Noradrenergic Transmission at Alpha1-adrenergic Receptors in the Ventral Periaqueductal Gray Modulates Arousal

Supplemental Information

Surgery

Mice were anesthetized with isoflurane (3% induction, 1-2% maintenance) and administered the analgesic meloxicam (5 mg/kg, s.c.). For experiments requiring intracranial drug infusions, a guide cannula (Plastics One, Roanoke, VA) was positioned above the vPAG (coordinates relative to bregma: AP: -2.7 mm, ML: ± 0.25 mm, DV: -1.8 mm for vPAG), lateral ventricle (AP: -0.6 mm, ML ±1.0 mm, DV: -1.7 mm), or VTA (AP: -3.1 mm, ML: ±0.5 mm, DV: -2.0 mm) and permanently secured with screws and dental cement. Obturators were inserted into guide cannulae to prevent clogging. For viral infusion experiments, the Cre-dependent viral vector AAV8-hSyn-DIO-hM3Dq-mCherry (aliquots purchased from the University of North Carolina Vector Core and generously gifted from Thomas Kash) was delivered into the LC (0.3 µL; AP: -5.4 mm; ML: ± 0.9 mm; DV: -3.7 mm) or rostral vPAG (0.3-0.5 µL; AP: -2.8 mm; ML: ±0.4 mm; DV: -3.6 mm) with a 5 µL Hamilton syringe and Stoelting Quintessential Stereotaxic Injector pump at a rate of 0.15 µL/min. Mice were monitored daily and given at least 7 days to recover from surgery before behavioral testing began. Behavioral testing of DREADD subjects began 3 weeks post-surgery to allow ample time for DREADD expression.

For EEG recordings, 4 EEG electrodes were implanted into the skull surface (coordinates relative to bregma: AP: +1.0 mm, ML: ±2.0 mm; AP: -2.0 mm, ML: ±2.0 mm). Two silver pad electrodes were implanted overlying the neck muscle to obtain EMG recordings.

EEG

Mice received 72 h of habituation to the commutator (Pinnacle Technologies, KS) before recording of video-EEG (Pinnacle Technologies), as previously described (1). Data was sampled at 1 kHz, with hardware high-pass filtering of EEG at 0.5 Hz and EMG at 10 Hz, with offline low-pass filtering of EEG at 55 Hz (Butterworth, 4th order, Spike2 Version 8.09b, Cambridge Electronic Design, Cambridge, UK). Data were then automatically sleep-scored with 20-sec epochs, with manual over-reading of the entire record, with typical 2-epoch criteria for scoring NREM, using Spike2 ('RatSleepAuto' script, Cambridge Electronic Design, Cambridge, UK). The spectrogram (Figure 1B) was generated by fast Fourier transform of EEG channel1 with a Hanning window and 8192 points. Video-only scoring was performed following an i.p. saline injection, with both EEG scorer (NPP) and video scorer (KAPS) blinded to each other's scoring.

Slice electrophysiology

Mice were decapitated under deep isoflurane anesthesia, and brains were quickly removed from the skull and placed in ice-cold oxygenated sucrose-based aCSF cutting solution [containing (in mM): 183 sucrose, 20 NaCl, 0.5 KCl, 1 MgCl2, 1.4 NaH2PO4, 2.5 NaHCO3, 1 glucose, and 0.4 ascorbic acid]. Three hundred μm thick brain slices containing the vPAG were prepared from adult male TH-eGFP mice with a VT1000 Vibratome (Leica) and then stored in a holding chamber with oxygenated normal aCSF [containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2.5 CaCl2, 2 MgSO4, 26 NaHCO3, 11 glucose, 0.4 ascorbic acid] at 28°C for 45 min prior to their use in electrophysiology experiments. For recordings, slices were transferred to a submerged recording chamber, where they were constantly perfused with normal aCSF at a rate of 2 mL/min and allowed to equilibrate for at least 10 min prior to recordings. For whole-cell voltage-clamp recordings, AMPA receptor-mediated spontaneous EPSCs (sEPSCs) were recorded in voltage-clamp with a holding potential of −70 mV, and pharmacologically isolated by adding 25 μM

picrotoxin to the aCSF. sEPSC recordings were acquired in 2-min gap-free blocks. Recording electrodes were filled with (in mM): 118 CsOH, 117 D-gluconic acid, 20 HEPES, 0.4 EGTA, 2 MgCl2, 5 tetraethylammonium chloride, 4 ATP, 0.3 GTP, pH 7.2–7.3, 285–295 mOsmol.

For experiments in which the effects of phenylephrine were determined, a stable baseline was recorded for 10 min prior to application of phenylephrine (3 μM). Phenylephrine was then administered to the slice for 20 min. A 10-min washout period was recorded following phenylephrine administration. In a separate group of cells, phenylephrine bath was applied in the presence of TTX (1 μM), followed by the addition of CNQX (10 μM) to the phenylephrine/TTX cocktail.

Cells in which the access resistance varied by >20% were deemed unstable and discarded from the overall analysis. All electrophysiology recordings were made using MultiClamp 700B Commander along with Clampex 10.4 and analyzed using Clampfit 10.4 (Molecular Devices).

Drugs

Terazosin and phenylephrine (Sigma-Aldrich, St. Louis, MO) were dissolved into aCSF, and behaviorally-relevant doses were chosen based on previous studies (2-5). CNO was generously provided by the National Institute on Drug Abuse, and doses were chosen based upon our pilot experiments and previous studies (6-9). For systemic administration, CNO was dissolved in 2.5% DMSO and saline, and for intracranial administration CNO was dissolved in aCSF. For behavioral pharmacology and DREADD experiments, drug treatment order was counterbalanced across subjects.

Intracranial infusions

Mice were handled prior to the start of experiments to habituate them to the gentle restraint required for brain microinfusions. To site-specifically infuse drug into the vPAG, VTA, or

ventricles on the day of testing, obturators were removed and a 33Ga injector (Plastics One, Roanoke, VA) which projected 2 mm below the guide cannula into brain was inserted. Injectors were connected to PE20 tubing and a 5 μL Hamilton syringe loaded in a KD Scientific pump, which delivered 0.3 μL of drug (aCSF, terazosin, phenylephrine, or CNO) into the brain. The infusion needle remained in the brain for 60 sec post-injection to ensure that the drug diffused away from the tip of the injector. Obturators were reinserted to the cannulae. Behavioral testing began immediately after infusion.

Histology

Following the conclusion of behavioral testing, mice were transcardially perfused with 4% paraformaldehyde. Brains were extracted and sliced in 40 μm sections. For intracranial infusion experiments, injector placements were verified with light microscopy and the Mouse Brain Atlas (10). For DREADD experiments, immunohistochemistry was performed to verify DREADD expression. Following 3 washes in phosphate-buffered saline (PBS), brain sections were incubated in 10% normal goat serum (NGS) and 2% bovine serum albumin (BSA) for 1 h. Sections were incubated in primary antibody at 4°C overnight. Anti-tyrosine hydroxylase primary antibody (chicken polyclonal, Abcam ab76442, 1:500 concentration) was used to identify DA neurons, and anti-dsRed primary antibody (rabbit polyclonal, Clontech 632496, 1:500 concentration) was used to identify the mCherry-tagged DREADDs. The following day, brain slices were incubated in the secondary antibodies that corresponded to the animal the primary antibody was raised in (goat anti-chicken 488, Abcam ab150169, 1:600 concentration; goat anti-rabbit 594, Life Technologies A-11012, 1:600 concentration). Tissue was mounted onto slides, coverslipped with fluoromount, and imaged on a Leica fluorescence microscope.

To determine the extent of viral transfection, the proportion of TH cells co-expressing mCherry was counted from 3 vPAG slices per DREADD-expressing animal. The mean from each set of 3 slices was generated to determine the average percentage of rostral vPAG TH cells expressing hM3Dq (Figure 2B).

Immunoelectron microscopy (EM)

Preparation of tissue

Mice were deeply anesthetized with a ketamine (100 mg/kg) and butorphanol (2 mg/kg) cocktail prior to being transcardially perfused with 4% paraformaldehyde (Electron Microscopy Sciences (EMS), Hatfield, PA) and 0.1% glutaraldehyde (EMS) in PB (0.1M, pH = 7.4). Following perfusion, brains were removed and post-fixed for 72 h in 4% paraformaldehyde. Brain tissue was then cut into 60 μm sections using a vibrating microtome and prepared for immunohistochemistry by exposing the tissue to $NaBH_4$ (ThermoFisher Scientific, Waltham, MA) and cryoprotectant.

Primary antibodies

Table S1 lists all antibodies used for EM. The specificity of each antibody was previously validated [α1AR: (4, 11); vGluT1 & vGluT2: (12, 13); TH: (14) and Manufacturer quality control on mouse brain lysates; GFAP: (15) and Manufacturer quality control on mouse brain lysates].

Single immunoperoxidase labeling for light microscopy

To confirm the presence of the α1AR and the various markers in the vPAG, as well as the working dilutions for each antibody, single immunoperoxidase labeling for light microscopy was performed for all antibodies listed in Table S1. Following exposure to $NaBH_4$, tissue containing the vPAG was pre-incubated in 10% NGS (Vector Laboratories, Burlingame, CA), 0.3% Triton, and 1% BSA (bovine serum albumin, Sigma-Aldrich) in PBS (pH = 7.4) for 1 h. All tissue was then exposed to 1% NGS, 1% BSA, 0.3% triton, and each of the primary antibodies overnight at

room temperature. The next day, tissue was exposed to secondary biotinylated antibodies (1:200, Vector) that corresponded to the animal the primary antibody was raised in, followed by the avidin-biotin complex (ABC, 1:100, Vector) and lastly a 3,3' diaminobenzidine tetrahydrochloride (DAB, 0.025%, Sigma) solution with 0.005% H_2O_2, and 10 mM imidazole in TRIS buffer (pH = 7.6). Tissue was examined using a light microscope (Aperio ScanScope; SS5115). Initially, serial dilutions were used [based on (4) and manufacturer information] to obtain labeling patterns seen previously (data not shown). The final concentrations listed in Table S1 were used for the EM studies below.

Single pre-embedding immunoperoxidase labeling for EM: α1AR

Following exposure to $NaBH_4$ and decreasing concentrations of cryoprotectant, brain sections containing the vPAG [AP coordinate -2.7 from Bregma, based on (10)] were pre-incubated as described above, with the exception of Triton. Tissue was incubated with the primary antibody for the α1AR for 48 h at 4ºC with 1% NGS, 1% BSA in PBS. After exposure to the biotinylated secondary goat anti-rabbit IgGs (1:200, Vector), ABC and DAB, tissue was then processed for EM. This involved osmification, dehydration and resin embedding, described previously (16). After incubating the tissue on slides with resin at 60ºC for 48 h, blocks of the vPAG (Figure 6A) were removed from the larger sections and put onto resin blocks to obtain 60 nm sections using an ultramicrotome and then placed on copper-mesh grids (EMS) to then be observed on the EM.

Analysis of single pre-embedding immunoperoxidase for α1AR

Thirty-forty electron micrographs were taken from 5 animals each (5 blocks) from the vPAG that contained immunoreactive elements for the α1AR. Micrographs were acquired at 40,000x using a CCD camera controlled by either Digital Micrograph (Gatan, Inc.) or AMT Image

Capture (version 5.42.498). Elements were classified as dendrites, unmyelinated axons, axon terminals, myelinated axons or glial elements as described (17). In total, 31 dendrites labeled for the α1AR were identified: 246 unmyelinated axons, 37 axon terminals, 1 myelinated axon and 182 glial elements from the 5 animals examined. The percentage of each of the elements listed was then calculated and averaged across the number of animals. Micrographs were edited only for contrast and brightness using Adobe Photoshop (version 12.0.4x32) and then compiled into figures using Adobe Illustrator (version 15.0.2).

Double pre-embedding immunogold and immunoperoxidase labeling

Four markers were used to determine the neurochemical identity of the α1AR expressing elements: TH (dopaminergic cells), vGluT1 [glutamatergic terminals originating from neurons in the cortex and hippocampus (13, 18), vGluT2 glutamatergic terminals originating from the thalamus and other hindbrain and midbrain structures (18)], and GFAP [astrocytes (19)].

Following exposure to $NaBH_4$ and decreasing concentrations of cryoprotectant, sections containing the vPAG were pre-incubated for 30 min in 5% dry milk in PBS at RT. The sections were then moved to the primary antibody solution containing the antibodies for the α1AR and one of the markers (TH, vGluT1, vGluT2 or GFAP) in 1% dry milk and TBS-gelatin buffer (pH = 7.6) overnight at room temperature. The next day, sections were rinsed and transferred to the secondary antibody solution, which contained goat anti-rabbit IgG conjugated with 1.4 nm gold particles for the α1AR (1:100, Nanoprobes, Yaphank, NY) and biotinylated goat anti-mouse (for TH, vGluT1 and GFAP) or goat anti-guinea pig (for vGluT2) IgGs (1:200, Vector). Following incubation with secondary antibodies, sections were rinsed with 2% $C_2H_3NaO_2$ (pH = 7.0), and the gold particles were silver intensified using a HQ kit (Nanoprobes), followed by ABC and DAB as described for single labeling. Immediately following DAB, tissue was osmified, dehydrated and embedded in

resin overnight and placed on slides the following day. The tissue on slides was then baked for 48 h at 60ºC. Finally, blocks of tissue were removed from the larger sections that contained the vPAG and placed on resin blocks. Tissue was trimmed and sliced at 60 nm using an ultramicrotome and placed on copper mesh grids for EM observation.

Analysis of double pre-embedding immunogold and immunoperoxidase labeling

Thirty to forty electron micrographs were taken at 40,000x of tissue stained for the α1AR revealed with immunogold and one of the 4 markers revealed with immunoperoxidase from 5 animals each for a total of 20 blocks of tissue. Micrographs were taken when both immunogold for the α1AR and immunoperoxidase for one of the markers were in the observable field. Pictures were captured using a CCD camera controlled by either Digital Micrograph (Gatan, Inc.) or with AMT Image Capture (version 5.42.498). Initially, all elements containing the α1AR were identified, classified and quantified as described for single labeling EM data above. Similar percentages of α1AR-immunogold containing dendrites, unmyelinated axons, axon terminals and glial elements were found in the vPAG, as with single immunoperoxidase labeling. Because only one myelinated axon was found in single labeled tissue, it was not included in the double-labeling analysis. Elements containing immunoperoxidase for either TH, vGluT1, vGluT2, or GFAP were also classified and quantified. To be considered positive for the α1AR, dendrites, axon terminals and glial elements had to contain at least two gold particles; for unmyelinated axons, only one gold particle was needed. In total, for tissue that also contained TH labeling, 73 α1AR-labeled dendrites, 110 unmyelinated axons, 48 axon terminals and 69 glial elements were analyzed. In tissue that also contained vGluT1 labeling, 62 α1AR-labeled dendrites, 199 unmyelinated axons, 77 axon terminals and 114 glial elements were analyzed. In tissue that also contained vGluT2 labeling 81 α1AR-labeled dendrites, 178 unmyelinated axons, 145 axon terminals and 74 glial elements were

analyzed. Lastly, in tissue that also contained GFAP labeling 24 α1AR-labeled dendrites, 125 unmyelinated axons, 54 axon terminals and 147 glial elements were analyzed. The percentage of elements that contained the α1AR immunogold labeling with one of the markers was determined and then averaged across the number of animals for each grouping. Micrographs were edited only for contrast and brightness using Adobe Photoshop (version 12.0.4x32) and then compiled into figures using Adobe Illustrator (version 15.0.2).

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 7 and IBM SPSS Statistics version 24. For all statistical tests, an α value of 0.05 was used to determine significance. Behavioral and EEG scoring of sleep tests were compared using a Pearson's correlation. Drug- or DREADD-induced changes in sleep latency were analyzed with paired t-tests. An unpaired t-test was used to analyze sleep latency between *Dbh* +/- and *Dbh* -/- mice. Locomotor activity was analyzed with mixed model ANOVAs (with genotype or drug as the between-subjects factor and time as the within-subjects factor) or repeated measures ANOVAs (when both time and drug were repeated measures variables). Phenylephrine-induced changes in sEPSC frequency were analyzed using a paired t-test, and phenylephrine-induced changes in sEPSC frequency in the presence of TTX and CNQX were analyzed using a one-way ANOVA and Dunnett's post hoc test. While male and female mice were included in the study, sex was not included as a factor in the analyses, because the experiments were not sufficiently powered to statistically test for sex differences.

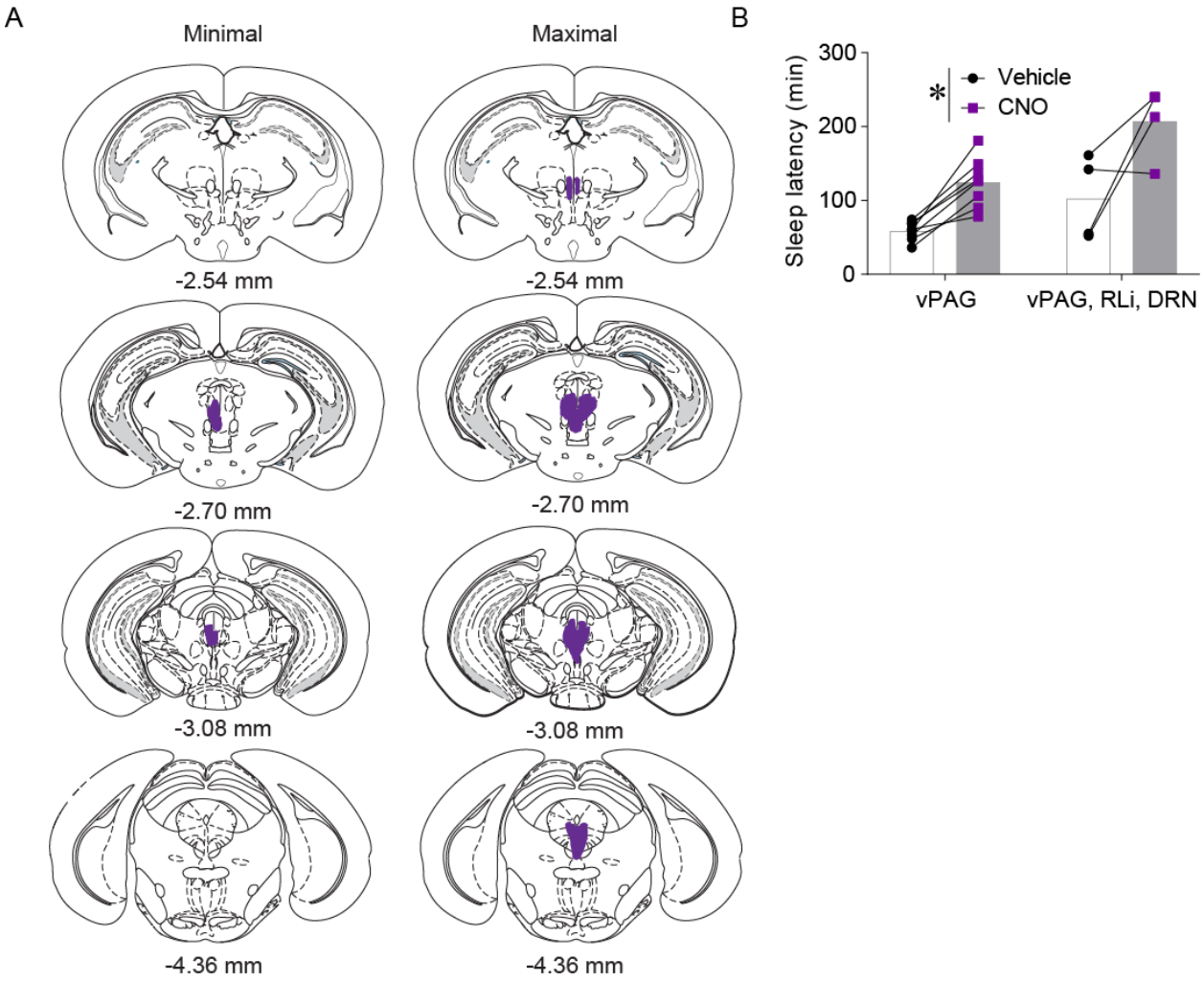

Figure S1. Viral spread of hM3Dq and effects on sleep latency. A) Approximated maximal and minimal virus spread was determined with microscopy and is depicted in purple. **B)** CNO significantly increased sleep latency in mice with DREADDs primarily confined to the vPAG as well as in subjects with DREADD expression extending into the RLi and DRN (main effect of drug, $F_{(1,10)} = 26.857$, $p = 0.0004$; interaction, $F_{(1,10)} = 1.405$, $p = 0.263$) Note that this analysis may be underpowered to detect subtle differences in the magnitude of wake-promoting effects between vPAG and vPAG, RLi, DRN. Regardless, stimulation of only rostral vPAG^{DA} is sufficient to promote wakefulness.

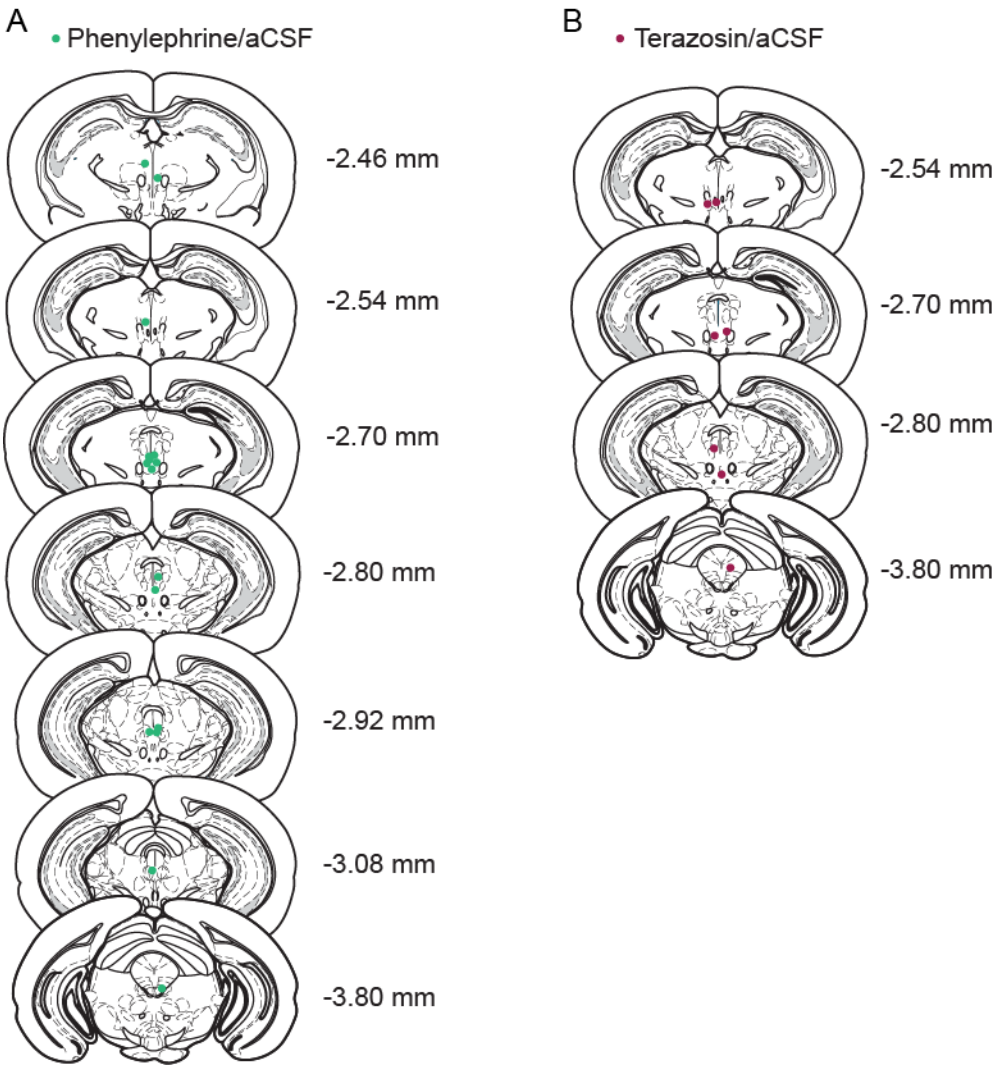

Figure S2. Histology for intra-vPAG infusion. A-B) Approximated injector placements of intra-vPAG phenylephrine (A) and terazosin (B) determined from brain slice histology.

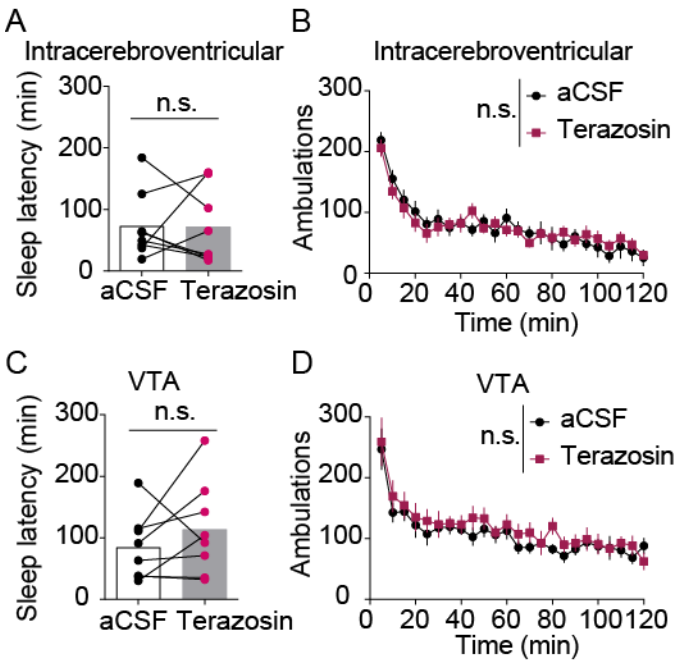

Figure S3. Reductions in arousal following intra-vPAG terazosin administration are not attributable to off-target effects. A-B) Infusion of the same dose of terazosin that promoted sleep in the vPAG (3 μg/0.3 μL) did not have behavioral effects on sleep latency (A; n=8) or locomotion (B; n = 8) when infused into the ventricles. **C-D)** Similarly, terazosin administered into the VTA did not alter sleep latency (C; n = 8) or locomotion (D; n = 8). n.s. = not statistically different.

Table S1. Antibodies used for electron microscopy.

Antigen	**Immunogen**	**Manufacturer Data**	**Dilution Used**
Alpha-1 adrenergic receptor (α1AR)	Synthetic peptide corresponding to residues K(339) FSREKKAAKT(349) of the third intracellular loop of human alpha-1ARs; approximately 60 kDa.	ThermoFisher Scientific, Invitrogen, Rabbit Polyclonal, #PA1-047	1:1000
Tyrosine hydroxylase (TH)	Clone LNC1. Recognizes an epitope on the outside of the regulatory N-terminus; approximately 59-61 kDa. Purified from PC12 cells.	Millipore, Mouse Monoclonal, #MAB318	1:750
Vesicular glutamate transporter 1 (vGluT1)	Clone N28/9, Produced against fusion protein amino acids 493-560 of the C-terminus; approximately 52 kDa.	UC Davis/NIH NeuroMab Facility, Mouse Monoclonal, #75-066	1:1000
Vesicular glutamate transporter 2 (vGluT2)	KLH-conjugated linear peptide corresponding to the C-terminal sequence of rat vGluT2; approximately 56 kDa.	Millipore, Guinea Pig Polyclonal, #AB2251-I	1:12,000
Glial fibrillary acidic protein (GFAP)	Clone GA5. Purified GFAP from porcine spinal cord; approximately 51 kDa.	Millipore, Chemicon, Mouse Monoclonal, #MAB360	1:3,000

Supplemental References

1. Pedersen NP, Ferrari L, Venner A, Wang JL, Abbott SBG, Vujovic N, et al. (2017): Supramammillary glutamate neurons are a key node of the arousal system. *Nature Communications*. 8:16.

2. Stone E, Zhang Y, Rosengarten H, Yeretsian J, Quartermain D (1999): Brain alpha 1-adrenergic neurotransmission is necessary for behavioral activation to environmental change in mice. *Neuroscience*. 94:1245-1252.

3. Mansur SS, Terenzi MG, Neto JM, Faria MS, Paschoalini MA (2011): Phenylephrine into the median raphe nucleus evokes an anxiolytic-like effect in free-feeding rats but does not alter food intake in free feeding rats. *Behav Brain Res*. 217:209-214.

4. Mitrano DA, Schroeder JP, Smith Y, Cortright JJ, Bubula N, Vezina P, et al. (2012): Alpha-1 adrenergic receptors are localized on presynaptic elements in the nucleus accumbens and regulate mesolimbic dopamine transmission. *Neuropsychopharmacology*. 37:2161-2172.

5. Schmidt KT, Schroeder JP, Foster SL, Squires K, Smith BM, Pitts EG, et al. (2017): Norepinephrine regulates cocaine-primed reinstatement via α1-adrenergic receptors in the medial prefrontal cortex. *Neuropharmacology*. 119:134-140.

6. Wang S, Tan Y, Zhang J-E, Luo M (2013): Pharmacogenetic activation of midbrain dopaminergic neurons induces hyperactivity. *Neuroscience Bulletin*. 29:517-524.

7. Li C, Sugam JA, Lowery-Gionta EG, McElligott ZA, McCall NM, Lopez AJ, et al. (2016): Mu opioid receptor modulation of dopamine neurons in the periaqueductal gray/dorsal raphe: A role in regulation of pain. *Neuropsychopharmacology*. 41: 2122–2132.

8. Vazey EM, Aston-Jones G (2014): Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. *Proc Natl Acad Sci*. 111:3859-3864.

9. Mahler SV, Vazey EM, Beckley JT, Keistler CR, McGlinchey EM, Kaufling J, et al. (2014): Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. *Nature Neuroscience*. 17:577-585.

10. Franklin KB, Paxinos G (2008): *The mouse brain in stereotaxic coordinates*. 3 ed.: Elsevier.

11. Nakadate K, Imamura K, Watanabe Y (2006): Cellular and subcellular localization of alpha-1 adrenoceptors in the rat visual cortex. *Neuroscience*. 141:1783-1792.

12. Montana V, Ni Y, Hua X, Parpura V (2004): Vesicular glutamate transporter-dependent glutamate release from astrocytes. *J Neurosci*. 24:2633-2642.

13. Raju DV, Smith Y (2005): Differential localization of vesicular glutamate transporters 1 and 2 in the rat striatum. In: Bolam JP, Ingham CA, Magill PJ, editors. *Basal Ganglia VIII*, pp 601-610.

14. Masilamoni G, Votaw J, Howell L, Villalba RM, Goodman M, Voll RJ, et al. (2010): 18 F-FECNT: Validation as PET dopamine transporter ligand in parkinsonism. *Exp Neurol*. 226:265-273.

15. Dottori M, Leung J, Turnley AM, Pebay A (2008): Lysophosphatidic acid inhibits neuronal differentiation of neural stem/progenitor cells derived from human embryonic stem cells. *Stem Cells*. 26:1146-1154.

16. Mitrano DA, Smith Y (2007): Comparative analysis of the subcellular and subsynaptic localization of mGluR1a and mGluR5 metabotropic glutamate receptors in the shell and core of the nucleus accumbens in rat and monkey. *J Comp Neurol*. 500:788-806.

17. Peters A, Palay SL (1991): *The fine structure of the nervous system: neurons and their supporting cells*. Oxford University Press, USA.

18. Fremeau RT, Troyer MD, Pahner I, Nygaard GO, Tran CH, Reimer RJ, et al. (2001): The expression of vesicular glutamate transporters defines two classes of excitatory synapse. *Neuron*. 31:247-260.

19. Eng LF (1985): Glial fibrillary acidic protein (GFAP) - The major protein of glia intermediate filaments in differentiated astrocytes. *J Neuroimmunol*. 8:203-214.