Supporting Information

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SI Materials and Methods

Subjects. Adult male Sprague–Dawley rats, weighing 250–275 g on arrival, were obtained from Hilltop Laboratory. Animals were allowed at least 1–2 wk of acclimation to the vivarium before surgeries. Rats were individually housed in transparent plastic high-efficiency particulate absorption (HEPA)-filtered cages and maintained on a 12/12-h light/dark cycle (lights on at 0700 hours) within a temperature- and humidity-controlled environment. Food and water were available ad libitum throughout the duration of the experiments. All experiments were conducted during the light cycle. All procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Experimental Animals* and were approved by the New York University Animal Care and Use Committee.

Stereotaxic Surgery. Rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), with supplementation as needed, along with buprenorphine–HCl (0.02 mg/kg, s.c.) for analgesia, and placed in a stereotaxic apparatus (David Kopf Instruments). Brain areas were targeted using coordinates (1). For behavioral pharmacology experiments, stainless steel guide cannulae (22 gauge; Plastics One) were lowered into position and secured to the skull using surgical screws and acrylic dental cement (Ortho-jet; Lang Dental Manufacturing Co.). Twenty-eight gauge dummy cannulae, cut to extend 0.5 mm from the guides, were inserted to prevent clogging. For amygdala experiments, cannulae were aimed at the lateral amygdala (LA) [from bregma: anterior/posterior (AP) −3.2; medial/lateral (ML) ± 5.3; dorsal/ventral (DV) −6.5 from skull for infusion 1.5 mm below the guide]. For intracerebroventricular (ICV) experiments, rats were implanted with single guide cannulae aimed at the right lateral ventricle (from bregma: AP −0.8; ML + 1.5; DV −3.4 from skull) for infusion 0.5 mm below the guide. For locus coeruelus (LC) experiments, double guide cannulae were used (from lambda: AP −0.8; ML ± 1.3, DV −5.3) for infusion 2 mm beyond guides. For pharmacological disconnection experiments, rats were implanted with one guide targeting the LC (from lambda: AP −0.8; ML +1.3, DV −5.3; 2-mm extension for infusion) and the other targeting LA (from bregma: AP −3.2; ML ± 5.4; DV −6.5 from skull; 1.5-mm extension for infusion). After surgery, rats were administered buprenorphine hydrochloride (0.02 mg/kg, s.c.) for analgesia and given 7–10 d to recover from surgery before behavioral manipulations.

For optogenetic experiments, virus was unilaterally injected into the perifornical hypothalamus (from bregma: AP −3.12; ML ± 1.3, DV −8.7) to a volume of 1 μL using a 10-μL Gastight Hamilton syringe (Hamilton Company) at a rate of 0.1 μL/min. Following viral infusion, a 20-gauge guide cannula was lowered to a position ∼2 mm above the LC (from lambda: AP −0.8; ML ± 1.3, DV −5.3) and secured to the skull using surgical screws and dental cement. Animals remained under quarantine in the BSL2 facility for 48 h following surgeries, after which they were returned to the vivarium. After 2–4 wk to allow for lentivirus infection and expression, animals were handled and subjected to behavioral conditioning or killed for slice physiology as described below.

Drug Preparation and Microinfusion. SB 334867 was obtained from Tocris and dissolved in 25% (wt/vol) β-cyclodextrin and 5–10% (vol/vol) DMSO in artificial cerebrospinal fluid (aCSF: 25 mM D-glucose, 115 mM NaCl, 24 mM Na_2PO_4, 3.3 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 25.5 mM $NaHCO_3$; pH 7.4) or 0.9% sterile saline. Concentrations were prepared for three dose groups: 0, 300 ng, 1.0 μg, or 5 μg/0.3 μL/side (5 μg/5 μL for ICV). TCS OX2 29 was obtained from Tocris and used at 5 μg/5 μL ICV in aCSF. For disconnection experiments, propranolol (± propranolol hydrochloride; Sigma-Aldrich) was dissolved in 0.9% sterile saline and administered at 1 μg/0.3 μL as in our previous study (2). For the drug infusions, internal infusion cannulae were attached to 10-μL Hamilton syringes via 0.015 × 0.043 × 0.014-in. polyethylene tubing obtained from A-M Systems. Tubing and syringes were backfilled with distilled water, and a small air bubble separated the water from the infusate. Rats were infused using an infusion pump (PHD 2000; Harvard Apparatus) that delivered drug at a constant rate of 0.1 μL/min for direct infusions or 2.5 μL/min for ICV infusions. Animals were allowed to move freely in their home cage during infusions. After infusion was complete, cannulae were left in place for an additional 1–2 min to allow drug diffusion away from the cannula tip. Following all ICV experiments, angiotensin II (50 ng/5 μL; Tocris) was infused to assess targeting. Only animals showing a rapid drinking response following infusion were included in the analysis (3).

Apparatus. For pharmacology experiments, rats underwent fear conditioning in 1 of 10 identical chambers (Rat Test Cage; Coulbourn Instruments) constructed of aluminum and Plexiglas walls, with metal stainless steel rod flooring that was attached to a shock generator (Model H13-15; Coulbourn Instruments). The chambers were lit with a single house light, and each chamber was enclosed within a sound isolation cubicle (Model H10-24A; Coulbourn Instruments). Long-term memory testing took place within a modified version of the context, with smooth black plastic flooring, mild peppermint scent, and a striped pattern on the Plexiglas door. An infrared digital camera, mounted on top of each chamber, allowed videotaping during behavioral procedures for subsequent behavioral scoring. A computer, installed with Graphic State 2 software and connected to the chambers via the Habitest Linc System (Coulbourn Instruments), controlled the presentation of stimuli during behavioral sessions. Apparati and procedures were slightly different for optogenetic experiments and are described in detail in a previous publication (4). Briefly, animals were trained in Med-Associates boxes without a houselight. All other conditions were similar to those described above.

Behavioral Pharmacology Procedures. Animals were allowed at least 1 wk of recovery following surgery. Animals were handled 1 d before training to minimize the stress of infusion. Drug infusions occurred 15–20 min before training, immediately after training (for testing consolidation) or 15–20 min before expression test. For disconnection experiments, propranolol was infused into LA simultaneously with SB 334867 infusion in LC. Vehicle-infused controls were included for both contralateral and ipsilateral animals and were combined for analysis due to no significant difference between groups ($P = 0.77$). Following infusion, cannulae were left in place for 1 min to allow for drug diffusion into the tissue. Conditioning procedures have been used extensively by our laboratory, and specific methods are detailed in available publications (5, 6). Briefly, following a 5-min acclimation period, animals received three conditioned stimulus (CS)–unconditioned stimulus (US) pairing in which the CS is a 30-s presentation of a 5-kHz, 80-db tone and the US is a standard, 1-s coterminating footshock (0.6 mA for ICV experiments, 0.7 mA for LC and LA experiments). Following training, animals were returned to their

home cage. Chamber floors, trays, and walls were thoroughly cleaned with water and dried between sessions. Twenty-four hours following training, animals were tested in a separate context (see Apparatus section) for long-term memory (LTM) (7). LTM test consisted of five CS presentations, and freezing time was quantified offline to assess threat (freezing) levels. Freezing behavior was blindly scored by an investigator and in some cases verified by other investigators. Data are presented as the mean percent of time freezing during all CS presentations. After experiments, animals were perfused with 10% formalin, and cannulae targeting was histologically verified as described (5, 6).

To confirm that our results were not due to shock reactivity in the LC or nearby sites, we assessed responses to increasing shock intensity following antagonism of OrxR1 in LC (8) (Fig. S1). For this experiment, 10 footshocks were delivered with increasing intensity (0.1–1 mA), each 1 s in duration with 30 s separating each trial. For quantification, responses to increasing shock intensity were assigned arbitrary numbers: 0 = unnoticed, 1 = noticed, 2 = flinch, and 3 = jump.

Channelrhodopsin Experiments. Procedures were similar to experiments described in a previous publication (4). The orexin neuron–specific lentivirus constructs pLV-Hcrt::ChR2-mCherry and pLV-Hcrt:: mCherry were generously provided by Luis De Lecea (Stanford University, Stanford, CA) (9), and the virus produced by the University of North Carolina Gene Therapy Center (Vector Core Services, Chapel Hill, NC). This virus effectively targets orexin neurons in rats (Fig. 4). Animals expressing a reporter virus (Hcrt::mCherry) controlled for channelrhodopsin (ChR) expression effects and animals expressing Hcrt::ChR-mCherry (with no light presentation) controlled for possible blue light effects. Animals were allowed 3–4 wk recovery following viral infusion and cannulation to allow for sufficient expression. Before training, a fiber optic cable (200 μm core diameter, 0.37 numerical aperture) fed through a cannula connector assembly (Plastics One) was inserted into the guide cannulae and extended 1.3–1.5 mm beyond the tip of the guide, placing the fiber ∼0.5 mm above the dorsal tip of the LC. The cable and connector assembly were painted with black nail polish or covered with black electrical tape to avoid possible conditioning effects to the flashing blue light. The fiber optic cable was attached to a 473-diode pumped solid-state laser (Laserglow Technologies), which output 20–30 mW from the tip of the fiber optic cable. A three CS–US pairing protocol was used where each CS consisted of a series of auditory pips (5-kHz tone pips at 1 Hz with 250 ms on and 750 ms off for 20 s) and the US was blue light laser stimulation (470-nm laser, 20 Hz, 10-ms pulse durations) combined with a weak, ∼0.5-mA footshock that coterminated with the last 2 s of the CS (4). Each trial was separated by a random intertrial interval (2–5 min). A weak training protocol was used to obtain lower baseline freezing levels (∼50%) and thus avoid ceiling effects on freezing levels. In another set of Hcrt::ChR2-mCherry–expressing animals, pretraining infusion of the OrxR1 antagonist SB 334867 (1 μg/0.3 μg) or vehicle preceded conditioning by 20 min. Twenty-four hours following training, animals were placed in a novel context (Apparatus section), and following a 3-min acclimation period, animals were exposed to five CSs (5-kHz tone pips at 1 Hz with 250 ms on and 750 ms off for 20 s) with a random intertrial interval no longer than 5 min.

Procedures for c-Fos detection are similar to our previous study (4). Briefly, animals were handled for 3 d before light stimulation to reduce baseline c-Fos levels. Ninety minutes following stimulation, animals were perfused as described below for histological processing.

Histology and Immunohistochemistry. Following behavior experiments, animals were overdosed with 25% chloral hydrate and transcardially perfused with either 10% formalin for histology to assess cannula placement or 4% paraformaldehyde in 0.01 M PBS for immunohistochemistry. Tissue processed for cannula placement was postfixed in 10% formalin or 4% paraformaldehyde at 4 °C until prepared for histological staining. For immunohistochemical processing, tissue was cryoprotected in a 30% sucrose–4% paraformaldehyde solution (in 0.01 M PBS) at 4 °C for at least 1 d and then stored in 0.01 M PBS. Brains were blocked coronally and cut on a freezing microtome. For histological verification of cannula targeting, tissue was cut at a thickness of 50 μm and kept in 0.01 M PBS until mounted on gelatin-coated slides and dried overnight. After standard histological Nissl staining and coverslipping, sections were examined on a light microscope for injector tip localization into LC or LA. Only data from rats with bilateral injector placements localized to the LC and LA were included in the study.

For immunohistochemistry, tissue was cut at 35 μm. Before antibody incubation, floating tissue was rinsed with agitation three times in 0.01 M PBS and blocked in 1% BSA in 0.01 M PBS for 1 h at room temperature. Immunohistochemical detection was achieved in primary antibody solutions containing 1% BSA, 0.2% Triton-X 100, and 0.02% NaAz. Sections were incubated overnight at room temperature in rabbit anti-dsRed (for detection of mCherry; 1:500; Clontech Laboratories) (10) and mouse anti–orexin-A (1:500; R&D Systems) antibodies for verification of targeting area and cell specificity of viral expression (11). For c-Fos experiments, LC sections were incubated in mouse anti-dopamine beta hydroxylase (DBH) (1:2,000; EMD Millipore) (12) and rabbit anti–c-Fos (1:5,000; Calbiochem) (13). Following primary antibody incubation, sections were rinsed with agitation three times for 5 min in 0.01 M PBS and incubated in Alexa-488 or -594 secondary antibody (Invitrogen) in 0.01 M PBS. Sections were rinsed three times for 5 min in PBS, mounted on gelatin-coated slides, and allowed to dry for several hours, followed by a brief wash in ddH_2O to remove excess salt (PBS), coverslipped in aqueous mount (ProLong Gold Antifade Reagent; Invitrogen), and allowed to cure overnight at room temperature before fluorescence imaging. For all experiments, animals were excluded from analysis if virus or cannulae targeting was outside the areas of interest.

Slice Preparation and Whole-Cell Recordings. We prepared 260- to 280-μm-thick acute coronal slices including the hypothalamus and 260- to 280-μm-thick horizontal brainstem slices including the LC from adult rats (>7 wk of age) expressing either the ChR2 construct or mCherry in orexinergic cells as described above. For LC experiments, we prepared acute horizontal brainstem slices to maximize synaptic connectivity between incoming fibers and dendritic arborizations of the LC cells. Slices were made at least 4 wk after injection of the virus to ensure adequate expression of ChR2 in axonal fibers for photoactivation within the slice preparation. Because the LC is a small structure, reliable responses could be obtained from only one high-quality slice per preparation. Although the amplitude of maximal depolarization varied from slice to slice, all four cells showed similar glutamatergic and orexinergic contributions to the fast and slow components of light-evoked synaptic responses. Animals were deeply anesthetized with 75–100 mg/kg ketamine + 10 mg/kg xylazine, and after confirmation of anesthesia, were transcardially perfused with ice-cold (4 °C) oxygenated slicing solution containing (in mM): 85 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 7 $MgSO_4$, 0.5 $CaCl_2$, 25 glucose, and 75 sucrose. Animals were decapitated, and their brains removed immediately after perfusion, and brains were immersed in the same ice-cold slicing solution continuously bubbled with carbogen (95% O_2 and 5% CO_2). After at least 5 min, the portions of the brain containing the hypothalamus and LC were blocked separately. One area was sliced at a time, whereas the other was maintained

in oxygenated ice-cold slicing solution, and slices were prepared using an OTS-5000 tissue slicer (Electron Microscopy Sciences). Slices were then transferred to a heated chamber (32–33 °C) and maintained submerged in a continuously oxygenated aCSF containing (in mM) 125 NaCl, 3.3 KCl, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 2 $CaCl_2$, 15 glucose, 0.01 D-serine, and 0.5 ascorbic acid. Slices were maintained at the heated temperature for 30–45 min and then kept at room temperature. We conducted all recordings at 32 ± 0.5 °C in the same aCSF used to maintain slices.

Whole-cell current clamp recordings were conducted in orexinergic projection cells of the dorsomedial hypothalamus (identified by fluorescence) or from principal cells of the LC on an upright Olympus BX51-W1 microscope. The LC was readily identified under IR-DIC imaging as a lucid area near the fourth ventricle containing large, densely packed cell bodies (14, 15). All recordings were obtained using 4- to 7-MΩ borosilicate glass pipettes filled with an internal solution containing (in mM) 136 potassium gluconate, 4 $MgCl_2$, 1 EGTA, 0.1 $CaCl_2$, 4 Na-ATP, 0.3 Na-GTP, 10 Hepes, and 15 μM Alexa Fluor-594 (pH 7.3; 291 mOsm). Orexinergic projection cells in the dorsomedial hypothalamus exhibited spontaneous firing and a resting membrane potential of approximately −50 mV and were held at −60 mV. Noradrenergic cells of the LC were characterized by low-frequency spontaneous firing (16, 17) and a depolarized resting membrane potential (approximately −45 mV) and were held at −55 mV. Reported potentials are uncorrected for a calculated liquid junction potential of 13 mV (PClamp Liquid Junction Calculator).

Photoactivation in acute slices was achieved using a 470-nm mounted high-power LED (Thorlabs) coupled to the microscope using a customized Siskiyou beamsplitter cube containing a sliding mirror to bring the LED beam in and out of the light path. Maximal light power was calculated as 4 mW after the 40× objective used for all recordings. For optically elicited synaptic responses in the LC, we delivered a 500-ms, 20-Hz train of light stimulation (10-ms pulse duration) every 30 s, interleaved with sweeps where no light was delivered. Values in baseline and drug conditions were calculated based on the average of the last five sweeps in each condition.

Data Acquisition for Slice Physiology. All electrophysiological data were acquired and analyzed using PClamp software (Molecular Devices). Signals were acquired using an AxoClamp 2B amplifier, digitized through a Digidata 1440A at a sampling rate of 25 kHz, and filtered online at 10 kHz. The fast component of light-activated transmission onto LC cells was measured as the maximal amplitude of the first excitatory postsynaptic potential (EPSP), measured in the initial 50 ms after the first light onset of a train. The slower component of light-activated depolarization onto LC cells was measured as the area under the response, in a window beginning at EPSP onset and ending 700 ms later. We conducted statistical analyses in Matlab (Mathworks) using standard resampling methods, including the commercially available Matlab Resampling Package.

Statistical Analysis. All data used in parametric tests were plotted and visually found to be compatible with the assumption of normal distribution. For behavioral data, a Student t test was used to analyze freezing levels when comparing two groups of averaged data, and one-way ANOVA was used for comparing more than one group followed by a Tukey's multiple comparison test. Bartlett's test for equal variances was used for one-way ANOVAs (18), whereas the F test was used for the Student t test to confirm that variances were not significantly different in compared groups (GraphPad Software). To analyze US sensitivity data, a repeated-measures ANOVA was used to compare freezing between vehicle and drug-treated groups and was followed by Bonferonni post hoc tests. Error bars in all figures represent ±SEM. Data were analyzed using GraphPad Prism.

For c-Fos experiments, to meet the assumption of equal variance across groups, the data were transformed using the equation $Y = \log(X + 1)$. A two-way ANOVA was subsequently used, followed by the Bonferonni posttest.

For electrophysiology experiments, we performed one-way ANOVAs and paired comparisons using resampling methods. Resampling was used because of the low sample size and inequality of variance due to low slice yield per animal and was intended to minimize the number of animals required for the experiment. Briefly, an F-statistic was calculated from the experimental data for the variable of interest, using the absolute value of the sum of errors. All data were then pooled and used to generate randomized data sets (100,000 iterations).

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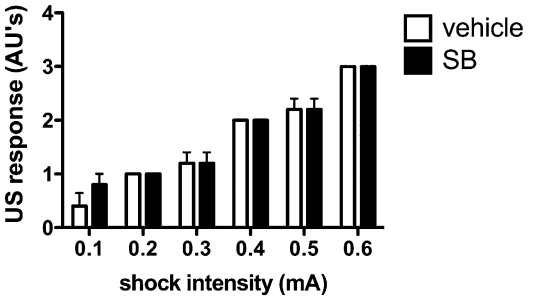

Fig. S1. SB 334867 in LC does not influence shock sensitivity. Amplitude was increased by 0.1 mA every 30 s from 0.1 to 1 mA. Responses were maximal by 0.6 mA [n = 5/group, two-way repeated-measures ANOVA; no significant interaction, $F(5,40) = 0.678$, $P = 0.64$; no main effect for drug treatment, $F(1,40) = 0.421$, $P = 0.53$; main effect of shock intensity, $F(5,40) = 80.81$, $P < 0.001$].

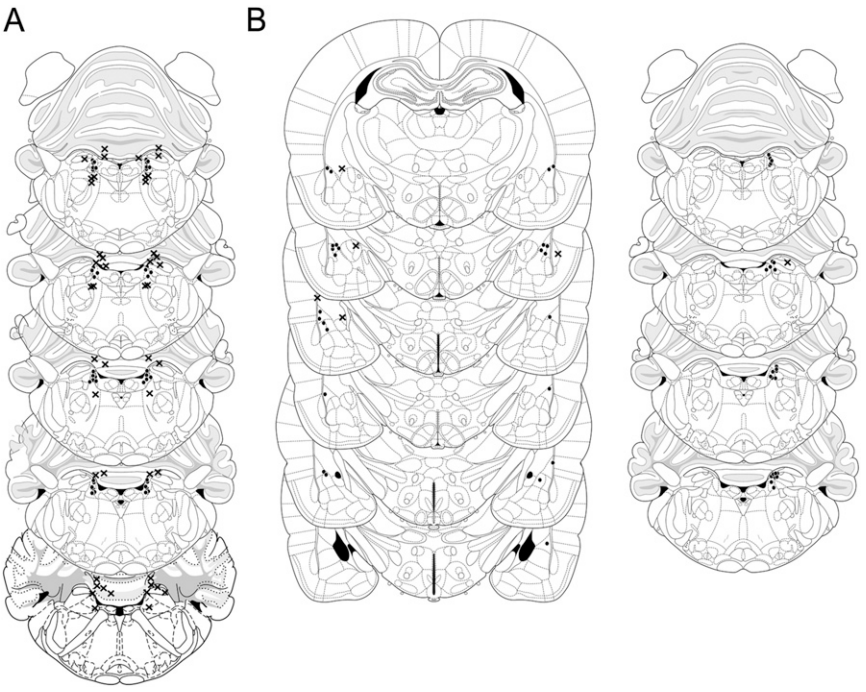

Fig. S2. Coronal rat sections depicting cannulae placement for microinfusion experiments. (*A*) Placements for bilateral LC infusion experiments. (*B*) Placements for disconnection experiments. Hits are denoted as black circles; misses are demarcated with an X.

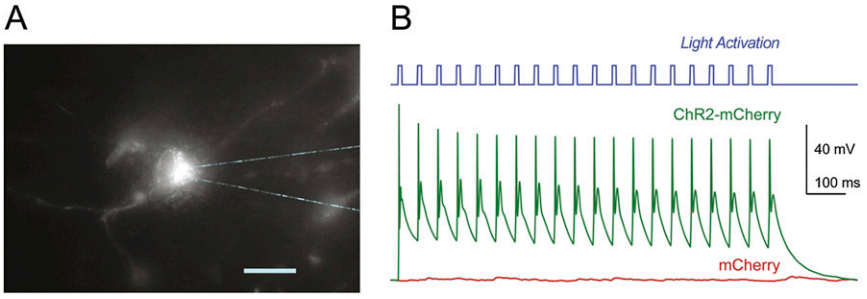

Fig. S3. Blue light activates neurons expressing ChR2. (*A*) Wide-field fluorescent image of a ChR2-expressing orexin cell before patching. (*B*) Robust spiking from a sample orexin cell (green) and no response from a control cell expressing mCherry alone (red) in response to 470-nm illumination. (Scale bar, 20 μM.)

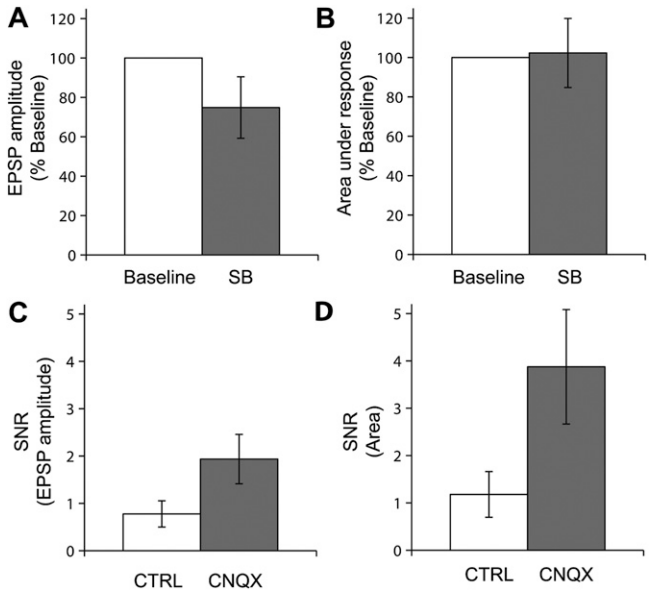

Fig. S4. (*A*) Bar plot indicating the effect of SB 334867 on initial EPSP amplitude ($n = 4$ cells from four animals). (*B*) Bar plot indicating the effect of SB 334867 on area under synaptic depolarization. (*C*) Signal-to-noise ratio (SNR) of the mean orexin contribution to EPSP amplitude (14%) relative to the SD of baseline responses in the presence (SD = 10 ± 4%, SNR = 1.94 ± 0.5) or absence (SD = 26 ± 7%, SNR = 0.78 ± 0.28) of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). (*D*) SNR of the orexin contribution to the area under the response (21%) relative to the SD of the area in the presence (SD = 8 ± 3%, SNR = 3.88 ±1.2) or absence (SD = 28 ± 8%, SNR = 1.18 ± 0.48) of CNQX.