potentiation (LTP).

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Still valid – Jouvet’s discovery that REM sleep is initiated in the pons (Priyattam J. Shiromani)

Soon after REM sleep was discovered in humans¹, Jouvet identified REM sleep in the cat and found that muscle atonia is one of its distinguishing features². Other indices such as pontogeniculo-occipital spikes, EEG theta waves, tumescence of the sex organs, dream mentation, irregular respiration and heart rate were also found to occur in REM sleep, indicating that brain activity in REM sleep is similar to waking³. Intrigued by REM sleep, Jouvet embarked on pioneering studies to identify the brain region(s) that trigger REM sleep. Employing the “high-

tech” method of the day, Jouvet cut the brain at various levels along the rostral-caudal axis and concluded that neurons in the pons generate REM sleep ². Other investigators have replicated Jouvet’s studies ^{4 3}. The transection method has drawbacks since making cuts anywhere in the brain causes profound behavioral changes that make it difficult to draw firm conclusions. Moreover, the cuts were never complete as the ventral portions of the brain, where the major brain arteries are located, had to be preserved. The dopamine neurons in the ventral tegmental area are located ventrally along with the sensory and motor pathways that connect the cerebrum to the brainstem and spinal cord (e.g., corticospinal tract). As these areas and tracks were most likely spared in Jouvet’s transection studies, the question about the role of dopamine neurons in the ventral tegmental area, or other areas rostral to the cut, in regulating one or more indices of REM sleep remains unsettled (comment: Jouvet did Pontine cat without the VTA and the ventral midbrain...).

Recently, Hasegawa et al., ⁵ used modern neuroscience tools to investigate the role of dopamine released from the ventral tegmental area (VTA) on REM sleep in mice. Hasegawa et al., recorded EEG theta activity coupled to muscle atonia, which are validated markers of REM sleep. In their first set of experiments, they used a new calcium-based fluorescence sensor ⁶ activated by dopamine signaling on the dopamine D2-receptor (GRAB-D2). The introduction of neural sensors, including one coupled to orexin ⁷, enables researchers to gauge neurotransmitter activity at specific receptors, although their cellular resolution is limited. Hasegawa et al., utilized a GRAB-D2 sensor and, with fiber photometry, measured fluorescence in discrete brain regions that are targets of VTA dopamine neurons. They noticed a spike in fluorescence in the basolateral amygdala (BLA) and nucleus accumbens (NaC) during the transition from non-REM to REM sleep. In subsequent experiments, they mechanistically tested the relevance of the surge in dopamine by optogenetically stimulating the dopamine fibers in the BLA and NaC. They found that optogenetic stimulation of the fibers or somata containing the D2-receptor in the BLA, but not the NaC, significantly reduced the latency to REM sleep. Next, in narcoleptic mice, they identified the role of the D2R-BLA neurons in cataplexy and found that increased dopamine levels in the BLA triggered cataplexy (verified by video). Their neuroanatomical experiments confirmed that BLA neurons projected to mesopontine regions implicated in regulating REM sleep. From these results, Hasegawa et al. concluded that REM sleep and atonia are initiated by BLA dopamine signaling.

I agree that Hasegawa et al., used modern tools to answer a difficult question. Hasegawa et al., noted a surge in dopamine in the BLA and NaC during the transition from non-REM to REM sleep. The increase in dopamine in both regions also occurred during chocolate-induced cataplexy in narcoleptic and wildtype mice. Narcoleptic mice also experience spontaneous bouts of cataplexy. It would be essential to determine if a surge in dopamine in the BLA and NaC occurs during spontaneous bouts of cataplexy. If the dopamine surge occurred only in response to chocolate, it would tightly link the BLA to emotion-induced cataplexy.

Nevertheless, the novel finding by Hasegawa et al., was that dopamine binding to the D2-receptor was increased at REM sleep onset in both the BLA and NaC. From the optogenetic studies, they proposed that the BLA to REM-generating pontine circuits influences REM sleep

and muscle atonia. I suggest that Hasegawa et al's conclusions would be more robust if they had genetically silenced the VTA neurons (for example with tetracycline-on-off method) or severed the BLA projections to the mesopontine regions with viral tracers. Such an experiment would be a modern equivalent of transecting the fibers to the mesopontine region. Lesions of the amygdala in primates does not alter REM sleep⁸, whereas, in humans⁹, cats, and rodents, a lesion in the dorsal pons abolishes one or more indices of REM sleep³. Pontine lesions also cause REM behavior disorder, indicating that neurons triggering atonia are located in the pons¹⁰. A pontine circuit responsible for muscle atonia has been described¹¹.

In the last decade, new genetic tools have made it feasible to dissect circuits regulating complex behaviors such as REM sleep¹². Optogenetic or pharmacogenetic stimulation of specific phenotypes of neurons has a predictable effect on non-REM, REM sleep or both^{13,14 15 16,17 18}. We suggest that the D2R-BLA neurons represent another neuronal phenotype that can influence REM sleep onset. I agree that Hasegawa et al., are the first to critically test the role of the VTA dopamine neurons in REM sleep. However, their conclusions do not undermine Jouvet's discovery that neurons in the dorsal pons initiate REM sleep.

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The increasingly complex case of REM sleep (Franz Weber)

A characteristic feature of mammalian sleep is the alternation between rapid eye movement (REM) and non-REM (NREM) sleep, constituting the NREM-REM or sleep cycle. Research over the past decade has identified multiple neural populations involved in the regulation of REM sleep at an unprecedented level of detail (Casaglia and Luppi, 2022; Peever and Fuller, 2017). This effort has resulted in an increasingly complex circuit map of the REM sleep circuitry comprising multiple neural populations located within brainstem and hypothalamus and their interactions. However, the neural mechanisms or signaling molecules that determine when REM sleep is initiated and thereby regulate the sleep cycle are still largely elusive. A recent study has identified another potential player in the regulation of REM sleep: dopamine receptor D2 (Drd2)-expressing neurons in the basolateral amygdala (BLA) (Hasegawa et al., 2022).

Using a G protein-coupled receptor activation-based (GRAB) sensor for dopamine (DA), *Hasegawa et al.* measured the extracellular dopamine (DA) concentration in the BLA and found that the DA levels transiently increased right before NREM-to-REM sleep transitions. Next, to test whether DA signaling in the BLA is causally involved in REM sleep regulation, the authors optogenetically stimulated the axons of dopaminergic neurons projecting from the midbrain ventral tegmental area (VTA) to the BLA. For optogenetic activation, the investigators expressed the bistable step-function opsin SSFO in dopaminergic VTA neurons, which causes a long-lasting (tens of minutes) excitation of neurons, when activated by a short laser pulse (Yizhar et al., 2011). Remarkably, photo-stimulation of the dopaminergic axons more than doubled the amount of REM sleep. In contrast, optogenetic inhibition of the VTA axons suppressed REM sleep.

The BLA contains neurons expressing Drd2, and previous studies demonstrated that systemic injection of DA agonists and direct injection into the amygdala promotes REM sleep (Dzirasa et al., 2006; Kumar Yadav and Mallick, 2021). *Hasegawa et al.* demonstrated *in vitro* that optogenetic activation of DA fibers in the BLA induces a long-lasting hyperpolarization of

Drd2-expressing neurons, leading to disinhibition of non-Drd2-expressing BLA neurons. Optogenetic silencing of Drd2-expressing BLA neurons *in vivo* strongly enhanced REM sleep, while their activation reduced REM sleep. Collectively, these data demonstrate that Drd2 neurons in the BLA strongly suppress REM sleep, whereas their inhibition by DA released from the VTA causes a disinhibition of BLA neurons, which in turn leads to an increase in REM sleep.

Previous studies have demonstrated a critical role of the amygdala in cataplexy, a sudden intrusion of muscle atonia into wakefulness, characteristic for narcolepsy (Burgess et al., 2013; Mahoney et al., 2017; Snow et al., 2017). *Hasegawa et al.* tested whether DA signaling in the BLA may also be involved in triggering cataplectic attacks. Using the GRAB sensor, the authors measured the DA levels in the BLA in narcoleptic mice lacking the neuropeptide orexin/hypocretin. Eating chocolate, a known trigger of cataplexy (Burgess et al., 2013), led to transient increases in DA, and activating Drd2 neurons in narcoleptic mice strongly increased the number of cataplectic attacks. Even in non-narcoleptic mice, stimulating dopaminergic fibers in the BLA during wakefulness could produce cataplexy, a striking finding because it indicates that DA release in the BLA can override orexin/hypocretin-dependent mechanisms normally protecting against cataplectic attacks in healthy wild-type mice.

In addition to providing convincing and compelling evidence for a role of BLA Drd2 neurons in controlling the amount of REM sleep, the authors further proposed that their findings revealed a novel mechanism for the generation of the mammalian sleep cycle. They argued that the spike in DA, which starts rising about 30 s before the NREM-to-REM transition and peaks right at the transition (**Figures 1B,C** in *Hasegawa et al.*), terminates NREM sleep and initiates REM sleep. The investigators showed that a single laser pulse (1 s) to optogenetically activate the dopaminergic axons using SSFO indeed induces a transient rise of DA within the BLA lasting about 90 s, mimicking the time course of the DA transient observed during spontaneous NREM-to-REM transitions (compare **Figures 1C** and **5E** in *Hasegawa et al.*). But surprisingly, the laser pulse was followed by REM sleep only after a delay of about 140 s, that is after the DA concentration has returned to baseline. The authors further demonstrated that a single laser pulse led to a minutes-long inhibition of Drd2 neurons in the BLA, lasting much longer than the DA transient itself (**Figures 4B,C** in *Hasegawa et al.*). Consequently, the light pulse and the resulting DA transient may not directly trigger REM sleep in a time-locked, one-to-one fashion, but the resulting long-lasting disinhibition of the BLA may more generally promote a state conducive to NREM-to-REM sleep transitions and facilitate the maintenance of REM sleep episodes (the increase in REM sleep was largely due to an elongation of REM sleep episodes). In this context, it will be an interesting question for future research to test whether other manipulations or molecular signaling pathways causing a long-lasting excitation of BLA (non-Drd2-expressing) neurons may similarly enhance REM sleep. For example, in addition to a Drd2 agonist, injection of an agonist for cholinergic receptors into the amygdala has been recently shown to also increase REM sleep (Kumar Yadav and Mallick, 2021).

How the Drd2 neurons in the BLA interact with other REM sleep-regulatory neurons in the hypothalamus and brainstem to facilitate REM sleep and cataplexy remains an important question for future studies. A possible pathway may include the excitation of neurons in the central nucleus of the amygdala (CeA) by the BLA neurons. Chemogenetic activation of the

largely inhibitory CEA neurons has been previously shown to promote cataplexy through inhibition of REM sleep-suppressing neurons in the midbrain (Mahoney et al., 2017; Snow et al., 2017). Alternatively, the authors propose that BLA neurons disinhibited by the DA release may directly excite REM sleep-promoting brainstem neurons. Identifying the presynaptic inputs that regulate the BLA-projecting VTA neurons and investigating the neural dynamics in pre- and postsynaptic populations during transitions into REM sleep will provide further important insights into how the Drd2 neurons are inter-connected with other REM sleep circuits. Of note, the increase of the DA levels about 30 s before a spontaneous switch to REM sleep is similar to the activity pattern observed for other REM sleep-regulatory neurons in hypothalamus and brainstem, which are typically activated during the transitional sleep stage leading to REM sleep (Blanco-Centurion et al., 2019; Boucetta et al., 2014; Izawa et al., 2019; Stucynski et al., 2022), suggesting that the DA levels in the BLA start rising in concert with other REM sleep-promoting neurons. Thus, it remains an open question for future research whether there exists a clear hierarchy in the REM sleep circuitry with specific first-order neurons initiating REM sleep, or whether multiple neural populations form a complex, recurrent network, in which each node can trigger REM sleep.

In summary, the study by *Hasegawa et al.* revealed a novel cell type for the regulation of REM sleep and highlights an important role of DA in REM sleep control and cataplexy. Their findings are of high translational significance to discover druggable targets for the treatment of narcolepsy and will certainly inspire future research to fully understand how BLA Drd2 neurons are anatomically and functionally integrated within the increasingly complex brain network regulating REM sleep.

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Summary (Antoine Adamantidis and John Peever).

The commentaries from Drs. Fraigne, Luppi, Mahoney, De Luca, Shiromani, and Weber highlight the strengths, limitations and new insights that Hasegawa *et al.*’s findings provide the field of sleep science, and our understanding of REM sleep control. A common theme that emerged was that Hasegawa *et al.*’s study adds to the growing body of evidence that REM sleep is controlled by a distributed network of circuits located throughout the brain. Research over the last 20 years has identified neurons in the medulla, pons, hypothalamus and midbrain in REM sleep control. Hasegawa *et al.*’s study now indicates that dopamine neurons in the ventral tegmental area and dopamine receptor-expressing neurons in the basolateral amygdala also contribute to the modulation of REM sleep.

Another common theme raised by commentors was – how do dopamine receptor-expressing neurons in the basolateral amygdala communicate with the pontine neurons that generate REM sleep? Although it has been more than 60 years since Jouvet’s seminal work suggesting that neural structures in the pons generate REM sleep, the principle pontine neurons responsible for generating REM sleep remain ambiguous. Although evidence indicates that neurons in the sublaterodorsal tegmental nucleus (a region that sits just beneath the locus coeruleus) are critical for REM sleep control, the transmitter phenotype(s) of these remains unclear. Evidence indicates that REM-generating neurons may be glutamatergic, GABAergic or cholinergic in nature. (comment, I believe it is clear muscle atonia is generated by glutamatergic neurons. What is still debatable is whether GABA and cholinergic local neurons play a role and whether outside SLD there are other groups playing such role... Tbc!). Personally I would be more generic than focusing on the pons there. Therefore, a central question in sleep biology is – which pontine neurons generate REM sleep? Answering this question will not only allow sleep scientists to understand how REM sleep is generated but it will also allow for a clearer determination of the functional hierarchy of the myriad of REM-modulating circuits that have been identified to date.