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## Noradrenergic transmission at alpha1-adrenergic receptors in the ventral periaqueductal gray modulates arousal

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### Abstract

**Background.**—Dysregulation of arousal is symptomatic of numerous psychiatric disorders. Previous research has shown that the activity of dopamine (DA) neurons in the ventral periaqueductal gray (vPAG) tracks with arousal state, and lesions of vPAG<sup>DA</sup> cells increase sleep. However, the circuitry controlling these wake-promoting DA neurons is unknown.

**Methods.**—The present study combines Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), behavioral pharmacology, electrophysiology, and immunoelectron microscopy in male and female mice to elucidate mechanisms in the vPAG that promote arousal.

**Results.**—DREADD-induced activation of locus coeruleus (LC) projections to the vPAG or vPAG<sup>DA</sup> neurons promoted arousal. Similarly, agonist stimulation of vPAG  $\alpha$ 1-adrenergic receptors ( $\alpha$ 1ARs) increased latency to fall asleep, while  $\alpha$ 1AR blockade had the opposite effect.  $\alpha$ 1AR stimulation drove vPAG<sup>DA</sup> activity in a glutamate-dependent, action potential-independent

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#### Disclosures

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manner. Compared to other dopaminergic brain regions,  $\alpha 1$ ARs were enriched on astrocytes in the vPAG, and mimicking  $\alpha 1$ AR transmissin specifically in vPAG astrocytes via Gq-DREADDs was sufficient to increase arousal. In general, the wake-promoting effects observed were not accompanied by hyperactivity.

**Conclusions.**—These experiments reveal that vPAG  $\alpha 1$ ARs increase arousal, promote glutamatergic input onto vPAG<sup>DA</sup> neurons, and are abundantly expressed on astrocytes. Activation of LC inputs, vPAG astrocytes, or vPAG<sup>DA</sup> neurons increase sleep latency but do not produce hyperactivity. Together, these results support an arousal circuit whereby noradrenergic transmission at astrocytic  $\alpha 1$ ARs activates wake-promoting vPAG<sup>DA</sup> neurons via glutamate transmission.

### Keywords

dopamine; norepinephrine; arousal; sleep; wakefulness; astrocytes; DREADDs

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### Introduction

Arousal is a fundamental component of adaptive behavior. Arousal increases when animals must stay alert to forage, mate, or avoid predation. In addition to simple goal-directed behaviors, arousal is an integral part of higher cognition including learning and decision making (1, 2). By contrast, suppression of arousal is required for rest, sleep, and energy conservation. Dysregulation of arousal is symptomatic of multiple neurological and neuropsychiatric disorders such as schizophrenia, depression, and substance abuse (3–7) and can have serious consequences. For example, 5% of adults surveyed reported falling asleep while driving during the past 30 days (8), and sleep disorders, sleepiness, and fatigue cause approximately 20% of car accidents resulting in serious injury or death (9). Therefore, an urgent need exists to understand how the brain mediates arousal and to develop better treatments for impairments in arousal.

In addition to playing critical roles in motivated and motor behaviors, dopamine (DA) is involved in arousal (10–12). Excessive sleepiness is a common non-motor symptom of Parkinson's disease (13). Recently, optogenetic stimulation of ventral tegmental area (VTA) or dorsal raphe (DRN) DA neurons was shown to promote wakefulness, whereas chemogenetic inhibition of these neurons increased sleep (14, 15). Over a decade ago, neighboring ventral (also known as ventrolateral) periaqueductal gray (vPAG) DA neurons (vPAG<sup>DA</sup>) were shown to be active during wakefulness and inactive during sleep, and lesioning these neurons resulted in significant increases in time spent asleep (16). While the vPAG is anatomically interconnected with numerous brain regions involved in arousal (16), the functional neural circuitry that modulates vPAG<sup>DA</sup> activity and its wake-promoting effects remain largely unknown.

Decades of research indicate that the noradrenergic locus coeruleus (LC) modulates arousal (17–19). The LC is active during wakefulness and relatively inactive during sleep (20), optogenetic stimulation of the LC promotes wakefulness (21), and noradrenergic manipulations can alter arousal (22). The LC is an ideal candidate to control wake-promoting vPAG<sup>DA</sup> neurons because 1) the LC projects to the vPAG (16, 23, 24), 2)  $\alpha 1$ -

adrenergic receptors ( $\alpha$ 1ARs) are present in the vPAG (25, 26), and 3) norepinephrine (NE) transmission via the Gq-coupled  $\alpha$ 1AR can modulate the activity of other DA neuron populations including the rostral linear nucleus (RLi) (27), substantia nigra (28, 29), and VTA (30).

The present study tested the hypothesis that LC-vPAG circuitry modulates arousal by examining 1) whether chemogenetic activation of vPAG<sup>DA</sup> and LC projections to the vPAG promote arousal, 2) whether vPAG  $\alpha$ 1ARs modulate arousal and vPAG<sup>DA</sup> activity, 3) the precise cellular identity and location of  $\alpha$ 1AR expression in the vPAG, and 4) the functional contribution of vPAG astrocytes, which contain  $\alpha$ 1ARs, in modulating arousal.

## Materials and Methods

### Subjects and behavioral testing

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University. To investigate the role of LC-vPAG circuitry on arousal, we examined latency to fall asleep following chemogenetic or pharmacological manipulations in adult male and female mice. We included measures of horizontal locomotion to serve as a control for potential hyperactivity following wake-promoting manipulations. C57BL/6J mice were used for EEG and terazosin experiments. The *Dbh*<sup>-/-</sup> mice used for phenylephrine experiments were generated as previously described (31–33) and maintained on a mixed 129/SvEv and C57BL/6J background. TH-Cre mice (originally obtained from Jackson Laboratories, Bar Harbor, ME) bred on a C57BL/6J background were used for Designer Receptors Exclusively Activated by Designer Drugs (DREADD) experiments to limit AAV-hSyn-DIO-hM3D(Gq)-mCherry expression to TH-containing cells (34). GFAP-hM3Dq mice (breeding pairs generously provided by Ken McCarthy) were bred on a C57BL/6J background and used to test the functional role of vPAG astrocytes in mediating arousal. All genotypes were confirmed by PCR.

Guide cannula were implanted over the vPAG, and AAV-hSyn-DIO-hM3D(Gq)-mCherry was virally expressed in the LC or vPAG (see Supplemental Information). Sleep latency testing was conducted in the home cage of individually-housed mice. Animals were kept on a 12:12 light/dark cycle, and testing began 2–3 h into the light cycle when pressure to sleep is high. A video camera recorded mouse behavior for at least 3 h. When mice fall asleep, they exhibit a distinct posture and breathing pattern that can readily be identified by a trained observer (12). Consistent with previous research (12, 35), sleep latency was quantified as the duration of time post intraperitoneal or intracranial injection that mice were awake until their first sleep bout, defined as exhibiting the sleep posture continuously for 2 min and 75% of the 10-min time period beginning at sleep onset.

To physiologically validate the behaviorally scored sleep posture, sleep latency was quantified independently through EEG/EMG and through behavior by a trained observer (Figure 1A-C; see Supplemental Information). Behavioral and EEG measures of sleep latency were nearly identical (Figure 1C;  $r = 0.9058$ ,  $p = 0.0019$ ), confirming that the behavioral scoring of sleep latency by a trained observer is a valid method of determining sleep onset (12, 35). The one “outlier” was due to the duration of the initial sleep bout; EEG

confirmed that the mouse was asleep during the time behaviorally scored as asleep but fell just short of the 2-min criteria.

Locomotor activity was measured via photobeam breaks in automated chambers (San Diego Instruments Inc., La Jolla, CA) as described (33, 36). Ambulations, defined as consecutive photobeam breaks, were quantified for 2 h. Testing began immediately following intracranial infusions or 20 min post intraperitoneal injection (to ensure drug reached the brain by the start of the test). For within-subject testing of drug and vehicle, locomotor tests were separated by a week to prevent habituation to the chambers. Drug order was counterbalanced across subjects. Additional methods including surgical details are described in Supplemental Information.

### Electrophysiology

Procedures were approved by the IACUC of Vanderbilt University. Electrophysiology experiments were conducted on brain slices from TH-eGFP mice [Tg(Th-EGFP)DJ76Gsat/Mmnc] on a C57BL/6J background. Breeding pairs were obtained from the Mutant Mouse Regional Resource Center (MMRRC; Chapel Hill, NC). Brain slices were prepared and electrophysiological recordings were conducted as previously described (27) (details in Supplemental Information).

### Immunoelectron microscopy

Procedures were approved by the IACUC of Christopher Newport University. vPAG sections containing DA cells were prepared from C57BL/6J mice. Immunoelectron microscopy was conducted as previously described (37, 38) and detailed in Supplemental Information.

## Results

### Chemogenetic activation of vPAG DA neurons promotes wakefulness

To test whether excitation of vPAG<sup>DA</sup> neurons increases arousal, Cre-dependent hM3Dq DREADDs were virally expressed in the vPAG (Figure 2A-D and S1A). DREADDs were expressed in approximately 75% of TH<sup>+</sup> cells in the vPAG of TH-Cre<sup>+</sup> mice, but were not detectable in wild-type littermates (Figure 2B-C), demonstrating that the virus is specific to Cre-containing cells. By targeting the rostral vPAG, we minimized overlap with neighboring dopaminergic nuclei such as the DRN and RLi (Figure 2D, S1); DREADD expression was contained to the vPAG in 67% of transfected mice. In 33% of DREADD-expressing subjects, transfection extended to the DRN and RLi; however, their behavioral data did not differ from subjects with DREADD expression confined to the vPAG (Figure S1B), indicating that activation of vPAG<sup>DA</sup> neurons alone is sufficient for the behavioral results described below. mCherry was not detected in the VTA or substantia nigra of any subject (data not shown).

CNO-induced activation of DREADDs robustly promoted wakefulness. Two out of 12 CNO-treated mice expressing DREADDs did not meet sleep criteria within 4 h, whereas all subjects fell asleep during the test when treated with vehicle (Figure 2E). CNO-induced activation of vPAG<sup>DA</sup> neurons significantly increased sleep latency in DREADD-expressing

mice, but had no effect on mice not expressing DREADDs (Figure 2F; controls,  $t_{(10)} = 1.822$ ,  $p = 0.098$ ; Gq DREADD-expressing mice,  $t_{(11)} = 4.987$ ,  $p = 0.0004$ ), demonstrating that the effects of CNO were due to DREADD activation and not any potential off-target effects of the ligand (39, 40). Activation of vPAG<sup>DA</sup> neurons did not increase locomotor activity, indicating that vPAG<sup>DA</sup> neurons can promote wakefulness without causing hyperactivity. CNO did not affect locomotion in controls (Figure 2G; main effect of drug  $F_{(1,10)} = 1.093$ ,  $p = 0.320$ ), and chemogenetic stimulation of vPAG<sup>DA</sup> neurons slightly reduced locomotor activity (Figure 2H; main effect of drug,  $F_{(1,11)} = 16.959$ ,  $p = 0.0017$ ).

### Chemogenic activation of the LC or LC terminals in the vPAG promotes wakefulness

hM3Dq DREADDs were expressed in the LC of TH-Cre mice (Figure 3A). Whereas all mice treated with vehicle fell asleep, 3 out of 8 CNO (1 mg/kg, i.p.)-treated subjects failed to meet sleep criteria within 3 h (Figure 3B). Overall, CNO-induced DREADD activation of the LC significantly increased sleep latency (Figure 3C;  $t_{(7)} = 2.444$ ,  $p = 0.0445$ ).

To test whether activation of LC projections to the vPAG is sufficient to promote wakefulness, CNO (1  $\mu$ g/0.3  $\mu$ L) or aCSF was infused into the vPAG of mice expressing hM3Dq DREADDs in LC neurons. Activation LC terminals in the vPAG significantly increased latency to sleep (Figure 3E;  $t_{(7)} = 2.464$ ,  $p = 0.0432$ ), and 2 out of 8 mice did not meet sleep criteria within 3 h (Figure 3D).

### $\alpha$ 1ARs modulate vPAG DA neuron activity and arousal

Electrophysiological recordings of vPAG<sup>DA</sup> neurons in brain slices from TH-GFP mice revealed that the  $\alpha$ 1AR agonist phenylephrine (3  $\mu$ M) significantly increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) onto vPAG<sup>DA</sup> neurons (Figure 4A-B; baseline compared to peak phenylephrine,  $t_{(6)} = 2.739$ ,  $p = 0.034$ ), suggesting that noradrenergic excitation of vPAG<sup>DA</sup> neurons is mediated by changes in glutamate transmission.

To determine whether  $\alpha$ 1AR-mediated excitation of vPAG<sup>DA</sup> neurons requires  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation or neuronal action potentials, we assessed the effects of phenylephrine (3  $\mu$ M) on sEPSCs in the presence of the AMPA antagonist CNQX (10  $\mu$ M) and the voltage-gated sodium channel blocker tetrodotoxin (TTX; 1  $\mu$ M). The facilitation of sEPSCs in vPAG<sup>DA</sup> neurons by phenylephrine persisted following bath application of TTX but was inhibited by CNQX (Figure 4C-D;  $F_{(2,14)} = 43.66$ ,  $p = 0.000001$ ; Dunnett's post hoc test compared to TTX: phenylephrine  $p = 0.0028$ , CNQX  $p = 0.0001$ ).

Because activation of vPAG  $\alpha$ 1ARs was sufficient to increase vPAG<sup>DA</sup> neuron activity, we next tested whether *in vivo* manipulations of NE/ $\alpha$ 1AR transmission alters arousal. To confirm that endogenous NE promotes arousal, we tested sleep latency and locomotor activity in dopamine  $\beta$ -hydroxylase knockout mice (*Dbh*  $-/-$ ) that completely lack NE. Consistent with previous research (12, 35, 41), *Dbh*  $-/-$  mice exhibited decreased sleep latencies (Figure 5A;  $t_{(15)} = 3.481$ ,  $p = 0.0034$ ) and blunted novelty-induced locomotion (Figure 5B; main effect of genotype,  $F_{(1,15)} = 14.496$ ,  $p = 0.0017$ ) compared to *Dbh*  $+/-$  littermates with normal levels of NE. To determine whether activation of vPAG  $\alpha$ 1ARs was

sufficient to promote arousal, aCSF or phenylephrine (4  $\mu\text{g}/0.3 \mu\text{L}$ ) was infused into the vPAG via a cannula immediately before the start of behavioral tests (Figures 5C and S2A). Stimulation of vPAG  $\alpha 1\text{ARs}$  with phenylephrine significantly increased sleep latency in *Dbh* +/- control mice as well as in *Dbh* -/- mice (Figure 5D; *Dbh* +/-,  $t_{(7)} = 2.562$ ,  $p = 0.037$ ; *Dbh* -/-,  $t_{(8)} = 3.937$ ,  $p = 0.004$ ) without causing hyperactivity (Figure 5E; Controls, main effect of drug,  $F_{(1,7)} = 0.149$ ,  $p = 0.711$ ; *Dbh* -/-, main effect of drug,  $F_{(1,8)} = 0.033$ ,  $p = 0.858$ ).

To test whether endogenous  $\alpha 1\text{AR}$  tone is important for arousal, aCSF or the  $\alpha 1\text{AR}$  antagonist terazosin (3  $\mu\text{g}/0.3 \mu\text{L}$ ) was site-specifically infused into the vPAG (Figure S2B). Blockade of vPAG  $\alpha 1\text{ARs}$  significantly decreased latency to fall asleep (Figure 5F;  $t_{(6)} = 4.235$ ,  $p = 0.0055$ ). Terazosin did not significantly alter novelty-induced locomotion (Figure 5G; main effect of time,  $F_{(1,10)} = 0.377$ ;  $p = 0.552$ ). Because the vPAG abuts the aqueduct, the arousal-altering effects of intra-vPAG infusions theoretically could be attributed to terazosin actions in other brain regions from the drug circulating through the ventricles following missed injections or diffusion. Therefore, we purposely infused terazosin into the ventricles but saw no alteration in sleep latency (Figure S3A;  $t_{(7)} = 0.038$ ,  $p = 0.971$ ) or locomotor activity (Figure S3B; main effect of drug,  $F_{(1,7)} = 0.001$ ,  $p = 0.992$ ). Additionally, to test whether antagonism of  $\alpha 1\text{ARs}$  reduces arousal in other wake-promoting dopaminergic nuclei, we also infused terazosin into the VTA, but this manipulation did not alter sleep latency (Figure S3C;  $t_{(7)} = 1.139$ ,  $p = 0.292$ ) or locomotor activity (Figure S3D; main effect of drug,  $F_{(1,7)} = 0.611$ ,  $p = 0.460$ ). Together, these results reveal that noradrenergic transmission at  $\alpha 1\text{ARs}$  specifically in the vPAG bidirectionally modulates arousal.

### vPAG $\alpha 1\text{ARs}$ are enriched on astrocytes

Although  $\alpha 1\text{ARs}$  are expressed in the vPAG (25, 26), their precise anatomical distribution is unknown. Therefore, we conducted immunoelectron microscopy on the same portion of the vPAG in which  $\alpha 1\text{AR}$  agonists and antagonists elicited changes in arousal (Figure 6A). In the vPAG,  $\alpha 1\text{AR}$  immunoreactivity was located primarily presynaptically in unmyelinated axons and in putative glial elements suspected to be astrocytes based upon morphology (Figure 6B-C).

Next, to determine the neurochemical identity of  $\alpha 1\text{AR}$ -containing elements, vPAG slices were double labeled for  $\alpha 1\text{ARs}$  using immunogold and either TH (DA cells), vGluT1 (glutamatergic terminals originating from neurons in the cortex and hippocampus), vGluT2 (glutamatergic terminals originating from neurons in the thalamus and other hindbrain and midbrain structures), or GFAP (astrocytes) using immunoperoxidase (37, 42–44). The most abundant  $\alpha 1\text{AR}$ -containing elements were vGluT2-positive axon terminals and GFAP-positive glia, whereas only modest levels of other combinations of co-labeling were evident (Figure 6D-H). Co-localization of  $\alpha 1\text{ARs}$  and TH was rare.

### Gq signaling in vPAG astrocytes is sufficient to promote arousal

Because the overwhelming majority of  $\alpha 1\text{ARs}$  in other dopaminergic brain regions are located on presynaptic neuronal elements (37, 38),  $\alpha 1\text{AR}$  stimulation can activate



astrocytes, and astrocytes in other brain regions modulate neuronal activity and sleep (45–48), we were particularly struck by the large proportion of vPAG  $\alpha 1$ ARs found on astrocytes (Figure 6). To test whether vPAG  $\alpha 1$ ARs modulate arousal, we mimicked local astrocytic  $\alpha 1$ AR-Gq transmission using intra-vPAG administration of CNO in GFAP-hM3Dq transgenic mice that express Gq-coupled DREADDs under the astrocytic GFAP promoter (49). Previous research established that expression of DREADDs in the brains of GFAP-hM3Dq mice is limited to astrocytes, and application of CNO increases astrocytic intracellular  $\text{Ca}^{2+}$  in these mice but not in wild-type littermates (49). aCSF or CNO (1  $\mu\text{g}/0.3 \mu\text{L}$ ) was infused through a guide cannula targeting the vPAG. CNO significantly increased sleep latency in DREADD-expressing mice but not littermate controls (Figure 7A-B; controls,  $t_{(8)} = 1.066$ ,  $p = 0.318$ ; GFAP-hM3Dq,  $t_{(6)} = 2.156$ ,  $p = 0.0385$ ). This increase in wakefulness was not due to hyperactivity (Figure 8C-D; controls, main effect of drug  $F_{(1,8)} = 0.808$ ,  $p = 0.395$ ; GFAP-hM3Dq, main effect of drug  $F_{(1,7)} = 7.302$ ,  $p = 0.0305$ ). Combined, these data show that stimulating Gq signaling in vPAG astrocytes is sufficient to promote wakefulness.

## Discussion

Using a combination of electrophysiology, immunoelectron microscopy, pharmacological manipulations, and chemogenetics, the present study elucidates mechanisms underlying noradrenergic control of wake-promoting vPAG<sup>DA</sup> neurons. Transmission at  $\alpha 1$ ARs increases the excitability of vPAG<sup>DA</sup> neurons in a glutamate-dependent, action potential-independent manner, and facilitates arousal. vPAG  $\alpha 1$ ARs are enriched on astrocytes, and Gq activation of vPAG astrocytes is sufficient to promote wakefulness. Together, these experiments support a novel circuit whereby NE increases arousal via astrocytic  $\alpha 1$ AR-mediated excitation of vPAG<sup>DA</sup> neurons (Figure 8).

### vPAG<sup>DA</sup> neurons promote wakefulness

Using c-Fos as a measure of activation, previous research found that vPAG<sup>DA</sup> neurons were active when rodents were awake and inactive when asleep, and lesioning vPAG<sup>DA</sup> neurons increased time asleep during both day and night (16). The present study demonstrates that chemogenetic activation of vPAG<sup>DA</sup> neurons promotes arousal by increasing latency to fall sleep without causing hyperactivity. A recent study reported that inhibition of DRN/caudal vPAG<sup>DA</sup> neurons promotes sleep, while activation of these neurons promotes wakefulness (15). Whereas those authors optogenetically targeted the DRN, we used a chemogenetic approach and targeted rostral vPAG<sup>DA</sup> neurons. Together, these studies demonstrate that vPAG<sup>DA</sup> neurons can bidirectionally modulate arousal.

vPAG<sup>DA</sup> neurons project to multiple brain regions involved in arousal (16). One plausible functional target is the wake-promoting orexin neurons in the lateral hypothalamus (50, 51). Alternatively, orexin's effects may be upstream of vPAG<sup>DA</sup> neurons and function via a relay from the LC, because the LC mediates some aspects of orexin-induced arousal (52, 53). The ventrolateral preoptic nucleus facilitates sleep (54, 55) and reciprocally connects with vPAG<sup>DA</sup> (16), making it another potential functional neuroanatomical partner of vPAG<sup>DA</sup> neurons. Finally, because vPAG<sup>DA</sup> neurons can co-release glutamate (34), their wake-

promoting properties could involve dual neurotransmitter signal integration by downstream cells.

The finding that DREADD-induced stimulation of vPAG<sup>DA</sup> neurons promotes wakefulness and causes a small, but statistically significant, decrease in novelty-induced locomotion highlights that wakefulness and locomotor activity are dissociable behaviors. DRN/caudal vPAG<sup>DA</sup> neurons are activated by a variety of salient stimuli (15). If rostral vPAG<sup>DA</sup> cells similarly signal salient stimuli, DREADD-induced activation of these neurons may increase other behaviors that compete with horizontal ambulations.

### **Noradrenergic transmission via $\alpha$ 1ARs modulates vPAG<sup>DA</sup> neuron activity and arousal**

vPAG<sup>DA</sup> neurons receive input from multiple brain regions that control sleep-wakefulness patterns, such as the LC, lateral hypothalamus, ventrolateral preoptic nucleus, prefrontal cortex, substantia innominata, laterodorsal tegmental nucleus, and DRN (16, 24, 56); however, the functionally significant circuits had not been identified. The present study tested whether vPAG<sup>DA</sup> neuron-mediated arousal is modulated by noradrenergic transmission from the LC.

Consistent with previous work demonstrating that optogenetic stimulation of the LC promotes wakefulness (21), DREADD-induced activation of the LC also increases wakefulness (Figure 3B-C). CNO infused into projection areas of DREADD-expressing neurons can activate fibers/terminals (57), and we found that intra-vPAG administration of CNO significantly increased latency to sleep (Figure 3D-E) with a magnitude similar to activating all DREADD-expressing LC cells and fibers via systemic CNO administration (Figure 3B-C), demonstrating that specifically activating LC projections to the vPAG is sufficient to promote wakefulness.

Although NE signals through three different AR subtypes ( $\alpha$ 1,  $\alpha$ 2, and  $\beta$ ), we focused on the  $\alpha$ 1AR for two reasons. First, this subtype mediates noradrenergic excitatory drive onto DA neurons in the VTA (30), substantia nigra (28, 29), and RLi (27). Second,  $\alpha$ 1AR activity in other brain regions is implicated in arousal (58–60). Stimulation of vPAG  $\alpha$ 1ARs increased vPAG<sup>DA</sup> neuron activity via glutamate and promotes wakefulness in NE-deficient mice (Figures 4–5). Furthermore, local antagonism of  $\alpha$ 1ARs decreased arousal, consistent with previous research (61). Infusion of terazosin into the vPAG, but not the ventricles or VTA, attenuates arousal (Figures 5F, S3), demonstrating neuroanatomical specificity. Additionally, these experiments indicate that  $\alpha$ 1ARs do not regulate arousal in all dopaminergic populations; the wake-promoting effects of VTA DA neurons (14) are unlikely under the control of endogenous  $\alpha$ 1AR tone. Combined, these results illustrate that the vPAG is a locus where NE modulates DA neuron activity and promotes behavioral arousal through  $\alpha$ 1ARs.

Previous research has shown noradrenergic receptors in other regions modulate arousal. Agonism of  $\alpha$ 1ARs or  $\beta$ ARs in the medial septal area or medial preoptic area promotes wakefulness (60) as does agonism of  $\alpha$ 1ARs, and to a lesser extent  $\beta$ ARs, in the lateral hypothalamus (17); however, infusions in the substantia innominata do not (62). The present study reveals that endogenous noradrenergic tone at  $\alpha$ 1ARs modulates wakefulness, and



intra-vPAG phenylephrine elevates sleep latencies of NE-competent mice as well as mice completely lacking NE. The magnitude of intra-vPAG-induced wakefulness in *Dbh*<sup>-/-</sup> mice is noteworthy given that adrenergic receptors in other brain regions contribute functionally to wakefulness. Because arousal is important for adaptive behavior and survival, the brain may have evolved redundant mechanisms involving ARs. Alternatively, each brain region might contribute uniquely to arousal.

### **$\alpha$ 1ARs on vPAG astrocytes modulate wakefulness**

Most  $\alpha$ 1ARs in the VTA, substantia nigra, nucleus accumbens, and prefrontal cortex localize to neuronal elements, with negligible glial expression (37, 38). By contrast, an equally large proportion of  $\alpha$ 1ARs are found on glia and axons in the vPAG (Figure 6B). Most  $\alpha$ 1AR-positive glia co-labeled with GFAP, suggesting that astrocytes are a major target of noradrenergic transmission in the vPAG (Figure 6D,H). Importantly, these astrocytes are functionally relevant in modulating arousal. Mimicking  $\alpha$ 1AR-Gq signaling specifically in vPAG astrocytes using Gq DREADDs promoted wakefulness without causing hyperactivity (Figure 7). Activation of vPAG astrocytes in fact slightly decreased locomotion, similarly to previously reported global activation of astrocytes (49). Recent studies reported that astrocytes in the suprachiasmatic nucleus (63), posterior hypothalamus (64), and hippocampus (47) modulate arousal. The present study reveals that the vPAG is also a critical node in the astrocytic network that controls arousal, adding to research endorsing the ability of astrocytes and gliotransmission to modulate behavior (65–67).

The LC projects broadly, and the LC/NE system is known to regulate astrocyte functions. NE can depolarize astrocytes and this effect is blocked by an  $\alpha$ 1AR antagonist (68). NE or phenylephrine increases calcium in hippocampal astrocytes (69), and electrical or behaviorally-induced stimulation of the LC provokes calcium transients in cortical astrocytes (70), whereas administering an  $\alpha$ 1AR antagonist or the LC-specific neurotoxin DSP-4 abolishes astrocytic calcium transients (71, 72). The present study supports the hypothesis that astrocytes respond to arousing events via noradrenergic transmission at  $\alpha$ 1ARs (73) and implicates vPAG astrocytes as behaviorally relevant targets of the LC.

Astrocytes can release gliotransmitters including glutamate, GABA, D-serine, and ATP to influence local neurons (46, 74). Phenylephrine increased glutamatergic sEPSCs onto vPAG<sup>DA</sup> neurons, this effect was preserved in the presence of TTX, and CNQX abolished phenylephrine-induced increases in sEPSCs on vPAG<sup>DA</sup> neurons (Figure 4). The magnitude of phenylephrine-induced sEPSCs was smaller in the presence of TTX. However, TTX reduces not only neuronal activity-dependent glutamate release but also all other neuronally-released neurotransmitters, likely altering the basal tone of numerous neurotransmitter systems that could modulate phenylephrine's effects. The fact that phenylephrine's excitatory effects persist in the presence of TTX are consistent with nonconical regulation of glutamate release, such as NE providing excitatory drive onto wake-promoting vPAG<sup>DA</sup> neuron activity through  $\alpha$ 1AR-induced activation of local astrocytes (Figure 8). Astrocytes could act on glutamatergic terminals to drive vPAG<sup>DA</sup> activity through gliotransmission (75) or other mechanisms (76). Because Gq signaling in astrocytes can cause glutamate release (77), a possible alternative is that astrocytes release glutamate directly onto vPAG<sup>DA</sup>

neurons. NE at  $\alpha 1$ ARs increases the amplitude of mEPSCs in hypothalamic neurons by inducing the release of ATP from astrocytes (78), and astrocytes can promote neuronal plasticity by releasing ATP which acts on purinergic receptors (79). Future experiments could investigate the involvement of ATP and other gliotransmitters in this circuit. Additionally, the potential contribution of axonally-expressed  $\alpha 1$ ARs remains to be determined.

### Technological considerations

Several of our conclusions rely on results of chemogenetic experiments, and we are cognizant of recent controversies surrounding CNO and the critical need for proper controls (39, 40, 80). We validated the specificity of the DREADD-CNO system in our study by including vehicle administration in DREADD-expressing mice and CNO administration in non-DREADD expressing mice. Only CNO administration in DREADD-expressing subjects produced behavioral effects.

### Conclusion

The present study demonstrates that wake-promoting vPAG<sup>DA</sup> neurons are physiologically and functionally under noradrenergic control. vPAG  $\alpha 1$ ARs drive glutamatergic excitation of vPAG<sup>DA</sup> neurons, are located on astrocytes, and promote wakefulness. This circuit could be a target for developing therapeutics to treat arousal disturbances and explain a mechanism for some currently available treatments. For example, modafinil (Provigil®) promotes wakefulness without causing hyperactivity (81) via contributions from both NE and DA (12). Future studies should examine the source and functional contributions of glutamatergic neuronal inputs to the vPAG, and which vPAG<sup>DA</sup> projections are critical for increasing arousal. Additionally, studies targeting DRN and vPAG<sup>DA</sup> neurons demonstrate that these cells are important in promoting wakefulness by salient stimuli (15), sociability (82), and antinociception (34). Mounting evidence suggests that vPAG<sup>DA</sup> neurons are not merely an extension of the VTA; further studies should continue to examine the functions of these understudied populations of DA neurons.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

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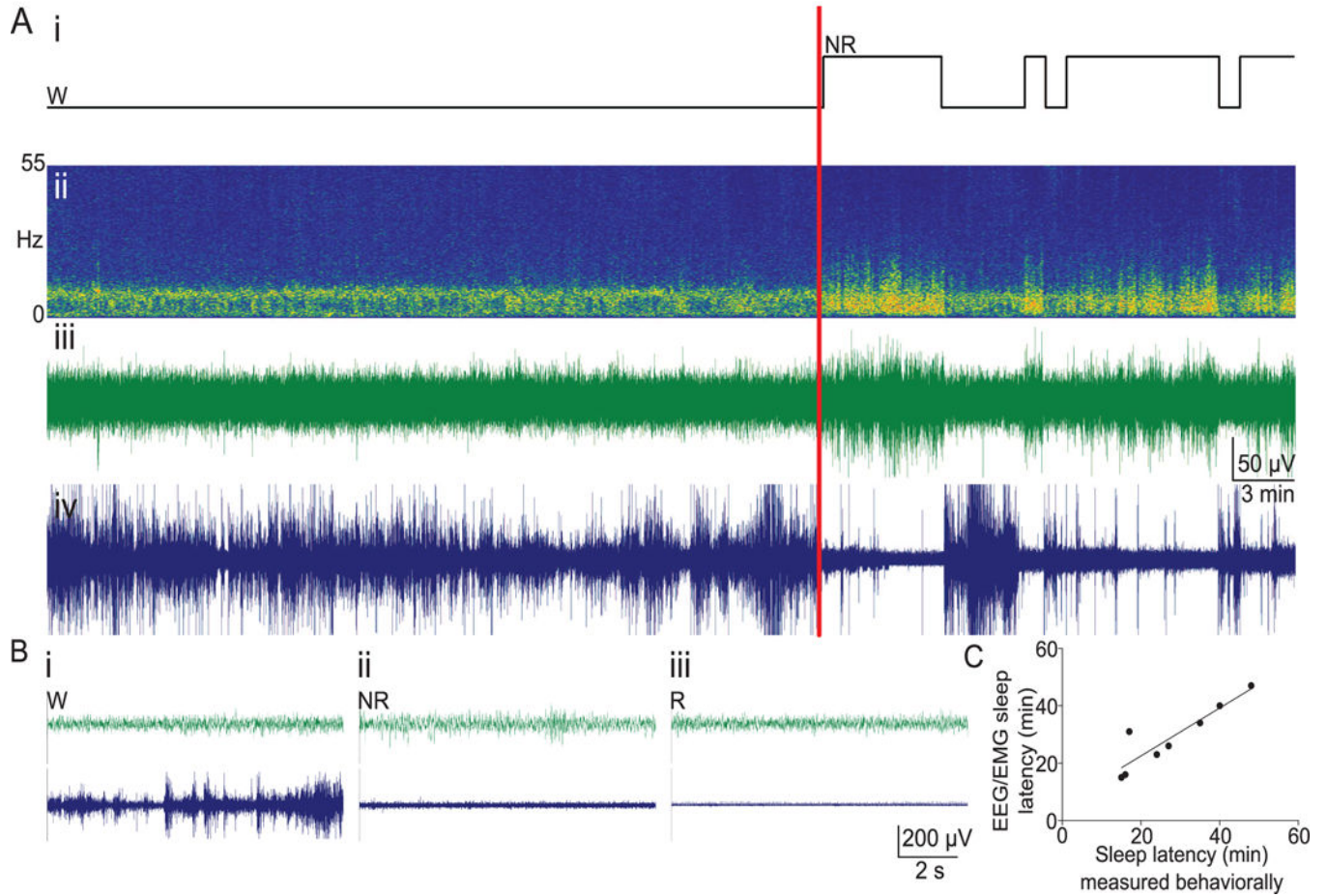
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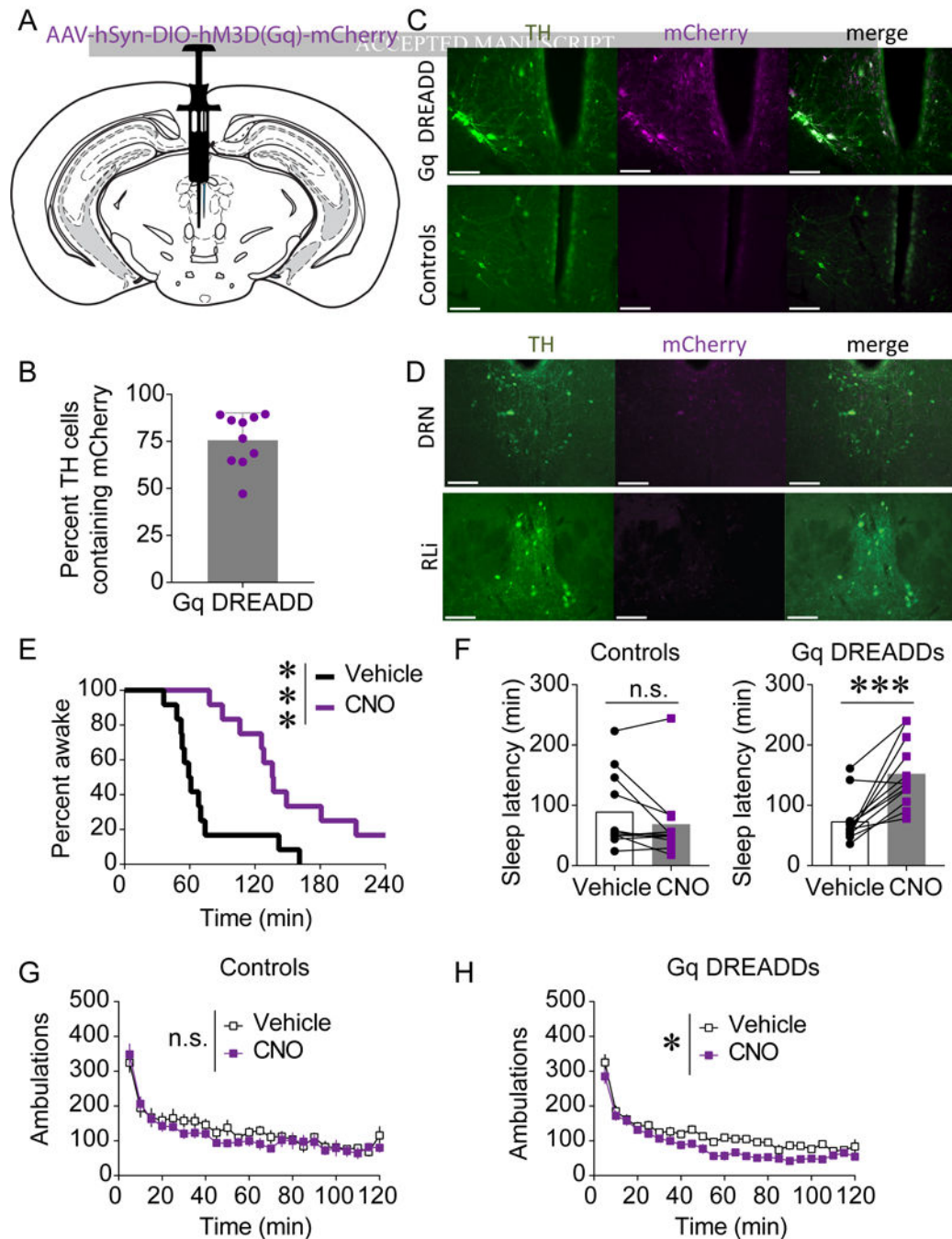
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**Figure 1. EEG validation of behavioral scoring of sleep-onset latency.**

**A)** Representative EEG/EMG data from one mouse. (i) Hypnogram of a 60 min segment of a mouse with video and EEG/EMG scoring. Wake (W) and non-REM sleep (NR) are shown, with a red vertical line indicating the time of the independently-scored behavioral onset of sleep from the video. (ii) Fast-Fourier-based spectrogram shows predominantly theta activity during wakefulness and an increased in low-frequency EEG power at the onset of sleep, correlating with changes in (iii) EEG and (iv) EMG. **B)** Example traces with a 30 sec time-base showing EEG and EMG activity during (i) wakefulness, (ii) non-REM sleep, and (iii) REM sleep. **C)** There is a significant correlation between EEG/EMG and behavioral/video scoring of sleep onset ( $n = 8$ ,  $r = 0.9058$ ,  $p = 0.0019$ ). One outlier resulted from using 20 sec epochs for EEG/EMG scoring and a 2-min requirement for sleep onset for video scoring. Voltage/time scales in (A) and (B) apply to both EEG and EMG traces.



**Figure 2. Activation of vPAG DA neurons promotes wakefulness without causing hyperactivity.** **A**) AAV-hSyn-DIO-hM3D(Gq)-mCherry was injected into the vPAG of TH-Cre<sup>+</sup> mice and wild-type littermates. **B**) mCherry was expressed in a large proportion of TH-containing vPAG<sup>DA</sup> cells. **C**) mCherry (magenta) was expressed in TH-Cre<sup>+</sup> mice, but not TH-Cre<sup>-</sup> mice, and overlapped with TH (green). **D**) DREADD expression in TH-Cre<sup>+</sup> mice did not extend into the DRN or RLi in 67% of subjects. All scale bars = 100  $\mu$ m. **E-F**) CNO (1 mg/kg, i.p.) promoted wakefulness in DREADD-expressing mice (**E-F**) but not in non-DREADD-expressing controls (**F**; n=11–12; n.s. = not statistically different; \*\*\* $p < 0.001$ ).

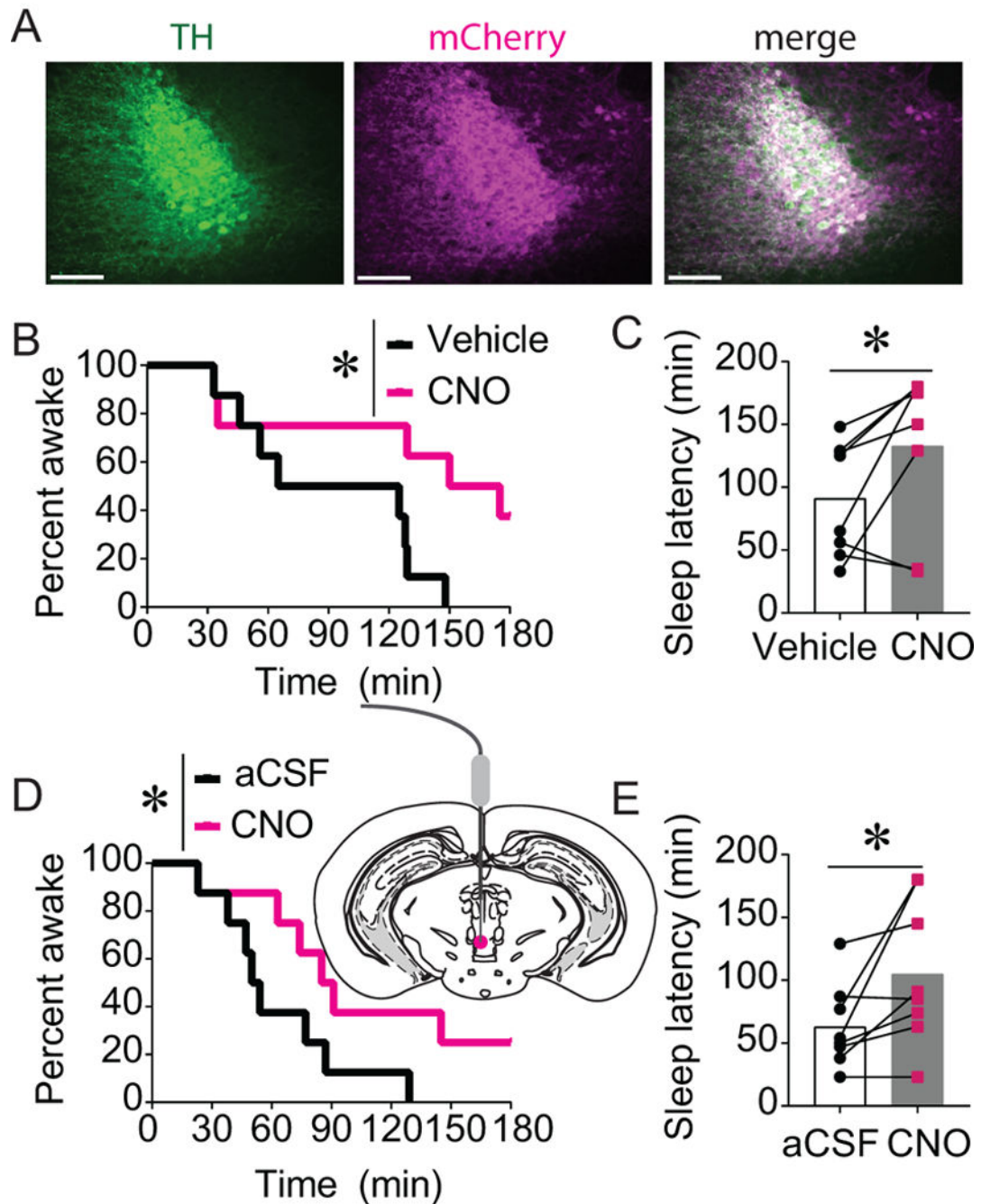
**G-H)** CNO modestly decreased locomotor activity in DREADD-expressing mice (H; n = 12, main effect of drug  $*p < 0.05$ ) but not in non-DREADD-expressing controls (G; n = 11, n.s. = not statistically significant).

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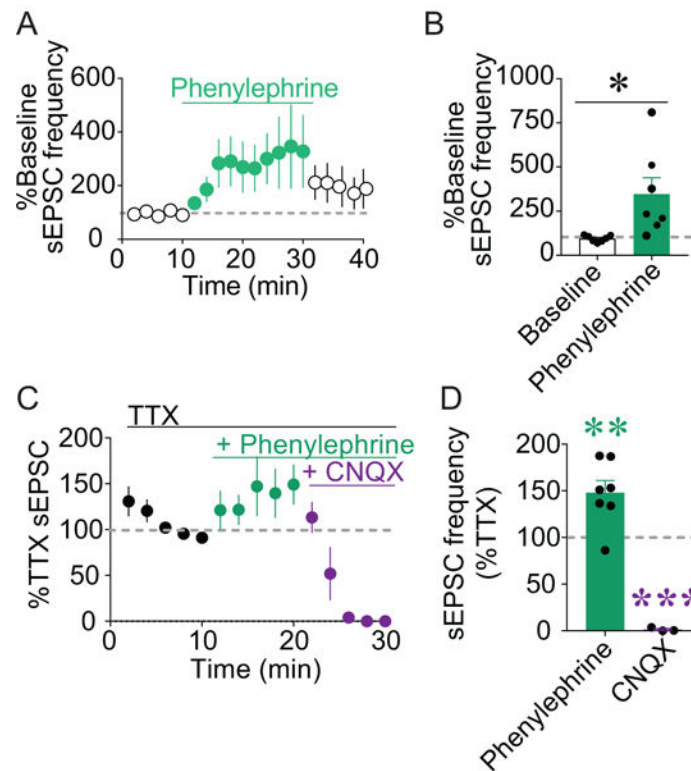
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**Figure 3. Chemogenetic stimulation of the LC or LC projections to the vPAG promotes wakefulness.**

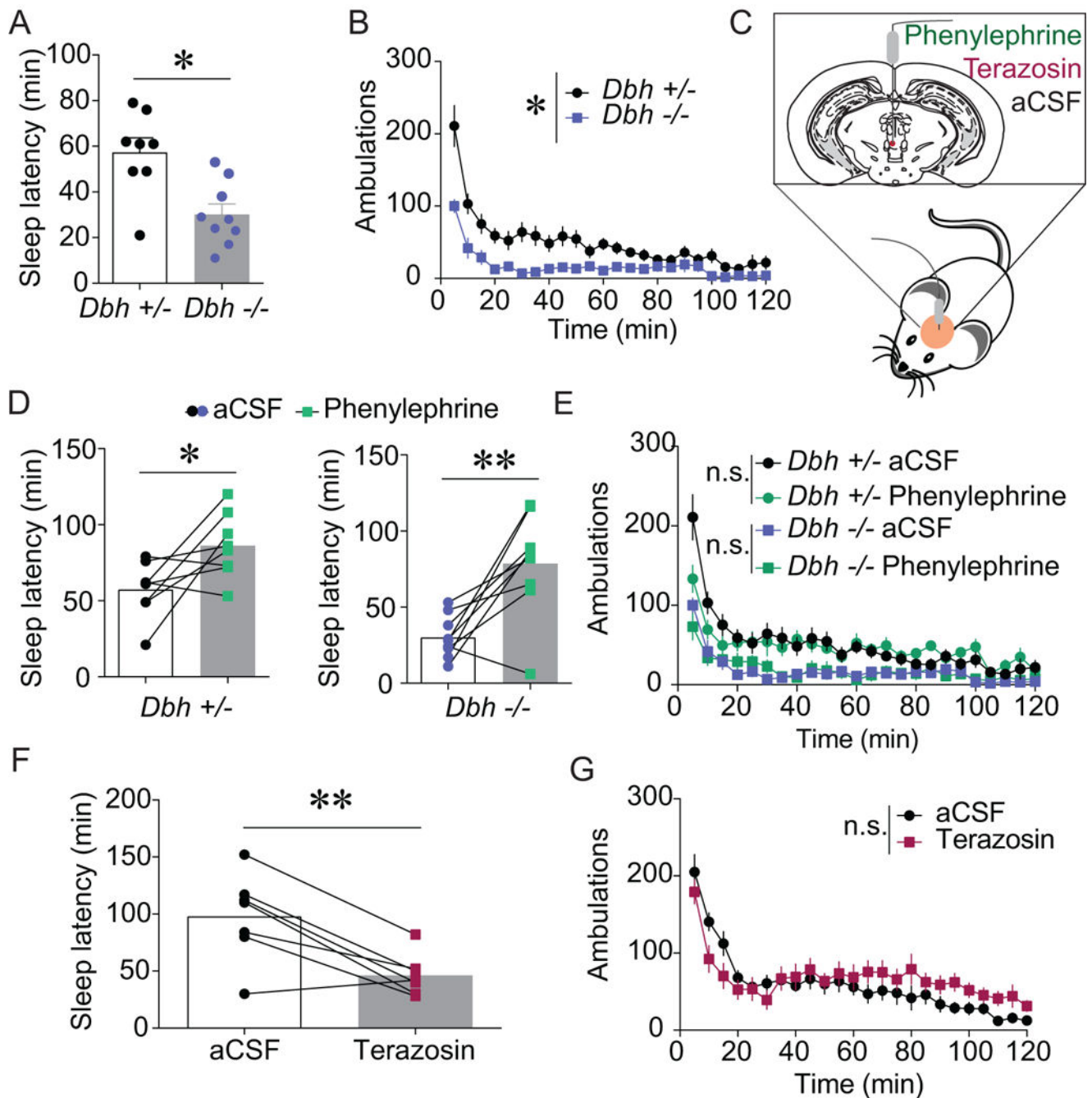
**A)** AAV-hSyn-DIO-hM3D(Gq)-mCherry was injected into the LC of TH-Cre+ mice. Scale bars = 100  $\mu$ m. **B-C)** CNO (1 mg/kg)-induced activation of Gq DREADDs in the LC significantly increased sleep latency ( $n = 8$ ;  $*p < 0.05$ ). **D-E)** Site-specific infusion of CNO (1  $\mu$ g/0.3  $\mu$ L) into the vPAG to stimulate LC projections to the vPAG similarly increased sleep latency ( $n=8$ ;  $*p < 0.05$ ).



**Figure 4. Stimulation of  $\alpha_1$ ARs increases glutamatergic drive onto vPAG<sup>DA</sup> neurons in an AMPA receptor-dependent, action potential-independent manner.**

**A-B)** Bath application of phenylephrine (3  $\mu$ M) significantly increased sEPSC frequency in vPAG<sup>DA</sup> neurons ( $n = 7$ ;  $*p < 0.05$ ). **C)** TTX (1  $\mu$ M), phenylephrine (3  $\mu$ M), and CNQX (10  $\mu$ M) were cumulatively applied to slices and sEPSCs were recorded from vPAG<sup>DA</sup> neurons. **D)** Phenylephrine in the presence of TTX increased sEPSC frequency compared to sEPSCs during TTX alone ( $n = 7$ ;  $**p < 0.01$ ), and CNQX abolished sEPSCs ( $n = 3$ ;  $***p < 0.001$ ).





**Figure 5. vPAG  $\alpha$ 1ARs bidirectionally modulate wakefulness.**

**A-B)** Mice lacking NE (*Dbh* -/-; *n* = 9) fell asleep faster than NE-competent littermates (*Dbh* +/-; *n* = 8) following a control injection and were less active in a novel environment ( $*p < 0.05$ ). **C)** An  $\alpha$ 1AR agonist, antagonist, or aCSF was site-specifically infused into the vPAG (D-G). **D)** Phenylephrine (4  $\mu$ g/0.3  $\mu$ L) significantly increased sleep latency in control (*n* = 8;  $*p < 0.05$ ) and *Dbh* -/- (*n* = 9;  $**p < 0.01$ ) mice compared to aCSF (0.3  $\mu$ L) infusion. **E)** Phenylephrine (4  $\mu$ g/0.3  $\mu$ L) site-specifically administered into the vPAG had negligible effects on novelty-induced locomotion (controls, *n* = 8; *Dbh* -/-, *n* = 9; main

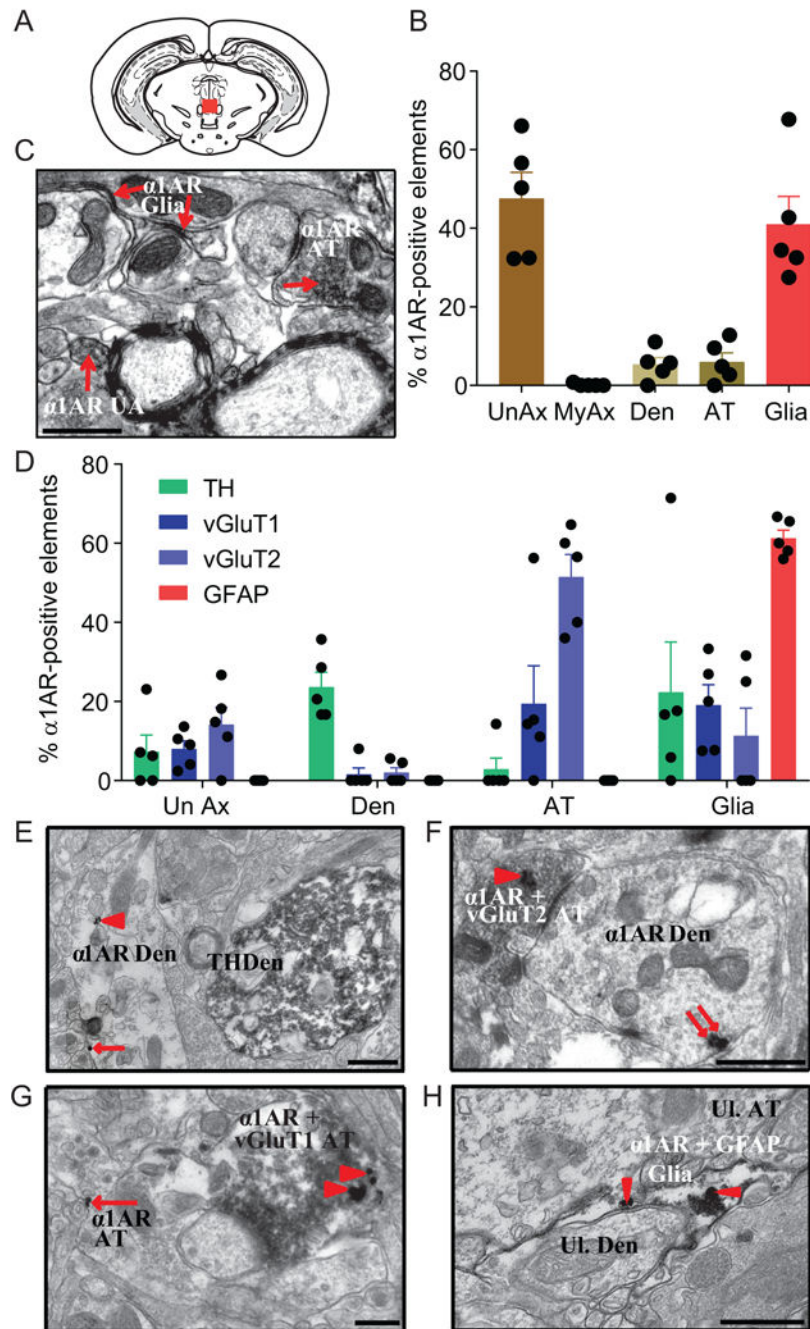
effects of drug, n.s. = not statistically different). **F-G**) Intra- vPAG terazosin (3  $\mu\text{g}/0.3 \mu\text{L}$ ) significantly decreased sleep latency (F;  $n = 7$ ;  $**p < 0.01$ ) but there was no main effect on novelty-induced locomotion (G;  $n=11$ ; n.s. = not statistically different) in C57BL/6J mice.

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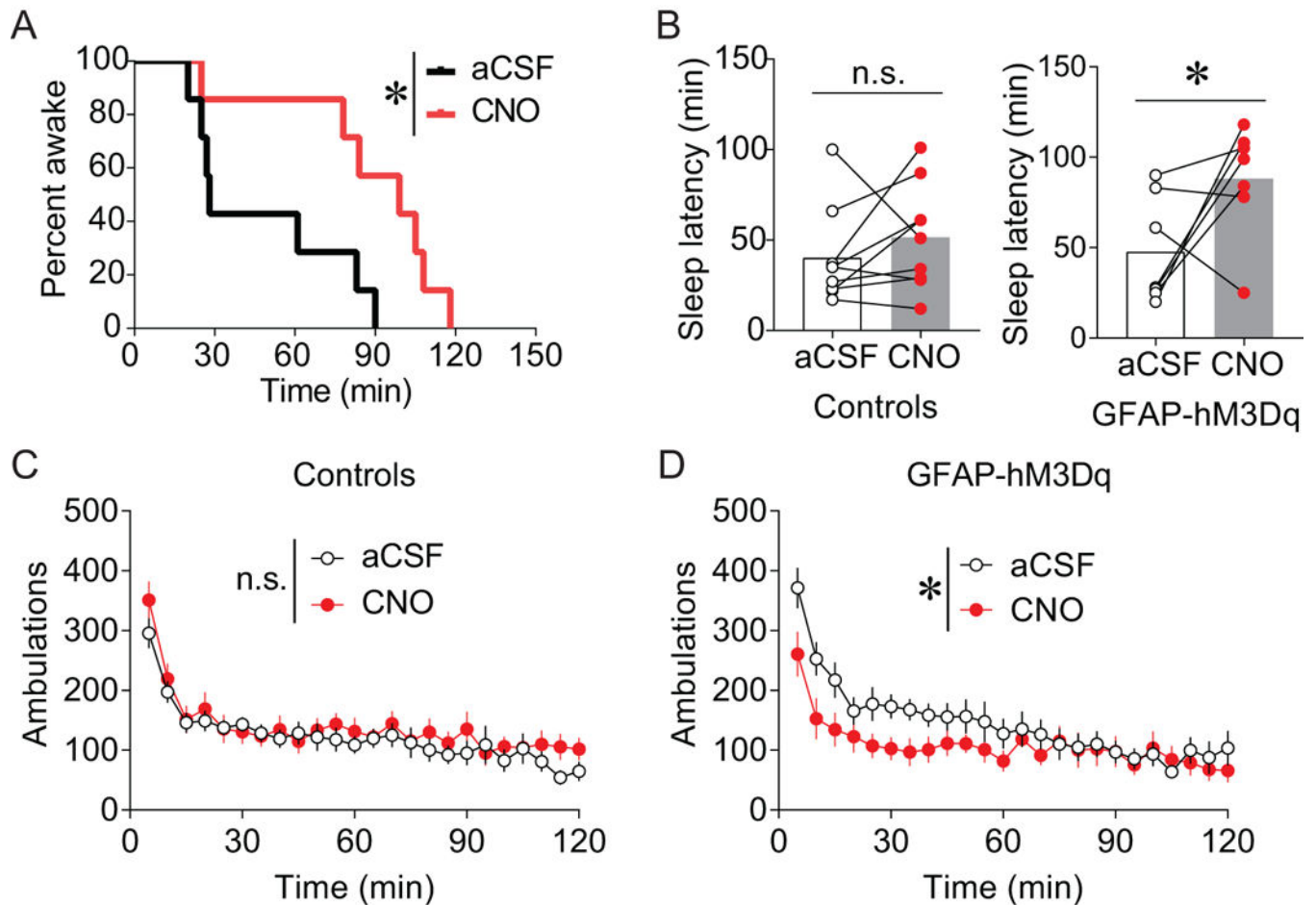
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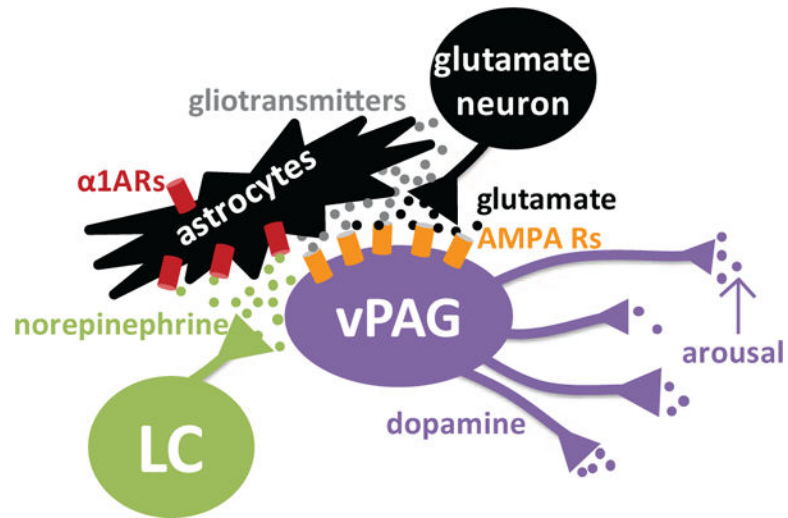
**Figure 6. Anatomical location of vPAG  $\alpha$ 1ARs determined with immunoelectron microscopy.** **A)** Red box signifies the portion of the vPAG used for EM experiments. **B)** Histogram depicting the mean percentage ( $\pm$ SEM) of unmyelinated axons (UnAx), myelinated axons (MyAx), dendrites (Den), axon terminals (AT), and glia found within the vPAG that contained immunoperoxidase labeling for the  $\alpha$ 1AR. **C)** Representative electron micrograph with immunoperoxidase labeling (arrows) for the  $\alpha$ 1AR in an unmyelinated axon (UnAx), axon terminal (AT), glial element. **D)** Percentage of  $\alpha$ 1AR-labeled elements co-labeled with TH, vGluT1, vGluT2, or GFAP. **E)** Representative micrograph of an  $\alpha$ 1AR-labeled dendrite

(Den) with immunogold (arrowheads point to intracellular gold particles; arrows point to plasma membrane bound gold particles) and a TH-positive dendrite filled with immunoperoxidase labeling. **F**) Representative micrograph of a double-labeled AT with intracellular gold particles for the  $\alpha$ 1AR and immunoperoxidase for vGluT2 synapsing on a  $\alpha$ 1AR labeled dendrite. **G**) Representative micrograph of a double-labeled axon terminal (AT) for the  $\alpha$ 1AR in immunogold and vGluT1 with immunoperoxidase synapsing on an unlabeled dendritic process. In addition, pictured is an  $\alpha$ 1AR-labeled AT with plasma membrane bound gold particles. **H**) Representative micrograph of a double-labeled astrocyte, containing GFAP with immunoperoxidase (patchy dark gray/black area within astrocyte) and intracellular  $\alpha$ 1ARs in immunogold (arrowheads point to  $\alpha$ 1ARs), wrapping around an unlabeled dendrite (Ul. Den). All scale bars = 0.5  $\mu$ m.



**Figure 7. Activation of Gq signaling vPAG astrocytes promotes arousal.**

**A-B)** aCSF or the DREADD ligand CNO (1  $\mu$ g/0.3  $\mu$ L) was site-specifically infused into the vPAG of mice expressing Gq-coupled DREADDs in GFAP positive cells (astrocytes). CNO/DREADD-induced activation of vPAG astrocytes increased latency to fall asleep in DREADD-expressing mice (A-B) but not in controls (B; controls,  $n = 9$ ; n.s. = not statistically different; GFAP-hM3Dq,  $n = 7$ ;  $*p < 0.05$ ). **C-D)** CNO-induced activation of DREADDs in astrocytes reduced novelty-induced locomotion (D;  $n = 8$ ;  $*p < 0.05$ ), and CNO had no effect in non-DREADD expressing littermates (C;  $n = 9$ ; n.s. = not statistically different).



**Figure 8. Hypothesized LC-vPAG arousal circuit.**

The LC releases NE in the vPAG, which activates astrocytic  $\alpha 1$ ARs. Astrocytes could release gliotransmitters or otherwise act upon neuronal glutamate terminals to elicit glutamate release in an action potential-independent mechanism, or astrocytes could release glutamate to directly activate DA neurons. Then, glutamate via AMPA receptors drives the activity of wake-promoting vPAG<sup>DA</sup> neurons to promote arousal. These vPAG<sup>DA</sup> neurons project to and modulate other brain regions involved in arousal.