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Supplementary Information for

The hypothalamic link between arousal and sleep homeostasis in mice

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Other supplementary materials for this manuscript include the following:

Materials and Methods

Mice

The experiments were performed in male and female Gad2-IRES-Cre mice (Jackson Laboratory 019022; B6N.Cg-*Gad2*$^{tm2(cre)Zjh}$/J). Optogenetic photoactivation experiments were performed on adult mice (2-9 months of age when surgery was performed, LPO: 124 days ± 16 SEM, nonLPO: 107 ± 27, GFP 135 ± 14, one-way ANOVA, p=0.5879), 19.5-37.4 g of body weight (LPO: 26.6 g ± 0.9 SEM, nonLPO: 26.8 ± 2.5, GFP 27.0 ± 2.0, one-way ANOVA, p=0.9832). Each group included at least 1 female mouse (LPO, n=1, nonLPO, n=2, GFP, n=2). Optogenetic inhibition experiments were performed on adult mice (2-7 months of age when surgery was performed, Arch: 164 days ± 23 SEM, GFP 79 ± 9, one-way ANOVA, p=0.08), 18.6-32.4 g of body weight (Arch: 25.5 g ± 1.7 SEM, GFP 25.5 ± 3.8, one-way ANOVA, p>0.9999). The number of male and female mice was the same in each group (Arch: 3 males, 3 females, GFP: 2 males, 2 females). Mice were singly housed with food and water available *ad libitum* under a 12-hour:12-hour light-dark cycle (lights on 9:00, ZT 0, and off at 21:00, ZT 12, light levels 120–200 lux). Room temperature and relative humidity were maintained at 21.4 ± 1.4°C and 58 ± 20%, respectively. All experimental procedures were performed under UK Home Office Project License #P828B64BC in accordance with Animal (Scientific Procedures) Act 1986 and the guideline of the University of Oxford.

Virus strains

AAV5-EF1a-DIO-ChR2(H134R)-EYFP (titer 1.772e13), AAV1-EF1a-DIO-EGFP (titer 2.96e13), rAAV2-EF1a-DIO-eArch3.0-EYFP (titer 2.6e12) were obtained from UNC vector core.

Device and surgery

For chronic electroencephalogram (EEG) and electromyogram (EMG) recordings, we used custom-made headmounts composed of three stainless-steel screw electrodes (Fine Science Tools) and two stainless-steel wires attached to an 8-pin surface mount connector (8415-SM, Pinnacle Technology Inc.), as previously described (58). All surgeries were performed using aseptic surgical technique and body temperature was monitored and maintained throughout surgical procedures. Analgesics were provided peri- and post-surgery (Buprenorphine and Meloxicam) and animals were monitored closely post-surgery. For *in vivo* optogenetic experiments, the animals were anesthetised with isoflurane (3-5% for induction and 1.5-2% for maintenance) and placed in a stereotaxic frame. Bregma and Lambda were adjusted to be horizontally aligned. The virus was loaded into a Hamilton syringe attached to a 32-gauge needle and injected slowly (50-100 nL/min) using an infuse/withdrawal pump aiming for the left lateral preoptic area (LPO: AP 0, ML 0.7 mm, DV 5.4–5.7 from Bregma) or left hypothalamic area posterior to LPO (nonLPO: AP -0.1, ML 0.7 mm, DV 5.4–5.7 from Bregma). For photoactivation, all animals in all groups were injected with 400 nL of virus, except for 4 mice in the LPO group for optical, which were injected with 150 nL. For photoinhibition, all animals in all groups were injected with 200 nL of virus. For optical stimulation, either an optic fiber of 400 um diameter (either 0.48 NA, Doric Lenses Inc., or 0.50 NA, Thorlabs) or a custom made optrode, consisting of an optic fiber glued with tungsten wires, was inserted at 0.2 mm above the virus injection site. The optrode was implanted in one single animal in an attempt to confirm that stimulation leads to local changes in neural activity; however, as behavioral effects were obvious, we opted against optrode implantation to minimise damage to the tissue. For EEG recording, two screws were fixed into the skull above the right frontal cortex (AP +2, ML +2 from Bregma) and the right occipital cortex (AP -3.5, ML +2.5 from Bregma) and a reference screw was inserted into the skull above the cerebellum (-1.5 mm posterior from Lambda, ML 0). For EMG recording, two wire electrodes

were inserted into the neck muscle. The optic fiber, screws and EMG electrodes were secured to the skull using non-transparent dental cement (Super-bond) to minimise the leaking of light during optical stimulation. Data from animals in which either the implantation of the optical fiber (i.e., fiber penetrated through the hypothalamus) or the virus transfection was not successful were excluded from the analyses (2/10 in LPO group and 3/9 in nonLPO group).

Electrophysiological recordings

4 to 7 weeks post virus injection, animals were individually housed in a clear Plexiglas home cage, placed inside a sound-attenuated recording chamber (maximum 2 cages per chamber). After habituation to the recording cage for at least 2 days, the EEG/EMG recording device mounted on the mouse skull was connected to flexible recording cables, and a patch cord was connected to the optic fiber implanted on the skull. Recordings started after at least 1 day of habituation to the cables. For data acquisition, a TDT RZ2 (Tucker-Davis Technologies) recording system was used. The EEG/ EMG signals were digitized at 305 Hz and amplified with a PZ5 amplifier (Tucker-Davis Technologies) and filtered with a 128 Hz low-pass filter.

Optogenetic manipulations

To examine the involvement of hypothalamic GAD2 neurons in vigilance state switching and homeostatic regulation, blue LED stimulation (470 nm, 10.8–13.2 mW at fiber tip) of 10 ms per pulse was applied at various frequency and train durations (Fiber-Coupled LED: Thorlabs). For stimulation across 24 hours, we stimulated at 20, 10, 5, 2 or 1 Hz, for a 2-minute train duration, with a 20-minute inter-trial interval with a jitter of 10% (i.e. 2-min stimulation every 18-22 minutes) (Fig. 1, 4, 5A-C, S1, S2, S4-S7). During baseline days, no stimulation was given. Sham stimulation days (sham) were identical to a baseline day, but for analysis the timing of 2 min for sham stimulation episodes was taken from a day with 10 Hz stimulation. To examine the effects of sleep pressure on awakening latency (Fig. 3D-G), the effects of stimulation after 4-h of undisturbed sleep were compared to 4-hour of sleep deprivation (SD) starting from light onset (ZT0). In the high sleep pressure (HSP) condition, SD was performed by providing novel objects such as paper towels, wood pieces, Styrofoam and cardboard, and also by gentle cage tapping if necessary. At ZT4, all animals were left undisturbed and after 10-20 minutes (to allow the animals to fall asleep) photostimulation was started. Stimulation consisted, as above, of, 2-min long stimulation sessions at 10 Hz, delivered every 20 minutes across 4 hours (12 stimulation sessions in total). In the low sleep pressure (LSP) condition, the animals were undisturbed between ZT0-4 and stimulation was performed during the corresponding 4-hour period between ZT4-8, as in the high sleep pressure condition.

In the dexmedetomidine sedation experiment (Fig. 3A-C, Fig. S3), dexmedetomidine (Dex), a selective alpha2-adrenoceptor agonist (DEXDOMITOR, Zoetis), was diluted with saline and injected s.c. (100 µg/kg) at ~ZT1. EEG, EMG and peripheral body temperature (see below) were continuously recorded before and after injection. Typically, within 2-3 minutes after Dex injection, the EEG started to show high amplitude, synchronised slow waves, and within approximately 5 minutes the animals became immobile. Once fully sedated (typically after 10 min) optical stimulation was commenced as above (10 Hz, 2-min long stimulation sessions) every 15 minutes across 4 hours. Note that stimulation during sedation was more frequent than the previously used 20 min inter-trial intervals to examine more trials under sedation. For examining behaviour during stimulation upon sedation (Suppl. Movie 2), 5-min stimulation sessions were performed every 15 minutes over a 2-hour period. All animals were injected with Atipamezole (ANTISEDAN, Zoetis) 4 hours after the Dex injection, which induced recovery from sedation.

To examine the effect of stimulation during waking on subsequent recovery sleep (Fig. 5D, E), trains of stimulation at 10 Hz for 20 seconds were applied every 2 minutes during a 2-hour

sleep deprivation protocol. Sleep deprivation was achieved by providing novel objects. As in other experiments, novel objects included paper towels, wood pieces, Styrofoam, cardboard, bedding etc. Stimulation was stopped 10 minutes before the end of sleep deprivation (55 stimulation sessions over a 2-h period in total). In a subset of animals, continuous stimulation at 10 Hz was performed for 1 hour, starting at ZT0 or ZT12.
For continuous stimulation (Fig. S8) we performed 10 Hz photostimulation for 1 hour starting at ZT0 for the light period condition and at ZT12 for the dark period condition.
To examine the suppression effect of GAD2 neurons in LPO on vigilance state switching and homeostatic regulation, green LED stimulation (550 nm, 18.8–24.0 mW at fiber tip) of continuous pulse was applied at various frequencies and train durations (Fiber-Coupled LED @ MINT: Thorlabs). For stimulation over 24 hours, we delivered 5-minute continuous pulses every 30 minutes with a jitter of 10% (48 times in total). To prevent the burst activity at the stimulation offset, the intensity of photostimulation was ramped down for approximately 30 seconds from stimulation offset. On sham stimulation day, the timing of 2 min for sham stimulation episodes was taken from a day with stimulation. To examine the effect of stimulation during waking on subsequent recovery sleep, 2-min continuous stimulation with ramp-down at stimulation offset were applied every 5 minutes during a 2-hour sleep deprivation protocol. Sleep deprivation was achieved by providing novel objects as described above. Stimulation was stopped 10 minutes before the end of sleep deprivation, resulting in 22 stimulation sessions delivered in total.

Peripheral body temperature and metabolic rates

In a subset of animals and wherever body temperature measures are reported (Fig. 3, S3, S8), skin temperature was measured using infrared thermal imaging cameras Optris Xi 70 and Xi 80 (Optris GmbH, Berlin, Germany) attached to the ceiling of the recording chamber. The change in skin temperature was calculated by taking the maximum value over 4 seconds and averaging over 30 minutes using a moving average. In two non-implanted mice in a separate experiment, metabolic rates were measured using indirect calorimetry, CaloBox (PhenoSys, Berlin, Germany) with a sampling rate of approximately 4 seconds (Fig. S3B, C). The animals were single housed in standard IVC cages and O_2 consumption, CO_2 production and water vapor were measured. On the experimental day, after baseline metabolic rates were recorded between ZT0-3, the animals were taken out of the IVC cage and injected with Dexmedetomidine (100 µg/kg in saline, s.c.). Immediately after the injection, the animal was returned to their homecage and the metabolic rates were monitored for the subsequent 4 hours. At around ZT7, the lid of the IVC cage was briefly opened to administer Atipamezole and the metabolic rates were monitored until the animal was fully recovered. Reference air was analyzed for 1-minute every 15 minutes and measurements during these intervals were excluded from the analyses.

Vigilance state scoring

Vigilance states were scored manually by visual inspection of electrophysiological signals using SleepSign software (Kissei Comtec). In most cases the scorers were not blinded to the genotype of the animal. For scoring, recordings were resampled at 256 Hz with custom MATLAB (MathWorks) scripts and the data format was transformed to edf format, for compatibility with SleepSign. EEG and EMG traces were displayed simultaneously on the screen, to allow visualisation of state sequences and transitions. Wakefulness was defined by high EMG and low amplitude of the EEG signal, which was dominated by theta (6-9 Hz) activity, especially in the occipital derivation. During NREM sleep, the EEG amplitude was increased, and slow waves (0.5-4Hz) and spindle events (10-15 Hz) were prominent, while the EMG signal was low. During REM sleep, EEG resembled signals during wakefulness, but EMG tone was low. In all cases, REM episodes were initiated from NREM sleep state, characterised by frequent spindles, and

terminated by a brief awakening. After scoring, EEG power spectra were computed every 4-sec by fast Fourier transformation (Hanning window, 0.25 Hz resolution).

To compute awakenings based on EMG variance we computed a histogram of EMG variance across a 12h recording session (in 250ms-long bins) for each mouse and separated out NREM and wake occurrences (Fig S1D). Based on this, we computed a likelihood ratio that an animal is awake, given a specific EMG variance value. This ratio was then used in each mouse to determine the EMG variance threshold value above which an animal was considered awake. The specific value of this ratio we used was 2; however, the results are very robust towards other values. In order to score an awakening, a mouse had to cross the EMG variance threshold for at least 4 seconds.

Histology

At the end of the experiment, mice were deeply anaesthetised and transcardially perfused with 0.1M PBS followed by 4% PFA in PBS. For fixation, brains were placed in 4% PFA for at least 24 hours and then placed in PBS with 0.05% sodium-azide. For sectioning, brains were placed in 30% sucrose in PBS for at least 3 days for cryoprotection and were then sectioned into 50 µm coronal slices using a microtome. Slices were placed on glass slides and mounted in VECTASHIELD HardSet with DAPI to stain for DNA (H-1500, Vector Laboratories). Fluorescence images were taken using a confocal microscope (Olympus Fluoview FV1000).

Slice recording

Ex vivo experiments were conducted 5-10 weeks after bilateral injection of AAV5-EF1a-DIO-ChR2(H134R)-EYFP into LPO. Acute slice preparation was performed as described previously (91). Briefly, mice were deeply anesthetised using isoflurane and rapidly dislocated and decapitated into ice cold carbogenated NMDG solution containing (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 5 Na-ascorbate, 2 thiourea and 3 Na-pyruvate, 10 MgSO4 and 0.5 CaCl2, titrated to pH 7.2-7.4 with HCl. Coronal slices (300 µm) were cut using a vibratome (Leica Vibratome VT1200S) in ice cold NMDG solution. Slices including the LPO were recovered in NMDG solution for 10 min at 30-34 °C, and then placed into an interface chamber in carbogenated aCSF containing (in mM) 126 NaCl, 3.5 KCl, 1.25 NaH2PO4, 1 MgSO4, 2 CaCl2, 26 NaHCO3, and 10 glucose at pH 7.2-7.4 when bubbled with carbogen gas (95% O_2, 5% CO_2), at RT for 1-6 h, before being transferred to the recording chamber (aCSF at 32-34°C).

For whole-cell patch-clamp recordings, recording pipettes (standard borosilicate glass capillaries, 1.2 mm OD, 0.68 mm ID, WPI) of 3-8 MΩ resistance were filled with intracellular solution containing: 110 mM potassium gluconate, 10 mM HEPES, 4 mM NaCl, 0.2 mM EGTA, 2 mM MgATP, 0.3 mM GTP-NaCl, 8 mM phosphocreatine-bisodium, 20 µg/ml glycogen (Life-Tech, R0551), 0.5 U/µl recombinant RNase inhibitor (Takara, 2313A) and 4 mg/ml biocytin and adjusted to pH 7.2 with KOH (290-300 mOsmol l^{-1}). This intracellular solution is modified to allow for single cell RNA sequencing (92, 93).

Current-clamp recordings were carried out using an Axon Multiclamp 700B amplifier (Molecular Devices) low-pass filtered at 3 kHz and digitised using an Axon Digidata 1440A or Instrutech ITC-18 at 10 kHz. Data acquisition and stimulation protocols were controlled using Axon pClamp or custom-written procedures in IgorPro (WaveMetrics). The input resistance was determined by a hyperpolarising 20 pA step for 300 ms in each sweep. To activate ChR2, blue light was delivered by an LED through a 40x water immersion objective (Olympus, LUMPLFLN).

In order to sample both ChR2-expressing and non-expressing cells, we targeted cells in the LPO area without monitoring eYFP expression. Once a stable intracellular recording was obtained, we performed a step protocol to characterise basic electrophysiological properties of

the cell. Subsequently, all cells were subjected to 1 or 2 minutes of ChR2 stimulation at 1 Hz. If the cell exhibited suprathreshold responses, it was subsequently subjected to stimulation at 2 Hz, 5 Hz and 10 Hz. If a cell did not exhibit suprathreshold ChR2-responses, we sought to quantify the net effect of light stimulation on spontaneous firing. Therefore, a mild depolarising current was injected via the patch pipette to elicit spontaneous firing. Subsequently, the cell was subjected to 1 Hz, 2 Hz, 5 Hz, and 10 Hz stimulation for 1 or 2 minutes. Following these 4 stimulation protocols, stimulation at 1Hz before and after a 10-minute bath-application of CNQX (10 μM, Sigma) or bicuculline (10 μM, Sigma) was repeated.

Quantification and Statistical Analysis

Statistical analyses were performed in MATLAB, SPSS and GraphPad Prism. P-values less than 0.05 (after correction for multiple hypotheses except the comparison of power density in each frequency bins, if applicable) were considered significant. For comparisons of the means of two populations, two-tailed unpaired t-tests were generally used, or Welch's t-test if unequal variances were assumed. Two-tailed paired t-tests were used for comparisons of the means of two conditions in the same animal group. Comparison of multiple means was done *via* ANOVA. For all analyses, the statistical test used, the meaning and value of n, definition of centre, and dispersion and precision measures can be found in the relevant main text, the figure legend or in Method Details.

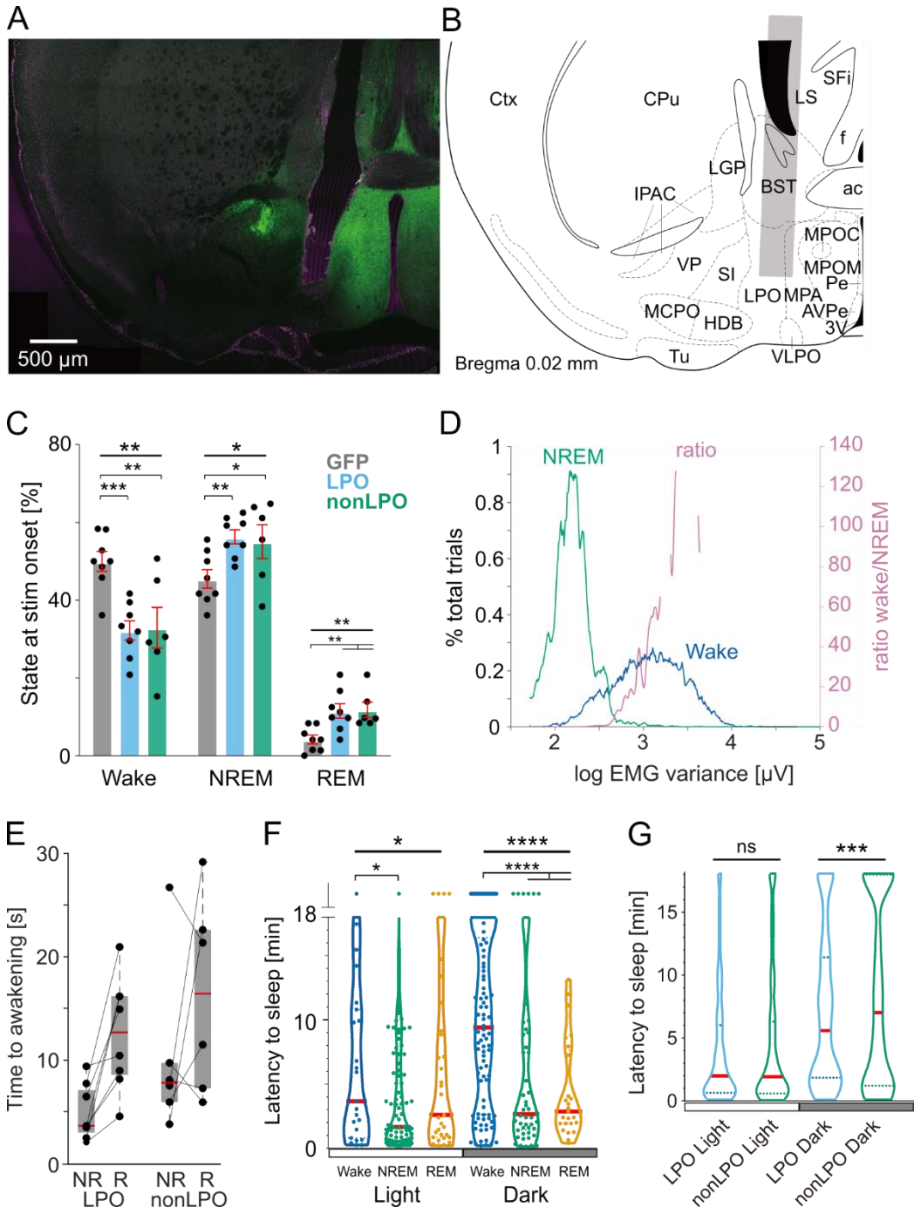

Figure S1. Effects of GAD2^{LPO} neurons photoactivation. (A) Low magnified image for figure 1A. Green: GFP, Magenta: DAPI. (B) Corresponding section from the mouse brain atlas. Grey bar indicates the location of the implanted fiber. (C) Percentage of photostimulation trials delivered during wake, NREM and REM sleep, averaged across animals for GFP (n=8), LPO (n=8) and nonLPO (n=6) groups. *p<0.05, **p<0.01, ***p<0.001, RM-ANOVA and post-hoc uncorrected Fisher's LSD. Error bar, SEM. (D) Representative probability distribution of EMG variance across 24h, separated according to vigilance states (blue: wake, green: NREM), which were derived from visual scoring. For analysis of awakenings based on EMG variance, a threshold in EMG variance was derived from the ratio between NREM and wake probabilities (pink line). Gaps in pink line are due to the lack of data. (E) Time to awakening after stimulation onset delivered during NREM and REM sleep in nonLPO and LPO animals. Black points represent individual animals, red bar represents median and gray box

represents ± 25/75 percentiles across all mice. Mixed ANOVA, p=0.002 for factor ‘sleep state’; p=0.078 for ‘sleep state’ x ’region’; p=0.079 for ‘region’. (F) Latency to sleep after wake induced by photostimulation in LPO (n=8). Latency was calculated separately based on the vigilance state when stimulation was triggered. Top dots represent the number of cases in which animals did not go to sleep until the next photostimulation. *p<0.05, ***p<0.001, ****p<0.0001, one-way ANOVA and post-hoc uncorrected Fisher’s LSD. Red bar: median, dotted line violin plot: 25/75% across animals. (G) Latency to sleep after wake induced by photostimulation in LPO and nonLPO. Latency plot at 18 min corresponds to the case in which an animal was awake until the next photostimulation. ns: p>0.1, ***: p<0.001 in Kolmogorov-Smirnov test. Red bar: median, dotted line violin plot: 25/75% across animals. Abbreviations, 3V: third ventricle, ac: anterior commissure, AVPe: anteroventral periventricular nucleus, BST: bed nucleus of stria terminalis, CPu: caudate putamen (striatum), f: fornix, HDB: nucleus of the horizontal limb of the diagonal band, IPAC: interstitial nucleus of the posterior limb of the anterior commissure, LGP: lateral globus pallidus, LH: lateral hypothalamic area, LPO: lateral preoptic area, LS: lateral septal nucleus, MCPO: magnocellular preoptic nucleus, MPA: medial preoptic area, MPOC: median preoptic nucleus, central part, MPOM; medial preoptic nucleus, medial part, Pe: periventricular hypothalamic nucleus, SFi: septofimbrial nucleus, SI: substantia innominata, Tu: olfactory tubercle, VLPO: ventrolateral preoptic nucleus, VMH: ventromedial hypothalamic nucleus, VP: ventral pallidum.

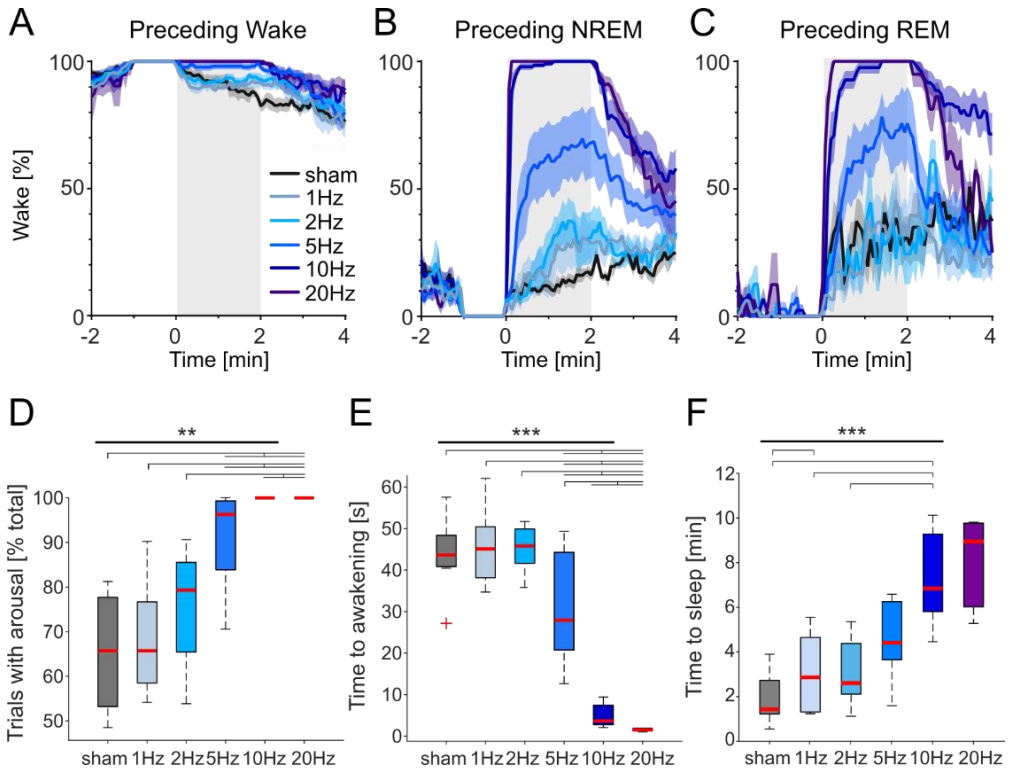

Fig. S2. Low frequency photoactivation of GAD2^{LPO} neurons has qualitatively similar effects on awakening latency as a 10Hz stimulation. (A-C) Effects of photostimulation (shaded area) on the amount of wakefulness. The trials are grouped according to the state of vigilance when stimulation started (wake and NREM sleep: last 1 minute, REM sleep: last 20 seconds before stimulation). Mean values, SEM; for sham, 1Hz, 2Hz, 5Hz, 10Hz: n=7 and for 20Hz: n=4. (D) Proportion of NREM stimulation trials (± SD), which result in an awakening within 2 min from stimulation onset. Stars (**) above bold line at the top denotes significance for RM-ANOVA ($p<0.01$). Lines above plots denote significant differences between conditions for pair-wise comparisons, Wilcoxon signed-rank test ($p<0.05$). (E) Average latency to an awakening (± SD) during those trials which resulted in an awakening. Stars (***) above bold line at the top denote significance for RM-ANOVA ($p<0.001$). Lines above plots denote significant differences between conditions for pair-wise comparisons, Wilcoxon signed-rank test ($p<0.05$). Note that the 20Hz stimulation was only performed in 4 animals and was therefore not included in statistical comparisons. (F) Average latency to sleep (± SD) during those trials where stimulation was performed during wakefulness. Stars (***) above bold line at the top denote significance for RM-ANOVA ($p<0.001$). Lines above plots denote significant differences between conditions for Wilcoxon signed-rank test ($p<0.05$).

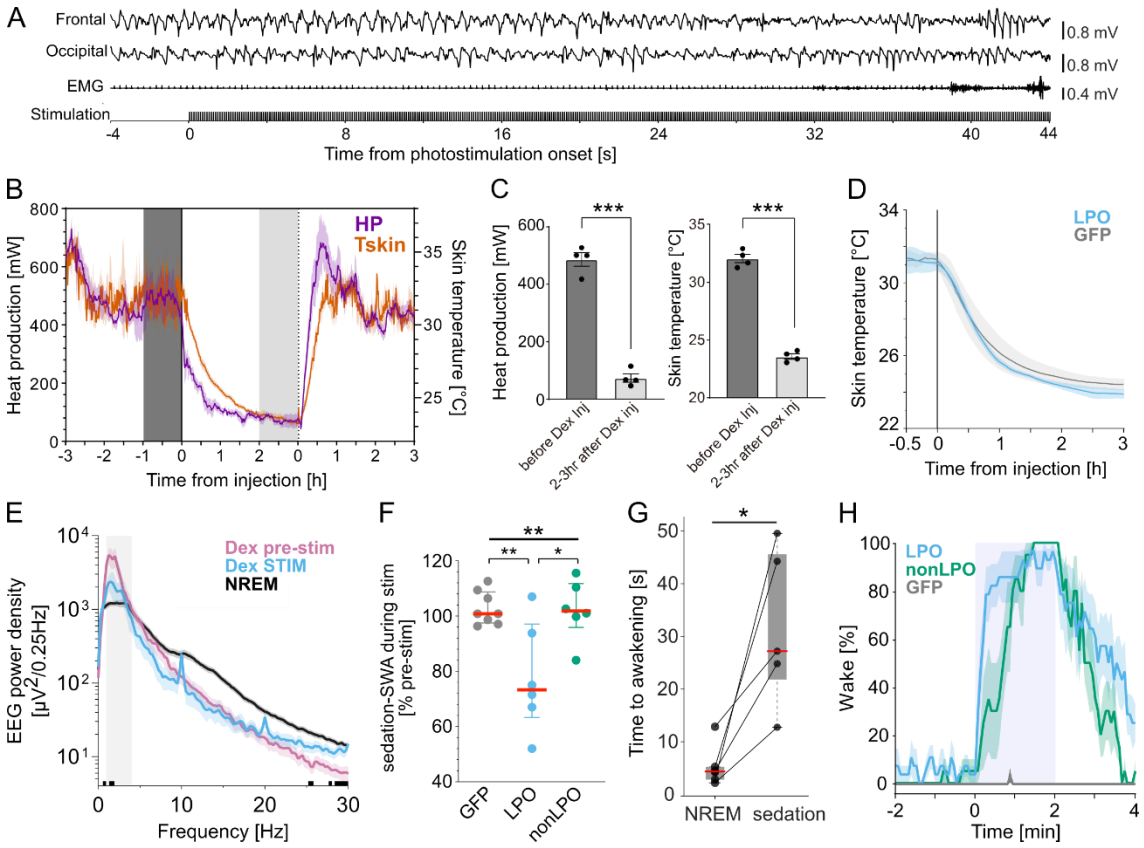

Fig. S3. GAD2^{LPO} neurons photoactivation during dexmedetomidine sedation. (A) Representative example of frontal and occipital EEGs and EMG traces before and during photo-stimulation in a representative animal injected with dexmedetomidine (Dex) showing the transition to wake. (B) Metabolic rates (heat production, HP) shown in purple were measured with indirect calorimetry, and skin body temperature (Tskin), shown in red, was recorded with a thermal imaging camera. In this experiment, naïve, non-implanted Gad2-cre mice (n=4) were injected with dexmedetomidine to induce sedation at ZT 3 (x=0 on the left), and were injected with Atipamezole to induce recovery at ~ZT 7 (dotted line, x=0 at right). Dark grey and light grey boxes correspond to the last hour before injection and last hour before sedation reversal, respectively, for which the analyses were performed as shown in C. Mean values ± SEM. Note the rate of decrease and recovery were significantly faster for MRs as compared to Tskin (calculated as the rate of change within the 1st hour after injection of dexmedetomidine and first hour after injection of Atipamezole). ***: p<0.005, paired t-test. (C) Mean values of MRs and Tskin averaged over 1-h intervals before dexmedetomidine injection, and 2-3 hours after injection. Note that both decreased significantly. (D) Peripheral body temperature change during sedation with photostimulation in LPO and GFP control animals. Blue, LPO (n=4). Grey, GFP control (n=2). Stimulation was given in 2-min or 5-min long trains in LPO animals, and over 2-min long trains in GFP controls. Baseline temperature was obtained from average temperature during sleep before Dex injection between ZT 0-1. (E) Average spectral power density of the frontal EEG during sedation in the LPO group (n=6). Pink: averaged over 2-min preceding photostimulations. Red: averaged over epochs after the onset of stimulation before awakening. Black, averaged 2-hour NREM sleep during ZT 1-3 in baseline day. Black bars at the bottom: p<0.05 in multiple t-test. Shaded area indicated SWA frequency band (0.5-4 Hz). (F) EEG spectral power in the slow wave frequency range (SWA, 0.5-4 Hz) during 2-min 10 Hz stimulation in sedated animals, from GFP

(n=8), LPO (n=6) and nonLPO (n=6) groups. Data are shown as percentage change from 2 minutes before stimulation. Wake states were excluded from sedation-SWA calculation. *: $p<0.05$, **: $p<0.01$ for one-way ANOVA and post-hoc Tukey's multiple comparisons test. (G) Awakening latency for stimulations during NREM sleep or Dex-induced sedation. Datapoints represent individual mice and box represents median across mice (± 25/75 percentiles). *$p<0.05$, two-tailed paired t-test (n=6). (H) Probability of awakening from dexmedetomidine induced hypothermia. Average of 4 stimuli that were triggered between 2-3 hours after Dex injection. Blue, LPO (n=7). Green, nonLPO (n=6), Grey, GFP control (n=3).

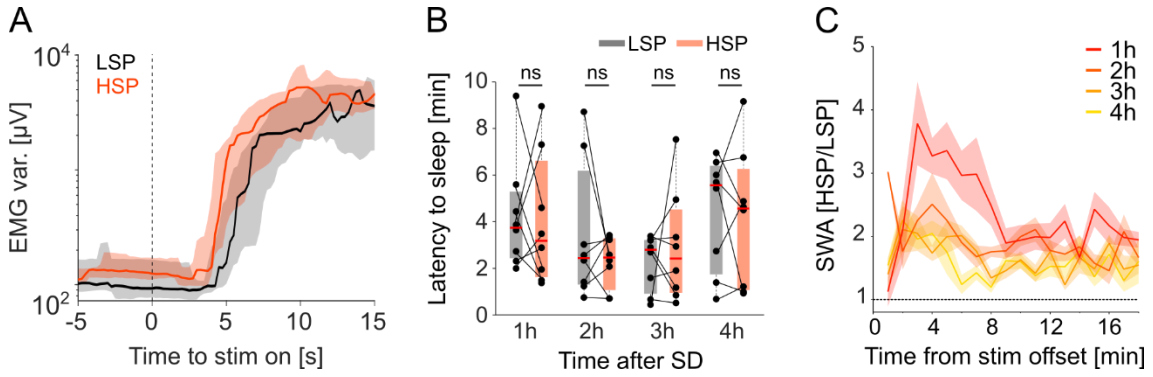

Fig. S4. GAD2^{LPO} photoactivation during high and low sleep pressure. (A) Representative stimulus-aligned EMG variance (median ± 25/75 percentiles) in one mouse during high and low sleep pressure conditions. LSP: low sleep pressure condition, HSP: high sleep pressure condition. (B) Latency to sleep after photostimulation during high and low sleep pressure conditions. Latencies were calculated in 1-h intervals during the 4-h recovery sleep after sleep deprivation. There were no significant differences between conditions at any time point (two-tailed paired t-tests). (C) Time course of NREM sleep EEG SWA during the 18-minute interval between stimulations in the high sleep pressure condition. Values are plotted relative to mean SWA in the low sleep pressure condition. Dotted line at 1 indicates equal SWA in the two conditions. Mean values ± SEM.

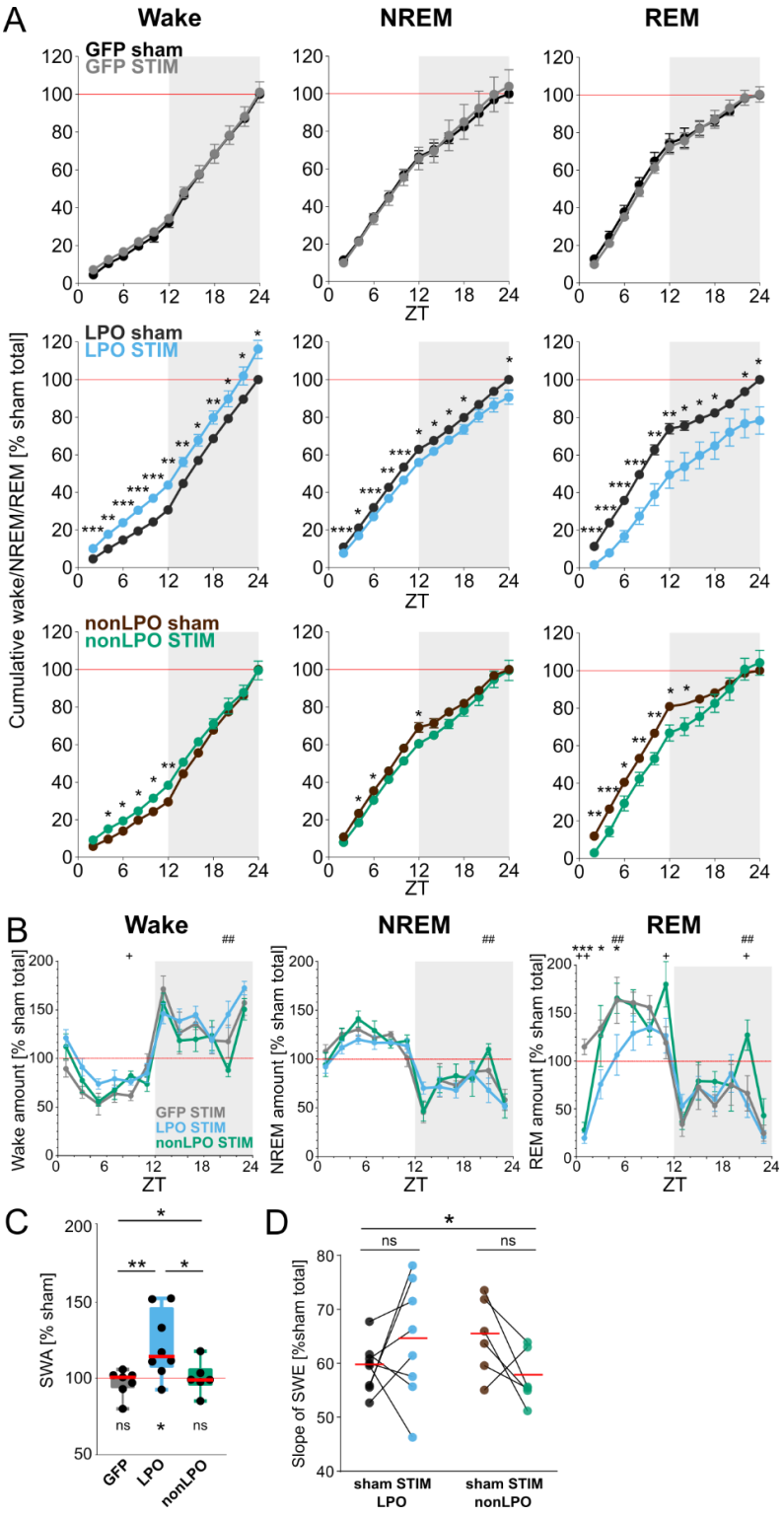

Fig. S5. Long-term, cumulative effects of GAD2^{LPO} photoactivation on sleep, SWA and SWE. (A) Cumulative amount of wake, NREM and REM sleep across a sham stimulation day and a day with photostimulation. The data are represented as percentage of the total amount of the corresponding state accumulated over the sham stimulation day (100 %). Top panels: GFP-

control (n=8), middle panels: LPO (n=8), bottom panels: nonLPO (n=6). *: $p<0.05$, **: $p<0.01$, ***: $p<0.01$, paired t-test. (B) Time spent in wake, NREM sleep, and REM sleep across 24 hours on the day with photostimulations. Amounts of vigilance states are plotted in 2-hour intervals. (Mean values, SEM). *: $p<0.05$, **: $p<0.01$, ***: $p<0.01$, Tukey's multiple comparisons test after RM-ANOVA. #: LPO vs nonLPO, *: LPO vs GFP, +: GFP vs nonLPO. (C) Frontal EEG SWA during NREM sleep on the day with photostimulation shown as percentage of SWA during sham stimulation day. Comparison of relative EEG-SWA during NREM sleep in GFP, LPO and nonLPO groups. *$p<0.05$, **$p<0.01$, one-way ANOVA, stars above bar plots depict significance in post-hoc uncorrected Fisher's LSD test. Statistical results at the bottom were comparison of SWA between photostimulation day and sham stimulation day in each group for Wilcoxon signed-rank test; *: $p<0.05$, ns: no significance. (D) Slope of the cumulative EEG slow-wave energy (SWE) during the light period (related to Figure 4G). Red bar depicts the median value within each condition. Significant differences are shown above: black bar at the top: $p<0.05$ for 2-way ANOVA for interaction between group and day. No significant differences (ns) were identified (post-hoc Sidak's multiple comparisons test).

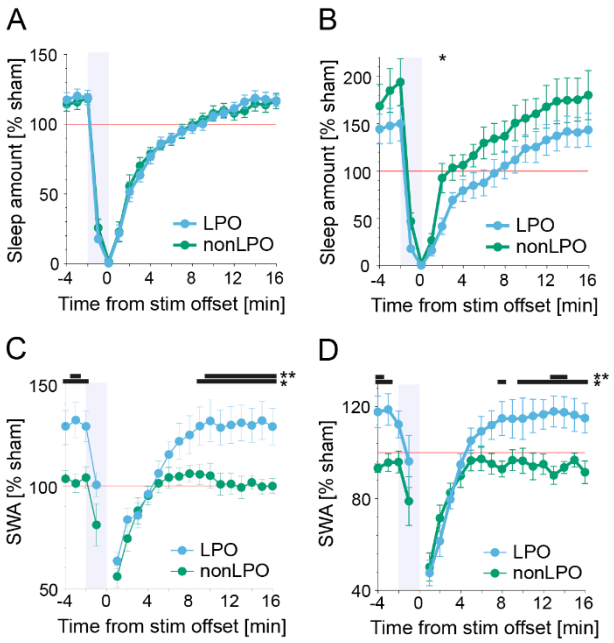

Fig. S6. Short-term effects of GAD2^{LPO} photoactivation on sleep and SWA. (A, B) Amount of sleep during the 20-min window aligned to stimulation offset during the light period (A) or the dark period (B) shown as percentage relative to the average sleep amount in sham stimulation day during the corresponding 12-hours. All stimulation trials occurring during the light period or the dark period are averaged for LPO (n=8) and nonLPO (n=6) animals. *: p<0.05 in RM-ANOVA, Uncorrected Fisher's LSD. Mean values ± SEM. (C, D) Time course of EEG over a 20-min window aligned to stimulation offset during the light period (C) or the dark period (D). SWA is shown as a percentage of SWA in sham stimulation day during the corresponding 12-hours. Black bars at the top of the figure denote 1-min bins where the difference between LPO and nonLPO was significant (*: p<0.05, **: p<0.01 in RM-ANOVA, uncorrected Fisher's LSD). Mean values ± SEM.

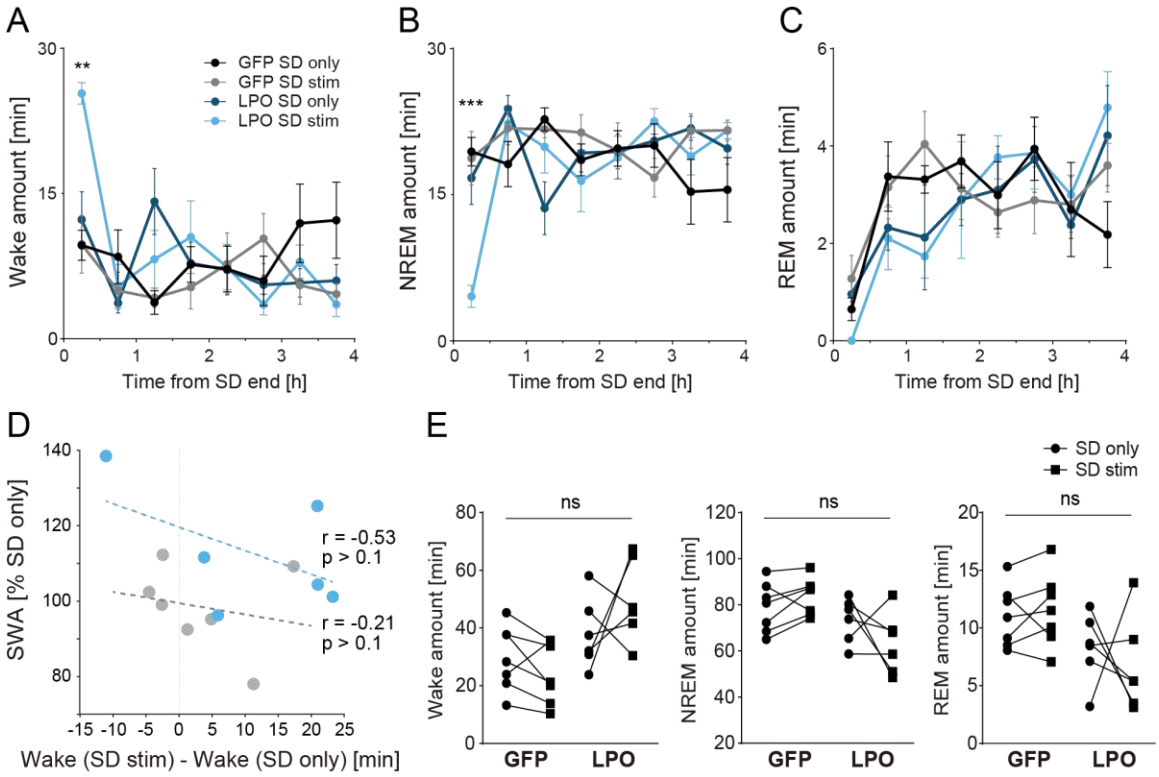

Fig. S7. Effects of SD combined with GAD2^{LPO} photoactivation on vigilance states. (A-C) Time spent in wake (A), NREM sleep (B), REM sleep (C) after the end of SD (SD only) and SD with photoactivation (SD stim). The values are plotted in 2-hour intervals for GFP (n=7) and LPO (n=6). **: p<0.01, ***: p<0.005, significant difference between LPO SD only vs SD stim in Tukey's multiple comparison test. (D) Correlation between stimulation-associated differences in wake amount and NREM SWA during the first 2h of recovery sleep. Wake amount was calculated from the start of SD until sleep onset. The values on the left depict r and p values of Pearson's correlation. Note that the difference in SWA rebound between the conditions was unrelated to the difference in the total wake duration. (E) Time spent in each vigilance state in the first 2-h recovery sleep in SD only and SD stim conditions. ns: no statistical difference in 2-way ANOVA.

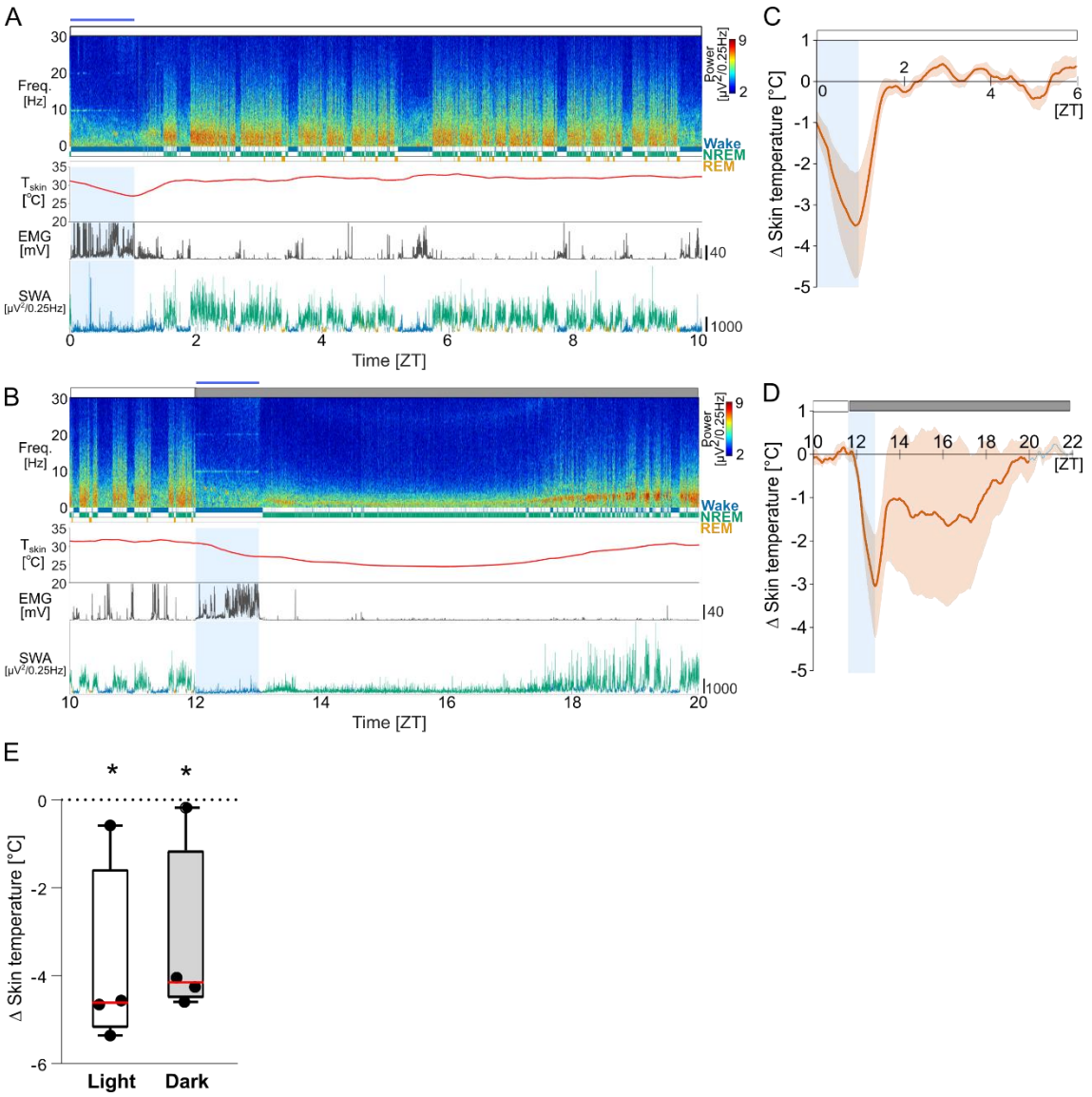

Fig. S8. Continuous strong photoactivation of GAD2^{LPO} neurons induced hypothermia. (A, B) Representative spectrogram, hypnogram, peripheral body temperature, EMG and hypnogram-colour-coded SWA during and after 1-hour 10 Hz stimulation starting at light onset (A) and dark onset (B). Data in A and B is from the same LPO animal recorded with an interval of 7-day. Note that in A, the peripheral body temperature (Tskin) decreased consistently during stimulation but increased when stimulation was stopped and returned to pre-stimulation levels. In B, however, the temperature decrease persisted after stimulation was stopped. Values for the color scale of the power spectrum: common logarithm. (C, D) Average skin temperature during and after 1-hour 10Hz stimulation compared to baseline levels in the LPO group (n=4). C) Stimulation between ZT0-1, D) between ZT12-13. Mean values ± SEM. Shaded area: photostimulation. Baseline is calculated in each animal by averaging skin temperature across 24 hours on the baseline day. (E) Change in minimum skin temperature during photostimulation. Datapoints represent individual mice in the LPO group (n=4). Box represents 25/75 percentile. Red, median. *p<0.05, one-sample *t*-test.

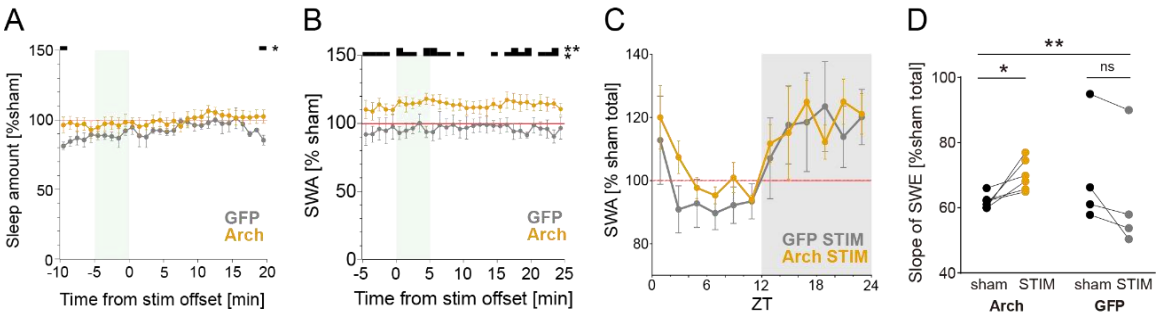

Fig. S9. Short-term and persistent effects of GAD2^{LPO} photoinhibition on sleep amount, SWA and SWE. (A) The amount of sleep during the 30-min window aligned to stimulation onset shown as percentage relative to the average sleep amount during sham stimulation day. All trials occurring during the light period are averaged among animal groups. *: p<0.05. post-hoc uncorrected Fisher's LSD test following one-way ANOVA. Mean values ± SEM. (B) Time course of EEG SWA during the 30-min window aligned to stimulation offset (time 0) during the light period. SWA is shown as a percentage of SWA in sham stimulation day during the light period. *: p<0.05, **: p<0.01, post-hoc uncorrected Fisher's LSD test following one-way ANOVA. Mean values ± SEM. (C) Time course of EEG SWA during NREM sleep across 24 hours on the day with photoinhibition. NREM SWA is plotted in 2-hour intervals and represented as percentage of average NREM SWA during sham stimulation day. Mean values ± SEM. (D) Slope of EEG slow-wave energy (SWE) during the light period (related to Figure 6H). Stars above a black bar at the top: p=0.0044 in 2-way ANOVA, interaction between group and stimulation. Stars and 'ns' above the plot indicate results of the post-hoc Sidak's multiple comparisons test.

Table S1. Summary of animal groups contributed to each experiment and the corresponding number of GAD2-cre mice.

	Figure	Activation			Inhibition		no AAV	Parameters required
		LPO	nonLPO	GFP	Arch	GFP		
Implanted animals		10	9	8	7	5		
Animal used for analysis *		**8**	**6**	**8**	**6**	**4**	**4**	
10Hz-2min stimulation, 24hr (VS amount analysis)	1, 4A S1	8	6	8				frontal or occipital EEG
10Hz-2min stimulation, 24hr (SWA, SWE analysis)	4B-G, S5, S6	8	6	7†				frontal EEG
10Hz-2min stimulation, 24hr (EEG spectrum analysis)	5A-C	7**	6	6††				frontal & occipital EEG
Dexmedetomidine	3A-C, S3A,D-H	7	6	8				frontal EEG
High/Low sleep pressure (stimulation after 4hSD)	3D-G, S4	8						frontal EEG
Wake Enhancement (2hSD+stimulation)	5D, E, S7	6***		7†††				frontal EEG
5, 2, 1Hz stimulation	S2	7						
20Hz stimulation	S2	4						
10Hz 1hr stimulation	S8	4						
5min stimulation every 30min across 24hr	6, S9				6	4		
Wake Suppression (2hSD+stimulation)								
Simultaneous metabolic and skin temperature recording	S3B, C						4	

Reason excluded from analysis: *, no virus expression or lost/damaged device. **, bad occipital EEG. ***, not performed or bad frontal EEG. †, bad frontal EEG. ††, bad frontal or occipital EEG. †††, bad frontal EEG. No symbol, experiment not performed.

Movie S1. Photoactivation of GAD2^{LPO} neurons during NREM sleep induces wakefulness. This movie shows a representative response to a photostimulation trial. 30 s before and after photostimulation are shown, with photostimulation indicated by the black square in the bottom right corner. EMG activity is shown below to indicate movement. The movie runs at 2x speed.

Movie S2. Photoactivation of GAD2^{LPO} neurons induces wakefulness in mice under Dexmedetomidine sedation. This movie shows the response of a Dexmedetomidine sedated mouse at different time points across the 5-minute photostimulation period. Specifically, video clips before stimulation (Pre stim), at the onset of stimulation (Stim on), 2-minutes into stimulation (During stim 2 min), 4 minutes into stimulation (During stim 4 min) and at the offset of stimulation (Stim off) are shown. The movie runs at 2x speed.