Supplemental Experimental Procedures

Animals

Pathogen-free, adult, male Sprague-Dawley rats (300-325 grams, Harlan) were individually housed and had free access to food and water. All animals were housed under controlled conditions (12 hr light starting at 7 am, 100 lux) in an isolated ventilated chamber maintained at 20-22 ºC. All protocols were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Surgery

Rat were anesthetized with ketamine/xylazine (100mg/kg and 10 mg/kg, respectively, i.p.) and then placed on a stereotaxic apparatus. To express the hM3Dq receptors in the neurons of PB, a burr hole was made and a fine glass pipette (1 mm glass stock, tapering slowly to a 10-20 micron tip) containing recombinant AAV_{10} carrying Ef1α::hM3Dq-mCherry (1-2 $\times 10^{12}$ infectious particles/ml) was lowered to the PB (AP = -9.0 mm, ML= ±2.0 mm, DV = -5.2 mm) bilaterally, as per the atlas of Paxinos and Watson [S1]. A total of 300 nl virus was delivered over a 5 min period per hemisphere via nitrogen gas pulses of 20-40 psi using an air compression system previously described [S2]. After 2 additional minutes, the pipette was slowly withdrawn. For testing the roles the specific PB projections in wake promotion, we injected AAV_6-CAG-Cre (150 nl, titers at 1-2 × 1012, AAV6 = AAV2/6: AAV serotype 2 genome packaged in the serotype 6 capsid) , which is taken up by the axonal terminals and retrogradely transported to the neuronal bodies [S3, S4], into lateral preoptic area (AP = -0.24 mm, ML= ±1.0 mm, DV = -8.2 mm), posterior lateral hypothalamus (AP = -3.72 mm, ML= ±1.3 mm, DV = -8.0 mm) and thalamus (AP = -3.12 mm, ML= ±1.0 mm, DV = -6.2 mm), respectively. And then we placed bilateral injections of recombinant AAV_{10} carrying hM3Dq-mCherry (AAV_{10}-hSyn-DIO-hM3Dq-mCherry) into the PB, which expression of hM3Dq receptor is dependent on the presence of Cre recombinase. Following these injections, rats were implanted with 4 screw electrodes for recording EEG (2 on the frontal bone and 2 on the parietal bone) and 2 wire electrodes on to the nuchal muscles for recording EMG. The other ends of the electrodes were connected to a 6-pin pedestal (Plastics One, USA) that was then secured on to the skull using dental cement. For postoperative care, mice were injected intraperitoneally with meloxicam (0.5 mg per kg). All injection sites were verified by immunohistochemistry. The 'misses' or 'partial hits' ones were excluded from data analyses.

Sleep-wake recording and analysis

After surgical procedures, rats were allowed to recover in individual housing for at least three weeks. The animals were then transferred to the recording room and habituated to the recording cables and conditions for 2 days. Following this habituation period, EEG/EMG activity from the beginning of the light period (7 a.m.) was recorded (AM systems, USA) from all the rats. The cortical EEG and EMG signal were amplified, digitized at a sampling rate of 256 Hz, and recorded using VitalRecorder (Kissei Comtec, Nagano, Japan). The behavior of the animals was recorded simultaneously with time-locked video recordings. EEG/EMG were filtered (EEG, 0.5-40 Hz band-pass; EMG 10 Hz high-pass) and automatically scored offline in 10-sec epochs as wake, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep in SleepSign (Kissei Comtec, Nagano, Japan) using established criteria . After automatic scoring, sleep-wake stages were examined and manually corrected. The amount of time spent in wake, NREM and REM sleep was determined from the scored EEG/EMG data. EEG power spectra for each epoch were analyzed offline using Fast Fourier Transformation (512 point, Hanning window, 0–24.5 Hz with 0.5 Hz resolution using SleepSign).

CNO injections

Clozapine N-oxide (CNO, 0.2 mg/kg, C0832, Sigma) in saline was injected by i.p. at 9 a.m., one

group rats was performed a single CNO injection, another group of rats was performed four consecutive days injection of CNO. For baseline data, rats were injected i.p, with saline vehicle (2ml/kg) at 9 a.m.

Body Weight Measurements, Blood Collection and Analysis:

Prior to EEG/EMG/video recording, and perfusion 4 hours after 4th CNO injections, body weight was measured. From each animal, approximately 5 ml of blood was obtained from the right atrium of each animal and stored in tubes containing heparin. Blood samples were then centrifuged at 5,700 rpm for 5 min at 4 ^{o}C. The plasma was transferred to polycarbonate tubes and frozen for further determination of plasma levels of metabolic parameters: insulin, cholesterol, TG, and leptin. All these assays were performed at the Specialized Assay Core Facility, Joslin Diabetes Center, Boston, USA using commercially available kits. ELISA kits for insulin (Cat. No. INSKR2010; Crystal Chem Inc, IL, USA), leptin (Cat. No. 90030; Crystal Chem Inc, IL, USA) were used for their respective assays. TG and cholesterol were determined by enzymatic and colorimetric methods (TG kit: Cat. No. 2200-430; Stanbio Laboratory. Cholesterol kit: Cat. NO. 1010-430; both are from Stanbio Laboratory TX, USA). Blood glucose was tested using test strips and a meter from Nipro Diagnostics, Inc, FL, USA (TRUEtrack).

Perfusion and ***immunohistochemistry***

For the single CNO injection group, after sleep-wake recording, the rats were returned to their normal housing room for another week, then the animals received CNO (0.2 mg/kg, i.p., 9 a.m.) and 4 h later they were deeply anesthetized with 7% chloral hydrate and perfused via the heart with saline followed by neutral phosphate buffered formalin (Fisher Scientific Co.) For the 4th CNO injection group, the rats were perfused at 1:00 pm on the 4th CNO injection day (4 h after the injection). The brains were harvested, post-fixed, and cryoprotected in 20% sucrose in PBS overnight, then sectioned in the coronal plane on a freezing microtome into 4 series of 40 μm sections.

Immunohistochemistry was performed in accordance with the free floating method described previously [S5]. Two series of sections were first processed for anti-c-Fos staining, briefly, sections were incubated with 0.3% H_2O_2 for 15 min to quench endogenous peroxidase activity. After washing in 0.1M PBS (pH 7.4), the sections were incubated with a rabbit polyclonal primary antibody against c-Fos (Ab5, Cat# PC38, Oncogene Research Products) at a 1:10000 dilution in PBS containing 0.25% Triton X-100 for 24 h at room temperature. On the second day, the sections were washed in PBS and incubated in biotinylated donkey anti-rabbit secondary antiserum (Jackson ImmunoResearch Laboratories, PA, USA; 1:1000 dilution) for 1 h, followed by a 1:1000 dilution of avidin-biotin-peroxidase (Vector Laboratories, CA, USA) for 1 h at room temperature. The peroxidase reaction was visualized with 0.05% 3, 3-diaminobenzidine tetrahydrochloride (Sigma, MO, USA) in PBS and 0.01% H_2O_2 and strengthened with 0.002% Ni, 0.001% $CoCl_2$. Then one of the two series was double stained with a rabbit polyclonal primary antibody against dsRed (Cat# 632496, Clontech, CA, USA) to value the AAV-M3-mCherry injection site and mCherry expression. Another series of sections were double stained with goat anti-choline acetyltransferase (ChAT, 1:1000, ab144, Chemicon), goat anti-Orexin A (1:1000, Cat# sc-8070, Santa Cruz Biotechnology), or mouse anti-tyrosine hydroxylase (TH, 1:50000, Cat#22941, Diasorin). For the PB-pathway experiment, one series of sections were stained with rabbit anti-Cre (1:5000, Cat# 69050-3, Novagen) immunohistochemistry to verify the injection locations of AAV_6-Cre. The procedures were performed as described above but without Ni and $CoCl_2$ strengthen at the last step for peroxidase reaction. After terminating the reaction by PBS-Azide, sections were mounted, dehydrated and cover slipped. As controls, adjacent sections were incubated without the primary antibody to confirm that no non-specific staining had occurred.

For immunofluorescence labeling, sections were blocked with 10% normal donkey serum/0.25%

Triton-X-100 in PBS for 1 h at room temperature and then incubated overnight at room temperature with the rabbit anti-c-Fos (1:5000, Ab5, Cat# PC38, Oncogene Research Products) diluted in blocking buffer, after washed three times with PBS, the sections were then incubated for 2 h at room temperature with Alexa Fluor-488 conjugated donkey anti rabbit secondary antibody (1:500, Cat# A21206, Molecular Probes, Invitrogen), following additional washes in PBS, sections were mounted to glass slides and cover slipped with VECTASHIELD mounting medium (Cat# H-1000, Vector Laboratories, CA, USA). Fluorescence images were captured with Olympus VS120 slide scanner microscope.

Cell counting

To evaluate the activity of the VLPO sleep center and wake-related nuclei, c-Fos positive neurons were counted in the VLPO, LC, TMN and PZ, respectively, and the number of c-Fos/orexin-positive and c-Fos/ choline acetyltransterase (ChAT)-positive neurons were counted in a 1.2 mm x 0.6 mm box. Cell counting was performed on three adjacent sections (separated by 160 μm) on both sides of the brain, the average counting per section per side was used to represent the data. $Orexin^+$ neurons activity in LH and $ChAT^+$ neurons activity in the horizontal diagonal band nucleus (HDB) were scored as the percentage of double-labeled cells per animal.

Statistical Analysis

The quantitative data were presented as the mean ± standard error of mean (Kaur et al.). Statistical significance was assessed with the paired t-test, with $P < 0.05$ taken as the threshold of significance.

Supplemental Reference

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S3. Towne, C., Raoul, C., Schneider, B.L., and Aebischer, P. (2008). Systemic AAV6 delivery mediating RNA interference against SOD1: neuromuscular transduction does not alter disease progression in fALS mice. Mol Ther 16, 1018-1025.

S4. Towne, C., Schneider, B.L., Kieran, D., Redmond, D.E., Jr., and Aebischer, P. (2010). Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6. Gene Ther 17, 141-146.

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