Archival Report

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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 $vPAG^{DA}$ cells increase sleep. However, the circuitry controlling these wake-promoting DA neurons is unknown.

METHODS: This study combined 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: Activation of locus coeruleus projections to the vPAG or $vPAG^{DA}$ neurons induced by DREADDs promoted arousal. Similarly, agonist stimulation of vPAG alpha1-adrenergic receptors (α1ARs) increased latency to fall asleep, whereas α1AR blockade had the opposite effect. α1AR stimulation drove $vPAG^{DA}$ activity in a glutamate-dependent, action potential–independent manner. Compared with other dopaminergic brain regions, α1ARs were enriched on astrocytes in the vPAG, and mimicking α1AR transmission 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 revealed that vPAG α1ARs increase arousal, promote glutamatergic input onto $vPAG^{DA}$ neurons, and are abundantly expressed on astrocytes. Activation of locus coeruleus inputs, vPAG astrocytes, or $vPAG^{DA}$ neurons increase sleep latency but do not produce hyperactivity. Together, these results support an arousal circuit whereby noradrenergic transmission at astrocytic α1ARs activates wake-promoting $vPAG^{DA}$ neurons via glutamate transmission.

Keywords: Arousal, Astrocytes, Dopamine, DREADDs, Norepinephrine, Sleep, Wakefulness

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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 nonmotor symptom of Parkinson's disease (13). Recently, optogenetic stimulation of ventral tegmental area (VTA) or dorsal raphe nucleus (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 ($vPAG^{DA}$) 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 $vPAG^{DA}$ activity and its wake-promoting effects remain largely unknown.

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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 $vPAG^{DA}$ neurons because 1) the LC projects to the vPAG (16,23,24); 2) alpha1-adrenergic receptors (α1ARs) are present in the vPAG (25,26); and 3) norepinephrine (NE) transmission via the Gq protein–coupled α1AR 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 $vPAG^{DA}$ and LC projections to the vPAG promote arousal; 2) whether vPAG α1ARs modulate arousal and $vPAG^{DA}$ activity; 3) the precise cellular identity and location of α1AR expression in the vPAG; and 4) the functional contribution of vPAG astrocytes, which contain α1ARs, in modulating arousal.

METHODS AND MATERIALS

Subjects and Behavioral Testing

All procedures were approved by the Institutional Animal Care and Use Committee 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 electroencephalography (EEG) and terazosin experiments. The $Dbh^{-/-}$ 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 (DREADDs) experiments to limit AAV-hSyn-DIO-hM3D(Gq)-mCherry expression to tyrosine hydroxylase (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 polymerase chain reaction.

Guide cannulas were implanted over the vPAG, and AAV-hSyn-DIO-hM3D(Gq)-mCherry was virally expressed in the LC or vPAG (see Supplement). Sleep latency testing was conducted in the home cage of individually housed mice. Animals were kept on a 12-hour light/dark cycle, and testing began 2–3 hours into the light cycle when pressure to sleep is high. A video camera recorded mouse behavior for at least 3 hours. 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 after intraperitoneal or intracranial injection that mice were awake until their first sleep bout, defined as exhibiting the sleep posture continuously for 2 minutes and 75% of the 10-minute time period beginning at sleep onset.

To physiologically validate the behaviorally scored sleep posture, sleep latency was quantified independently through EEG and electromyography and through behavior by a trained observer (Figure 1A–C; Supplement). Behavioral and EEG measures of sleep latency were nearly identical ($r = .9058$, $p = .0019$) (Figure 1C), 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-minute criterion.

Locomotor activity was measured via photobeam breaks in automated chambers (San Diego Instruments Inc., San Diego, CA) as described (33,36). Ambulations, defined as consecutive photobeam breaks, were quantified for 2 hours. Testing began immediately following intracranial infusions or 20 minutes after 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 1 week to prevent habituation to the chambers. Drug order was counterbalanced across subjects. Additional methods including surgical details are described in the Supplement.

Electrophysiology

Procedures were approved by the Institutional Animal Care and Use Committee 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 Resource and Research Center (Chapel Hill, NC). Brain slices were prepared and electrophysiological recordings were conducted as previously described (27) (details are in the Supplement).

Immunoelectron Microscopy

Procedures were approved by the Institutional Animal Care and Use Committee 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 the Supplement.

RESULTS

Chemogenetic Activation of vPAG DA Neurons Promotes Wakefulness

To test whether excitation of $vPAG^{DA}$ neurons increases arousal, Cre-dependent hM3Dq DREADDs were virally expressed in the vPAG (Figure 2A–D and Supplemental Figure S1A). DREADDs were expressed in approximately 75% of TH^+ cells in the vPAG of TH-Cre^+ 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 rostal vPAG, we minimized overlap with neighboring dopaminergic nuclei, such as the DRN and RLi (Figure 2D and Supplemental Figure 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

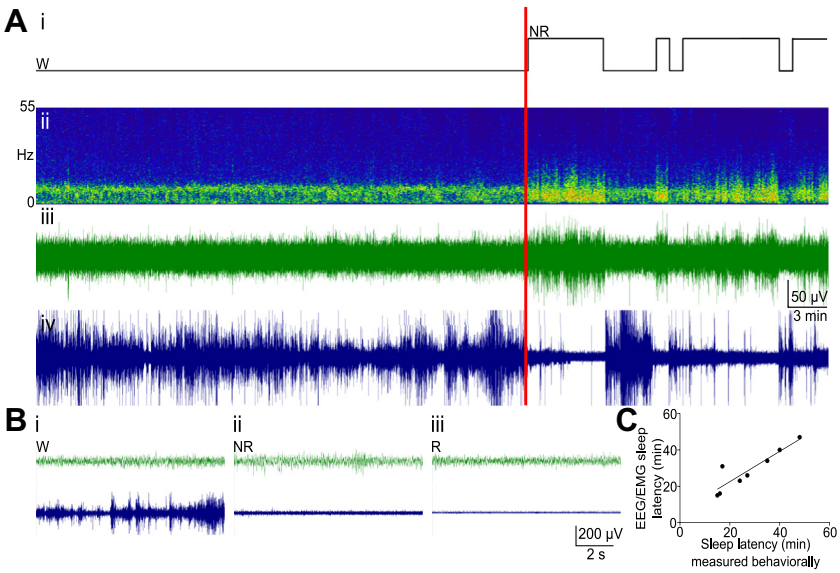

Figure 1. Electroencephalography (EEG) validation of behavioral scoring of sleep-onset latency. **(A)** Representative EEG and electromyography (EMG) data from one mouse. (i) Hypnogram of a 60-minute segment of a mouse with video and EEG/EMG scoring. Wakefulness (W) and non–rapid eye movement sleep (NR) 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-second time base showing EEG and EMG activity during (i) wakefulness, (ii) non–rapid eye movement sleep, and (iii) rapid eye movement sleep. **(C)** There is a significant correlation between EEG and EMG and behavioral and video scoring of sleep onset (n = 8, r = .9058, p = .0019). One outlier resulted from using 20-second epochs for EEG and EMG scoring and a 2-minute requirement for sleep onset for video scoring. Voltage and time scales in panels **(A)** and **(B)** apply to both EEG and EMG traces.

(Supplemental Figure S1B), indicating that activation of vPAG^{DA} 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).

Clozapine *N*-oxide (CNO)–induced activation of DREADDs robustly promoted wakefulness. Two of 12 CNO-treated mice expressing DREADDs did not meet sleep criteria within 4 hours, whereas all subjects fell asleep during the test when treated with vehicle (Figure 2E). CNO-induced activation of vPAG^{DA} neurons significantly increased sleep latency in DREADD-expressing mice but had no effect on mice not expressing DREADDs (control subjects: t_{10} = 1.822, p = .098; Gq DREADD–expressing mice: t_{11} = 4.987, p = .0004) (Figure 2F), 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^{DA} neurons did not increase locomotor activity, indicating that vPAG^{DA} neurons can promote wakefulness without causing hyperactivity. CNO did not affect locomotion in control subjects (main effect of drug: $F_{1,10}$ = 1.093, p = .320) (Figure 2G), and chemogenetic stimulation of vPAG^{DA} neurons slightly reduced locomotor activity (main effect of drug: $F_{1,11}$ = 16.959, p = .0017) (Figure 2H).

Chemogenetic Activation of 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 of 8 subjects treated with CNO (1 mg/kg, intraperitoneal injection) failed to meet sleep criteria within 3 hours (Figure 3B). Overall, CNO-induced DREADD activation of the LC significantly increased sleep latency (t_7 = 2.444, p = .0445) (Figure 3C).

To test whether activation of LC projections to the vPAG is sufficient to promote wakefulness, CNO (1 μg/0.3 μL) or artificial cerebrospinal fluid (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 (t_7 = 2.464, p = .0432) (Figure 3E), and 2 of 8 mice did not meet sleep criteria within 3 hours (Figure 3D).

α1ARs Modulate vPAG DA Neuron Activity and Arousal

Electrophysiological recordings of vPAG^{DA} neurons in brain slices from TH-GFP mice revealed that the α1AR agonist phenylephrine (3 μmol/L) significantly increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) onto vPAG^{DA} neurons (baseline compared with peak phenylephrine: t_6 = 2.739, p = .034) (Figure 4A, B), suggesting that noradrenergic excitation of vPAG^{DA} neurons is mediated by changes in glutamate transmission.

To determine whether α1AR-mediated excitation of vPAG^{DA} neurons requires alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor activation or neuronal action potentials, we assessed the effects of phenylephrine (3 μmol/L) on sEPSCs in the presence of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid antagonist cyanquixaline (CNQX) (10 μmol/L) and the voltage-gated sodium channel blocker tetrodotoxin (TTX) (1 μmol/L). The facilitation of sEPSCs in vPAG^{DA} neurons by phenylephrine persisted following bath application of TTX but was inhibited by CNQX ($F_{2,14}$ = 43.66, p = .000001; Dunnett post hoc test compared with TTX: phenylephrine p = 0.0028, CNQX p = 0.0001) (Figure 4C, D).

Because activation of vPAG α1ARs was sufficient to increase vPAG^{DA} neuron activity, we next tested whether in vivo manipulations of NE and α1AR transmission alters arousal. To confirm that endogenous NE promotes arousal, we tested sleep latency and locomotor activity in dopamine β-hydroxylase knockout mice ($Dbh^{-/-}$) that completely lack NE. Consistent with previous research (12,35,41), $Dbh^{-/-}$ mice exhibited decreased sleep latencies (t_{15} = 3.481, p = .0034) (Figure 5A) and blunted novelty-induced locomotion (main effect of genotype: $F_{1,15}$ = 14.496, p = .0017) (Figure 5B) compared with $Dbh^{+/-}$ littermates with normal levels of NE. To

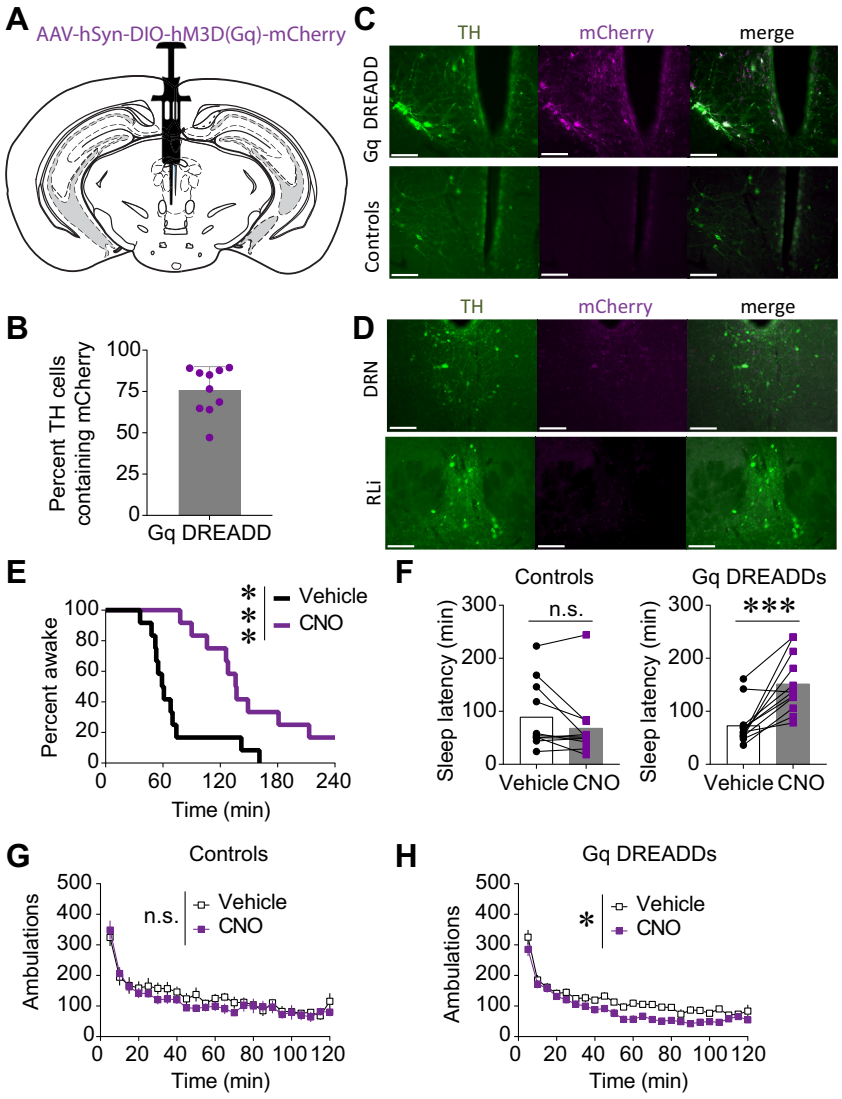

Figure 2. Activation of ventral periaqueductal gray dopamine neurons promotes wakefulness without causing hyperactivity. **(A)** AAV-hSyn-DIO-hM3D(Gq)-mCherry was injected into the ventral periaqueductal gray of TH-Cre^{+} mice and wild-type littermates. **(B)** mCherry was expressed in a large proportion of tyrosine hydroxylase (TH)–containing ventral periaqueductal gray dopamine cells. **(C)** mCherry (magenta) was expressed in TH-Cre^{+} mice, but not TH-Cre^{-} mice and overlapped with TH (green). **(D)** Designer receptors exclusively activated by designer drugs (DREADDs) expression in TH-Cre^{+} mice did not extend into the dorsal raphe nucleus (DRN) or rostral linear nucleus (RLi) in 67% of subjects. All scale bars = 100 μm. **(E, F)** Clozapine *N*-oxide (CNO) (1 mg/kg, intraperitoneal injection) promoted wakefulness in DREADD-expressing mice but not in non–DREADD-expressing control mice [panel **(F)**; n = 11–12; not statistically different [n.s.]; ***$p < .001$]. **(G, H)** CNO modestly decreased locomotor activity in DREADD-expressing mice [panel **(H)**; n = 12; main effect of drug; *$p < .05$] but not in non–DREADD-expressing control mice [panel **(G)**; n = 11; n.s.]. n.s., not statistically different.

determine whether activation of vPAG α1ARs was sufficient to promote arousal, aCSF or phenylephrine (4 μg/0.3 μL) was infused into the vPAG via a cannula immediately before the start of behavioral tests (Figure 5C and Supplemental Figure S2A). Stimulation of vPAG α1ARs with phenylephrine significantly increased sleep latency in *Dbh*$^{+/-}$ control mice as well as in *Dbh*$^{-/-}$ mice (*Dbh*$^{+/-}$: t_7 = 2.562, p = .037; *Dbh*$^{-/-}$: t_8 = 3.937, p = .004) (Figure 5D) without causing hyperactivity (control mice, main effect of drug: $F_{1,7}$ = 0.149, p = .711; *Dbh*$^{-/-}$, main effect of drug: $F_{1,8}$ = 0.033, p = .858) (Figure 5E).

To test whether endogenous α1AR tone is important for arousal, aCSF or the α1AR antagonist terazosin (3 μg/0.3 μL) was site-specifically infused into the vPAG (Supplemental Figure S2B). Blockade of vPAG α1ARs significantly decreased latency to fall asleep (t_6 = 4.235, p = .0055) (Figure 5F). Terazosin did not significantly alter novelty-induced locomotion (main effect of time: $F_{1,10}$ = 0.377; p = .552) (Figure 5G). 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 we saw no alteration in sleep latency (t_7 = 0.038, p = .971) (Supplemental Figure S3A) or locomotor activity (main effect of drug: $F_{1,7}$ = 0.001, p = .992) (Supplemental Figure S3B). Additionally, to test whether antagonism of α1ARs reduces arousal in other wake-promoting dopaminergic nuclei, we also infused terazosin into the VTA, but this manipulation did not alter sleep latency (t_7 = 1.139, p = .292) (Supplemental Figure S3C) or locomotor activity (main effect of drug: $F_{1,7}$ = 0.611, p = .460) (Supplemental Figure S3D). Together, these results reveal that noradrenergic transmission at α1ARs specifically in the vPAG bidirectionally modulates arousal.

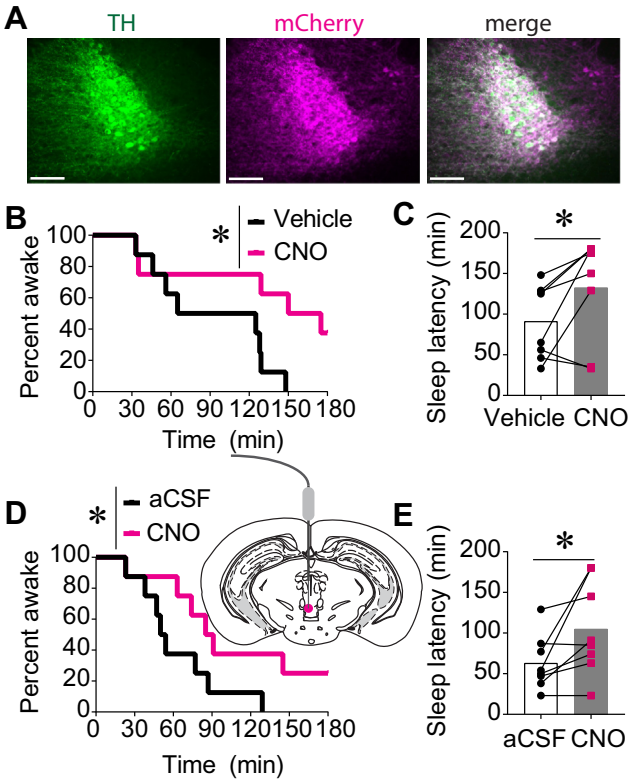

Figure 3. Chemogenetic stimulation of the locus coeruleus (LC) or LC projections to the ventral periaqueductal gray promotes wakefulness. **(A)** AAV-hSyn-DIO-hM3D(Gq)-mCherry was injected into the LC of TH-Cre^{+} mice. Scale bars = 100 μm. **(B, C)** Clozapine *N*-oxide (CNO) (1 mg/kg)–induced activation of Gq designer receptors exclusively activated by designer drugs in the LC significantly increased sleep latency (n = 8; $^{*}p < .05$). **(D, E)** Site-specific infusion of CNO (1 μg/0.3 μL) into the ventral periaqueductal gray to stimulate LC projections to the ventral periaqueductal gray similarly increased sleep latency (n = 8; $^{*}p < .05$). aCSF, artificial cerebrospinal fluid; TH, tyrosine hydroxylase.

vPAG α1ARs Are Enriched on Astrocytes

Although α1ARs 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 α1AR agonists and antagonists elicited changes in arousal (Figure 6A). In the vPAG, α1AR immunoreactivity was located primarily presynaptically in unmyelinated axons and in putative glial elements suspected to be astrocytes based on morphology (Figure 6B, C).

Next, to determine the neurochemical identity of α1AR-containing elements, vPAG slices were double labeled for α1ARs using immunogold and 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 glial fibrillary acidic protein (GFAP) (astrocytes) using immunoperoxidase (37,42–44). The most abundant α1AR-containing elements were vGluT2-positive axon terminals and GFAP-positive glia, whereas only modest levels of other combinations of colabeling were evident (Figure 6D–H). Colocalization of α1ARs and TH was rare.

Gq Signaling in vPAG Astrocytes Is Sufficient to Promote Arousal

Because the overwhelming majority of α1ARs in other dopaminergic brain regions are located on presynaptic neuronal elements (37,38), α1AR 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 α1ARs found on astrocytes (Figure 6). To test whether vPAG α1ARs modulate arousal, we mimicked local astrocytic α1AR-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 Ca^{2+} in these mice but not in wild-type littermates (49). aCSF or CNO (1 μg/0.3 μL) was infused through a guide cannula targeting the vPAG. CNO significantly increased sleep latency in DREADD-expressing mice but not littermate control mice (control mice: t_8 = 1.066, p = .318; GFAP-hM3Dq: t_6 = 2.156, p = .0385) (Figure 7A, B). This increase in wakefulness

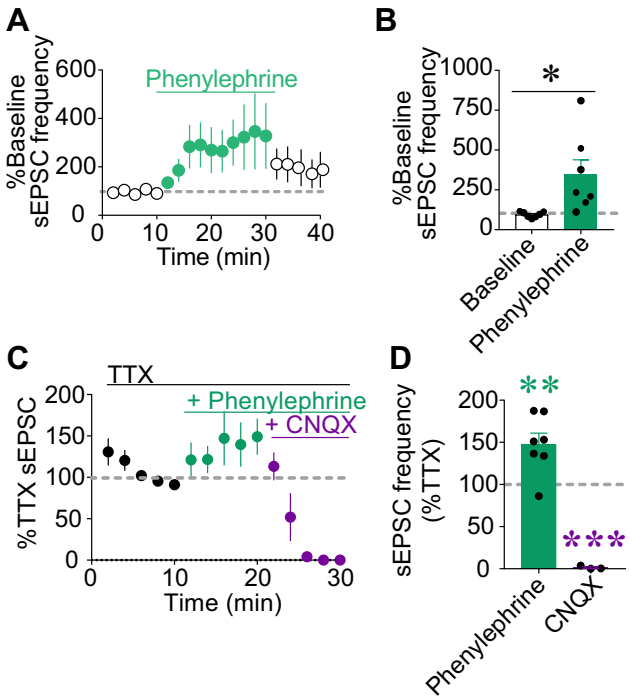

Figure 4. Stimulation of alpha1-adrenergic receptors increases glutamatergic drive onto ventral periaqueductal gray dopamine neurons in an alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor–dependent, action potential–independent manner. **(A, B)** Bath application of phenylephrine (3 μmol/L) significantly increased spontaneous excitatory postsynaptic current (sEPSC) frequency in ventral periaqueductal gray dopamine neurons (n = 7; $^{*}p < .05$). **(C)** Tetrodotoxin (TTX) (1 μmol/L), phenylephrine (3 μmol/L), and cyanquixaline (CNQX) (10 μmol/L) were cumulatively applied to slices, and sEPSCs were recorded from ventral periaqueductal gray dopamine neurons. **(D)** Phenylephrine in the presence of TTX increased sEPSC frequency compared with sEPSCs during TTX alone (n = 7; $^{**}p < .01$), and CNQX abolished sEPSCs (n = 3; $^{***}p < .001$).

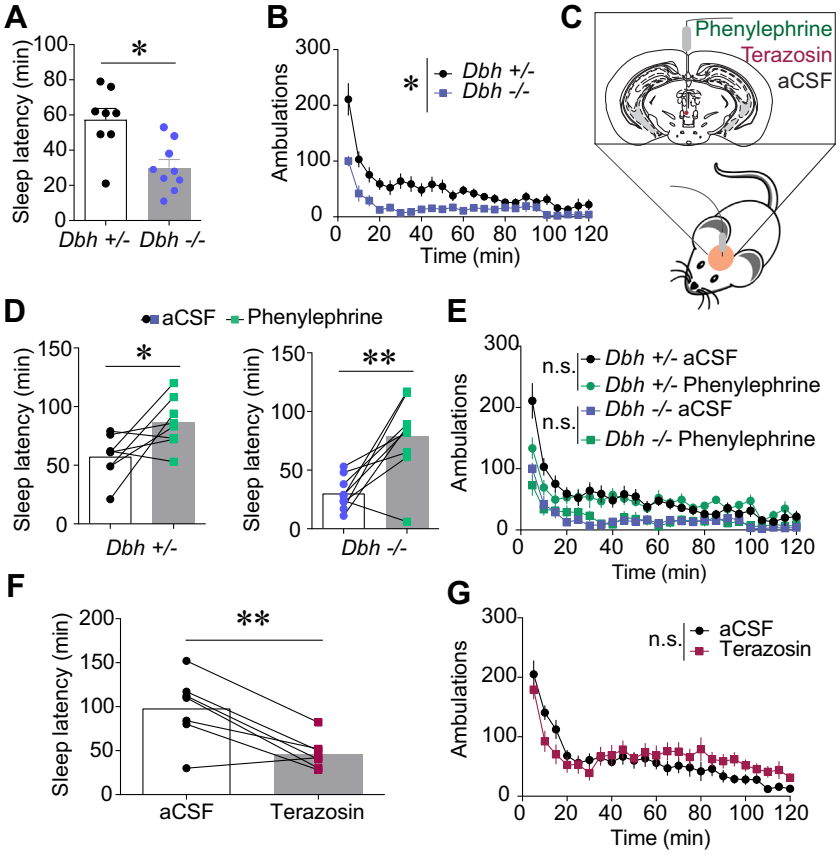

Figure 5. Ventral periaqueductal gray (vPAG) alpha1-adrenergic receptors bidirectionally modulate wakefulness. **(A, B)** Mice lacking norepinephrine ($Dbh^{-/-}$; n = 9) fell asleep faster than norepinephrine-competent littermates ($Dbh^{+/-}$; n = 8) following a control injection and were less active in a novel environment (*$p < .05$). **(C)** An alpha1-adrenergic receptor agonist, antagonist, or artificial cerebrospinal fluid (aCSF) was site specifically infused into the vPAG **(D–G)**. **(D)** Phenylephrine (4 μg/0.3 μL) significantly increased sleep latency in control (n = 8; *$p < .05$) and $Dbh^{-/-}$ (n = 9; **$p < .01$) mice compared with aCSF (0.3 μL) infusion. **(E)** Site-specific administration of phenylephrine (4 μg/0.3 μL) into the vPAG had negligible effects on novelty-induced locomotion (controls, n = 8; $Dbh^{-/-}$, n = 9; main effects of drug; not statistically different [n.s.]). **(F, G)** Intra-vPAG terazosin (3 μg/0.3 μL) significantly decreased sleep latency [panel **(F)**; n = 7; **$p < .01$], but there was no main effect on novelty-induced locomotion [panel **(G)**; n = 11; n.s.] in C57BL/6J mice.

was not due to hyperactivity (controls, main effect of drug: $F_{1,8}$ = 0.808, p = .395; GFAP-hM3Dq, main effect of drug: $F_{1,7}$ = 7.302, p = .0305) (Figure 7C, D). 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 $vPAG^{DA}$ neurons. Transmission at α1ARs increases the excitability of $vPAG^{DA}$ neurons in a glutamate-dependent, action potential–independent manner and facilitates arousal. vPAG α1ARs 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 α1AR-mediated excitation of $vPAG^{DA}$ neurons (Figure 8).

$vPAG^{DA}$ Neurons Promote Wakefulness

Using c-Fos as a measure of activation, previous research found that $vPAG^{DA}$ neurons were active when rodents were awake and inactive when rodents were asleep, and lesioning $vPAG^{DA}$ neurons increased time asleep during both day and night (16). The present study demonstrates that chemogenetic activation of $vPAG^{DA}$ neurons promotes arousal by increasing latency to fall asleep without causing hyperactivity. A recent study reported that inhibition of DRN and caudal $vPAG^{DA}$ 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 $vPAG^{DA}$ neurons. Together, these studies demonstrate that $vPAG^{DA}$ neurons can bidirectionally modulate arousal.

$vPAG^{DA}$ 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 $vPAG^{DA}$ neurons and function via a relay from the LC, as the LC mediates some aspects of orexin-induced arousal (52,53). The ventrolateral preoptic nucleus facilitates sleep (54,55) and reciprocally connects with $vPAG^{DA}$ (16), making it another potential functional neuroanatomical partner of $vPAG^{DA}$ neurons. Finally, because $vPAG^{DA}$ neurons can corelease glutamate (34), their wake-promoting properties could involve dual neurotransmitter signal integration by downstream cells.

The finding that DREADD-induced stimulation of $vPAG^{DA}$ 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 and caudal $vPAG^{DA}$ neurons are activated by a variety of salient stimuli (15). If rostral $vPAG^{DA}$ cells similarly signal salient stimuli, DREADD-induced activation of

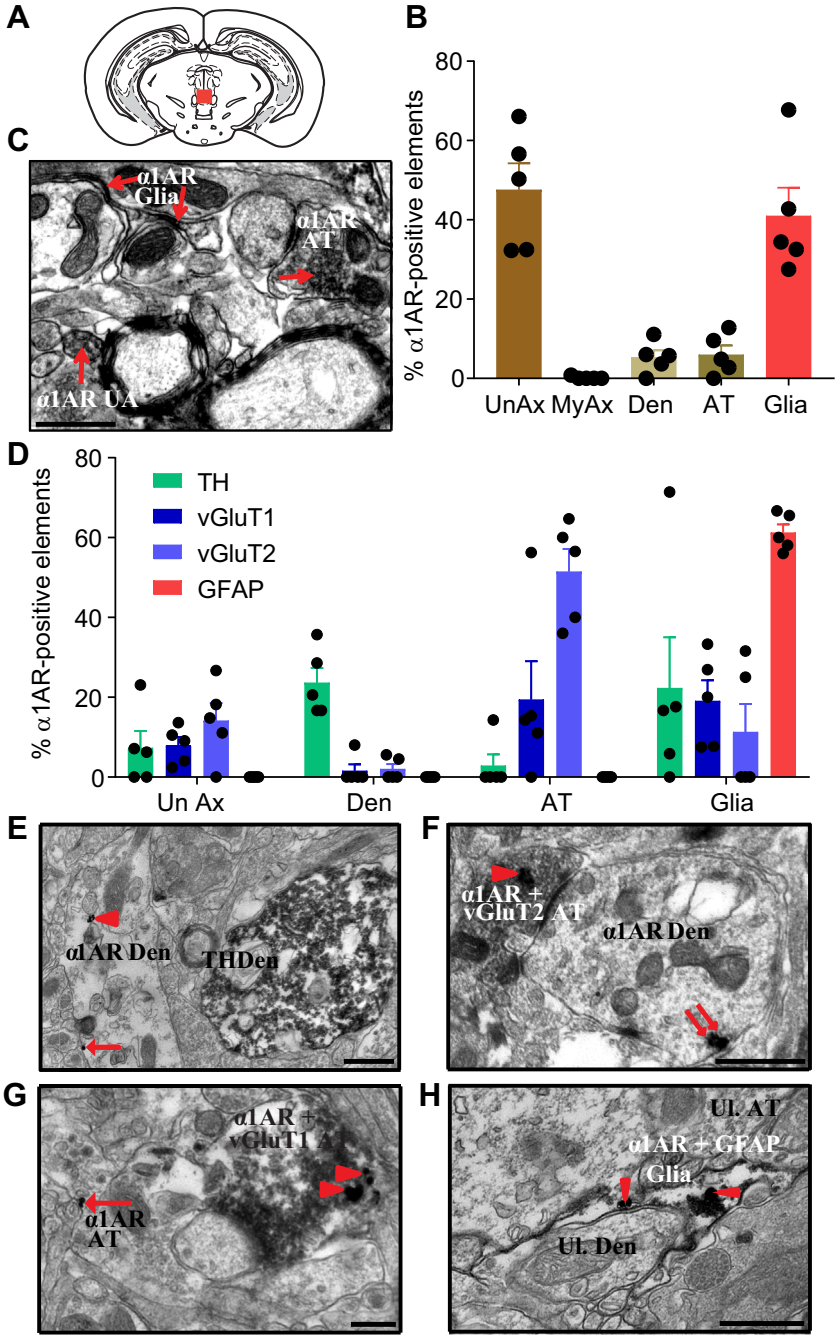

Figure 6. Anatomical location of ventral periaqueductal gray alpha1-adrenergic receptors (α1ARs) determined with immunoelectron microscopy. **(A)** Red box signifies the portion of the ventral periaqueductal gray used for electron microscopy experiments. **(B)** Histogram depicting the mean percentage (± SEM) of unmyelinated axons (UnAx), myelinated axons (MyAx), dendrites (Den), axon terminals (AT), and glia found within the ventral periaqueductal gray that contained immunoperoxidase labeling for the α1AR. **(C)** Representative electron micrograph with immunoperoxidase labeling (arrows) for the α1AR in UnAx, AT, and glia. **(D)** Percentage of α1AR-labeled elements colabeled with tyrosine hydroxylase (TH), vGluT1, vGluT2, or glial fibrillary acidic protein (GFAP). **(E)** Representative micrograph of α1AR-labeled dendrite (α1AR Den) with immunogold (arrowheads point to intracellular gold particles; arrows point to plasma membrane bound gold particles) and a TH-positive dendrite (TH Den) filled with immunoperoxidase labeling. **(F)** Representative micrograph of double-labeled AT with intracellular gold particles for α1AR and immunoperoxidase for vGluT2 synapsing on α1AR-labeled dendrite. **(G)** Representative micrograph of a double-labeled AT for α1AR in immunogold and vGluT1 with immunoperoxidase synapsing on an unlabeled dendritic process. In addition, α1AR-labeled AT with plasma membrane bound gold particles is pictured. **(H)** Representative micrograph of a double-labeled astrocyte containing GFAP with immunoperoxidase (patchy dark gray/black area within astrocyte) and intracellular α1ARs in immunogold (arrowheads point to α1ARs) wrapping around an unlabeled dendrite (Ul. Den). All scale bars = 0.5 μm.

these neurons may increase other behaviors that compete with horizontal ambulations.

Noradrenergic Transmission via α1ARs Modulates vPAG^{DA} Neuron Activity and Arousal

vPAG^{DA} 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 have not been identified. The present study tested whether vPAG^{DA} 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

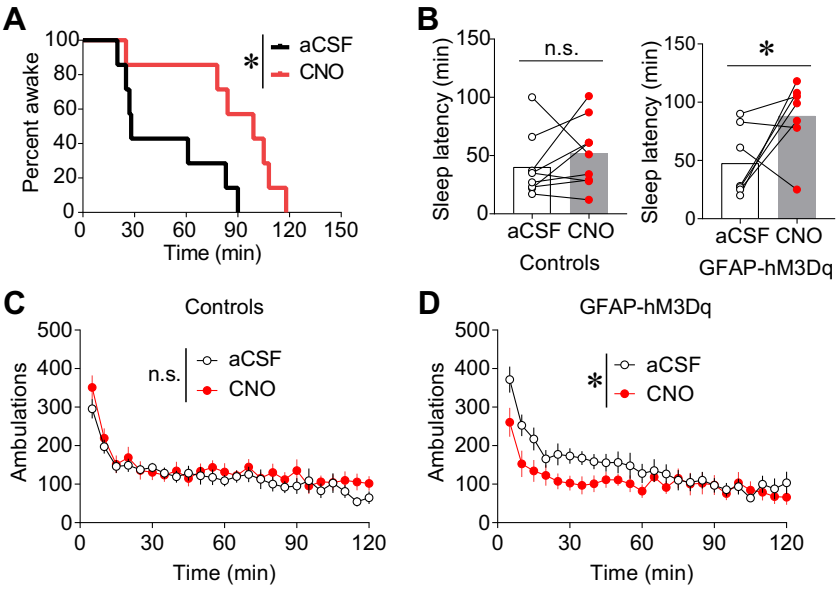

Figure 7. Activation of Gq protein signaling in ventral periaqueductal gray astrocytes promotes arousal. **(A, B)** Artificial cerebrospinal fluid (aCSF) or the designer receptors exclusively activated by designer drugs (DREADDs) ligand clozapine *N*-oxide (CNO) (1 μg/0.3 μL) was site specifically infused into the ventral periaqueductal gray of mice expressing Gq-coupled DREADDs in glial fibrillary acidic protein (GFAP)–positive cells (astrocytes). CNO and DREADD–induced activation of ventral periaqueductal gray astrocytes increased latency to fall asleep in DREADD-expressing mice **(A, B)** but not in controls [panel **(B)**; controls, $n = 9$; not statistically different [n.s.]; GFAP-hM3Dq, $n = 7$; $^{*}p < .05$]. **(C, D)** CNO-induced activation of DREADDs in astrocytes reduced novelty-induced locomotion [panel **(D)**; $n = 8$; $^{*}p < .05$], and CNO had no effect in non–DREADD expressing littermates [panel **(C)**; $n = 9$; n.s.].

DREADD-expressing neurons can activate fibers and 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 adrenergic receptor subtypes (alpha1, alpha2, and beta), we focused on the α1AR 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, α1AR activity in other brain regions is implicated in arousal (58–60). Stimulation of vPAG α1ARs increases vPAG^{DA} neuron activity via glutamate and promotes wakefulness in NE-deficient mice (Figures 4 and 5). Furthermore, local antagonism of α1ARs decreased arousal, consistent with previous research (61). Infusion of terazosin into the vPAG, but not the ventricles or VTA, attenuates arousal (Figure 5F and Supplemental Figure S3), demonstrating neuroanatomical specificity. Additionally, these experiments indicate that α1ARs do not regulate arousal in all dopaminergic populations; the wake-promoting effects of VTA DA neurons (14) are unlikely under the control of endogenous α1AR tone. Combined, these results illustrate that the vPAG is a locus where NE modulates DA neuron activity and promotes behavioral arousal through α1ARs.

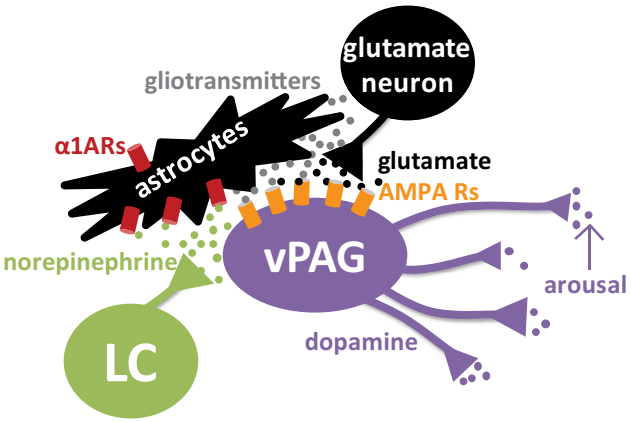

Figure 8. Hypothesized locus coeruleus (LC)–ventral periaqueductal gray (vPAG) arousal circuit. The LC releases norepinephrine in the vPAG, which activates astrocytic alpha1-adrenergic receptors (α1ARs). Astrocytes could release gliotransmitters or otherwise act on neuronal glutamate terminals to elicit glutamate release in an action potential–independent mechanism, or astrocytes could release glutamate to directly activate dopamine (DA) neurons. Then glutamate via alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA Rs) drives the activity of wake-promoting vPAG^{DA} neurons to promote arousal. These vPAG^{DA} neurons project to and modulate other brain regions involved in arousal.

Previous research has shown noradrenergic receptors in other regions modulate arousal. Agonism of α1ARs or beta-adrenergic receptors in the medial septal area or medial preoptic area promotes wakefulness (60), as does agonism of α1ARs and, to a lesser extent, beta-adrenergic receptors in the lateral hypothalamus (17); however, infusions in the substantia innominata do not (62). The present study reveals that endogenous noradrenergic tone at α1ARs 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*$^{-/-}$ 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 adrenergic receptors. Alternatively, each brain region might contribute uniquely to arousal.

α1ARs on vPAG Astrocytes Modulate Wakefulness

Most α1ARs 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 α1ARs are found on glia and axons in the vPAG (Figure 6B). Most α1AR-positive glia are colabeled 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 α1AR-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 α1AR 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 α1AR 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 α1ARs (73) and implicates vPAG astrocytes as behaviorally relevant targets of the LC.

Astrocytes can release gliotransmitters, including glutamate, gamma-aminobutyric acid, D-serine, and adenosine triphosphate (ATP), to influence local neurons (46,74). Phenylephrine increased glutamatergic sEPSCs onto $vPAG^{DA}$ neurons; this effect was preserved in the presence of TTX, and CNQX abolished phenylephrine-induced increases in sEPSCs on $vPAG^{DA}$ 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 the effects of phenylephrine. The fact that the excitatory effects of phenylephrine persist in the presence of TTX is consistent with nonconical regulation of glutamate release, such as NE providing excitatory drive onto wake-promoting $vPAG^{DA}$ neuron activity through α1AR-induced activation of local astrocytes (Figure 8). Astrocytes could act on glutamatergic terminals to drive $vPAG^{DA}$ 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 $vPAG^{DA}$ neurons. NE at α1ARs increases the amplitude of miniature EPSCs 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 α1ARs 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.

Conclusions

The present study demonstrates that wake-promoting $vPAG^{DA}$ neurons are physiologically and functionally under noradrenergic control. vPAG α1ARs drive glutamatergic excitation of $vPAG^{DA}$ 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; Teva Pharmaceutical Industries Ltd., Petah Tikva, Israel) 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 $vPAG^{DA}$ projections are critical for increasing arousal. Additionally, studies targeting DRN and $vPAG^{DA}$ neurons demonstrate that these cells are important in promoting wakefulness by salient stimuli (15), sociability (82), and antinociception (34). Mounting evidence suggests that $vPAG^{DA}$ neurons are not merely an extension of the VTA; further studies should continue to examine the functions of these understudied populations of DA neurons.

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