Knockdown of GABA_A alpha3 subunits on thalamic reticular neurons enhances deep sleep in mice

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Identification of mechanisms which increase deep sleep could lead to novel treatments which promote the restorative effects of sleep. Here, we show that knockdown of the α3 GABA_A-receptor subunit from parvalbumin neurons in the thalamic reticular nucleus using CRISPR-Cas9 gene editing increased the thalamocortical delta (1.5–4 Hz) oscillations which are implicated in many health-promoting effects of sleep. Inhibitory synaptic currents in thalamic reticular parvalbumin neurons were strongly reduced in vitro. Further analysis revealed that delta power in long NREM bouts prior to NREM-REM transitions was preferentially affected by deletion of α3 subunits. Our results identify a role for GABA_A receptors on thalamic reticular nucleus neurons and suggest antagonism of α3 subunits as a strategy to enhance delta activity during sleep.
Sleep is vital for maintaining physical and mental well-being. In particular, thalamocortical delta (1.5–4 Hz) oscillations present in deep non-rapid-eye-movement (NREM) sleep are implicated in a wide range of processes beneficial to health including synaptic homeostasis, cellular energy regulation, clearance of toxic proteins, cognitive performance and mood. Conversely, insomnia, traumatic brain injury, obstructive sleep apnea, and other brain disorders are associated with interrupted/fragmented sleep, reduced deep NREM sleep and decreased delta wave power. Although hypnotic agents which potentiate the activity of GABA<sub>A</sub> receptors promote sleep induction, they also reduce delta oscillations, suggesting that a subset of GABA<sub>A</sub> receptors prevents deep restorative sleep. Thus, identification and elimination of this confounding effect of the most widely used hypnotics, which target GABA<sub>A</sub> receptors, could be beneficial in developing drugs which boost the positive effects of sleep.

Delta oscillations during NREM sleep are primarily generated by thalamocortical relay neurons, which tend to discharge in bursts at delta frequencies when sufficiently hyperpolarized due to activation of a slow hyperpolarization-activated cation current and de-inactivation of low-threshold calcium channels. Hypo-polarization of thalamocortical relay neurons during NREM sleep is due to the withdrawal of excitatory neurmodulatory inputs and active inhibition mediated by the major GABAergic input from the thalamic reticular nucleus (TRN). Recent work suggested that increased activity of TRN neurons plays a role in promoting delta power and NREM sleep. However, the role of the GABAergic receptors on TRN neurons in controlling sleep oscillations is not well understood.

Here we used state-of-the-art CRISPR-Cas9 gene editing to test the hypothesis that GABA<sub>A</sub> receptors on TRN neurons suppress NREM sleep delta oscillations.

Results

Targeting the TRN for localized alpha3 (α3) KD. In the adult brain, most GABA<sub>A</sub>Rs consist of two α subunits (α1–6), two β subunits (β1–3), and one γ subunit (γ1–3). The α subunit is a necessary component of the GABA<sub>A</sub>R, required for assembling a functional receptor. In the mouse thalamus, all synaptic GABA<sub>A</sub>Rs in thalamocortical relay nuclei contain the α1 subunit, whereas the majority of GABA<sub>A</sub>Rs in the TRN contain the α3 subunit. To introduce a brain region and cell-type-specific ablation of the α3 subunit gene, we first generated mice which expressed the Cas9 endonuclease in the major subset of TRN neurons which contain the calcium-binding protein parvalbumin (PV) by crossing PV-Cre mice with Rosa26-Lox-stop-lox-Cas9-GFP mice to produce PV-Cas9-GFP offspring. Next, we analyzed the gene sequence of the α3 subunit and selected three loci close to the start codon as target regions expected to maximize CRISPR-Cas9 mediated ablation (Fig. 1a). Our target regions were within the extracellular domain (Fig. 1a), the major ligand binding component, forming parts of the GABA binding site and the benzodiazepine binding site. We then constructed an adeno-associated viral (AAV) vector to target the α3 subunit (AAV5-α3-sgRNA-mCherry) by introducing the sequences for the single-guide RNAs (sgRNAs) into an AAV vector plasmid, each driven by the U6 promoter paired with mCherry as a red fluorescent marker (Fig. 1b). To test the effect of α3 knockdown (α3KD) on sleep and spectral activity, we recorded cortical oscillations using frontal electroencephalographic (EEG) electrodes and nuchal muscle electromyographic (EMG) electrodes before and after we introduced AAV-α3-sgRNA-mCherry into the TRN via chronically implanted guide cannulae (Fig. 1b).

NREM delta wave power is enhanced by α3KD in the TRN. α3KD in TRN PV neurons resulted in a marked increase in NREM slow wave activity (0.5–4 Hz, which was most pronounced at the beginning of the mouse sleep period (ZT0–4, 47.5 ± 21.8% change, t (5) = −5.159, p = 0.0036). The increase in slow wave activity was similar in magnitude to the increase observed in the first four hours following six hours of sleep deprivation, even though the mice here were not sleep deprived.

The 0.5–4 Hz EEG band includes both cortically-generated slow waves (typically 0.5–1 Hz or 0.5–1.5 Hz) and delta oscillations (1.5–4 Hz), which are largely thalamically generated. More fine-grained analysis of our data to separate out these two components revealed that significant increases were seen in the 1.5–4 Hz delta range (Fig. 1d, e and Supplementary Fig. 1) as well as in narrower bands including the delta2 (2.5–3.5 Hz) band which is sensitive to sleep deprivation (Supplementary Table 1) whereas there was no significant change in slow oscillation (0.5–1.5 Hz, 0–1 Hz or 0.5–1.75 Hz) EEG bands (Supplementary Table 1). 6/6 mice with > 85% transduction of TRN PV neurons showed the increase in delta power (Supplementary Fig. 1). We found no significant change in any other frequency bands in NREM (Fig. 1e), and no changes in any frequency bands during wakefulness (NREM sleep (Supplementary Fig. 2)). Only modest changes in the amount of NREM sleep itself were observed, with more NREM only in the first four hours of the dark period when mice are mostly awake (BL: 23.2 ± 2.4% vs KD: 32.4 ± 2.8%, t (5) = −4.7472, p = 0.005). Moreover, we found no changes in the duration or frequency of NREM bouts (Fig. 2a). However, we observed a significant reduction in the proportion of shortest bout durations (Fig. 2c), suggesting more consolidated NREM sleep following α3KD. REM sleep was unchanged [duration: BL = 35(1) vs α3KD = 38(3.79); bouts/hour: BL = 6.59(0.41) vs α3KD = 6.42(0.59)]. Analysis of sleep spindles using a recently validated algorithm did not identify any difference in spindle density, frequency or duration (Supplementary Fig. 3). No NREM delta effects were observed in four negative control mice three of which showed no AAV-α3-sgRNA-mCherry transduction in TRN and one with only 66% transduction of TRN PV neurons (Supplementary Fig. 4).

In humans, delta oscillations are most prominent in the deepest stage of NREM sleep, N3. However, in mice NREM is not generally split into stages. Nevertheless, mouse NREM probably also has degrees of depth which are not evident using standard scoring approaches. In humans, arousal threshold increases with depth of NREM sleep; humans are more likely to awaken from the lighter stages N1 or N2. Thus, we analyzed delta oscillations prior to NREM sleep preceding transitions to REM sleep (Fig. 1f–h) [means (standard error (SEM)): BL = 2.98 (0.24), α3KD = 3.3 (0.26); 11.5% change for all transitions occurring during the light period (±5.56); t (5) = −2.14, p = 0.04]. No difference was apparent in NREM sleep before transitions to wake (Fig. 1i–k). Similarly, no change in delta power was seen during NREM sleep in the initial phase of transitions from wakefulness to NREM sleep (Supplementary Fig. 5). We also found that increased delta power prior to NREM → REM transitions was not apparent in the negative control group with either no or poor TRN targeting (Supplementary Fig. 4).

Enhanced NREM delta corresponds to longer NREM bouts. Sleep is more fragmented in mice than in humans. Thus, we reasoned that long NREM bouts might be required to observe large increases in delta. Further analysis of NREM-REM transitions revealed larger increases in delta power within sustained NREM periods prior to transitions to REM (Fig. 3a). Interestingly, heightened delta was seen at the onset and throughout the NREM bout, rather than gradually increasing as a NREM bout persists (Fig. 3a). Delta power levels were higher in longer NREM periods compared to shorter NREM periods (Fig. 3a). Thus, enhanced delta power associated with α3KD was only evident during NREM sleep preceding transitions to REM sleep (Fig. 1f–h) [means (standard error (SEM)): BL = 2.98 (0.24), α3KD = 3.3 (0.26); 11.5% change for all transitions occurring during the light period (±5.56); t (5) = −2.14, p = 0.04]. No difference was apparent in NREM sleep before transitions to wake (Fig. 1i–k). Similarly, no change in delta power was seen during NREM sleep in the initial phase of transitions from wakefulness to NREM sleep (Supplementary Fig. 5). We also found that increased delta power prior to NREM → REM transitions was not apparent in the negative control group with either no or poor TRN targeting (Supplementary Fig. 4).
bouts and were increased more by the α3KD. In fact, there was a highly significant linear relationship whereby the longer the NREM duration preceding a NREM-REM transition, the larger the increase in delta power, as measured by percent changes (Fig. 3b). These data also suggested the possibility that the difference in the delta increase observed between NREM-REM and NREM-wake transitions might be explained, at least in part, by a shorter NREM episode duration prior to NREM-wake transitions compared with the longer NREM preceding REM sleep. This prediction was confirmed since NREM episodes leading to wakefulness were shorter (76.3 ± 7.1 s) than those which transitioned into REM sleep (253.2 ± 30.9 s).
**Fig. 1** α3KD in PV + TRN neurons increased NREM 1.5–4 Hz delta power, especially at NREM to REM transitions. a The γ-amino butyric acid receptor type-A (GABA_A) is a pentameric heteromeric ion channel. CRISPR-Cas9 abscission was directed to three locations (insertion/deletion; INDEL sites) of the gene which correspond to the large extracellular region of the α3 subunit, a necessary structural as well as ligand binding component in GABA_A Rs of the TRN. b Adeno-Associated Viral (AAV) vectors encoding three separate single-guide RNAs (i, ii & iii), each driven by its own U6 promoter, and the marker protein mCherry driven by the human synapsin (hSyn1) promoter, were injected into the TRN region of PV-Cas9/GFP mice in vivo via guide cannulas. [Adapted from Franklin and Paxinos59, with permission from Elsevier]. c Examples of frontal electroencephalogram (EEG) and electromyogram (EMG) recordings during a NREM period (ZT0-4) in baseline (BL; blue) and following α3KD (red), in a single mouse. Panels d–k present grand average data from all 6 α3KD mice. d Compared with their baseline (BL) recordings, α3KD mice had higher non-rapid eye movement sleep (NREM) delta (δ, 1.5–4 Hz) power. ZT0-4, p = 0.0176; ZT4-8, p = 0.0355; ZT8-12, p = 0.0335; ZT12-16, p = 0.0388; ZT16-20, p = 0.0275; ZT20-24, p = 0.0221. Significance was determined using two-tailed paired t-tests with Hommel corrected p-values. Thick lines indicate mean; envelopes indicate SEM. The shaded area indicates the 12 h dark period. e Only NREM delta (δ, 1.5–4 Hz) power is significantly different between BL and α3KD records (p = 0.0035, two-tailed paired t test), slow waves (0.5–1.5 Hz), theta (θ, 5–9 Hz) and sigma (σ, 10–15 Hz) power were not affected. The power profile from NREM in ZT-0-4 is shown here, the time period where we saw the largest effect. Significance was determined using two-tailed paired t-tests. Thick lines indicate mean; envelopes indicate SEM. Line colors indicate time-frequency power dynamics present high delta power in NREM leading to a transition to rapid eye movement sleep (REM; data from the whole 12 h light period). f After α3KD, the high delta in NREM before a transition to REM was increased. Compared with their baseline (BL) recordings, α3KD mice (red) had higher delta power in the NREM before a transition to REM (t (5) = 2.14, p = 0.04). Significance was tested using a one-tailed paired t-test. Thick lines indicate mean; envelopes indicate SEM. g BL time-frequency power dynamics presents high delta power in NREM leading to a transition to wake as well. j α3KD did not increase this delta power that occurs during NREM before a transition to wake. k Compared with BL (blue), α3KD (red) did not lead to a change in delta power in the NREM before a transition to wake (t (5) = 0.23, p = 0.41). Significance was tested using a one-tailed paired t-test. Thick lines indicate mean; envelopes indicate SEM. * p < 0.05, n.s. indicates not significant. Color scales represent normalized power (power at time/power from wakefulness).

**Fig. 2** α3KD, but not α1KD, in PV + TRN neurons decreased time spent in the shortest (<60 s) NREM bouts. a Compared with their baseline conditions (BL; blue), α3KD (red) mice (n = 6) did not have altered durations or number of NREM bouts. b α1KD mice (n = 7) also did not have altered durations or number of NREM bouts. The proportion of time spent in short bouts lasting <60 s was significantly reduced in α3KD mice (two-tailed paired t-test, *p = 0.03), but no other bins showed any differences between BL and α3KD (n = 6). Box plots: center lines represent median values, 75th percentiles are box tops and 25th percentiles are box bottoms. + symbols represent outliers defined as >1.5x the interquartile range away from box tops or bottoms. Data from whole 12 h light period.

Widespread α3KD in TRN determines elevated NREM delta power. Selective deletion of α3 subunits in PV neurons requires the combination of selective expression of Cas9 in PV neurons and sgRNA targeting α3 subunits in the same cells. mCherry (red; marker of sgRNA) was expressed in the majority of TRN PV neurons (green) within the core of the injection site (Fig. 4a). In the six α3KD-confirmed mice, we found a high percentage of PV + TRN neurons (GFP+) were transduced by AAV–α3-sgRNA–mCherry (mCherry+: 93.9 ± 2.0%), and a large proportion of the TRN (94.4 ± 1.0%) area was covered (Fig. 4a; Supplementary Table 2). Analysis of off-target viral spread assessed by mCherry+ cells which were also GFP+ revealed only 5% medially and 3.9% laterally. All cases of off-target mCherry/GFP colocalization were found exclusively in the anterior dorsal thalamic nucleus and globus pallidus (Supplementary Table 3). Neither of these regions express substantial amounts of α3 subunit containing GABA_A receptors35–39. Thus, our functional effects are highly likely to be due to effects on the TRN.
preliminary work prior to in vivo experiments, we confirmed that Cas9 expression (marked by GFP co-expression) was selective for PV neurons by immunohistochemical staining for PV (Fig. 4b), consistent with the previously published validation of Cas9 selective expression in PV+ neurons in this mouse model40.

α3KD causes a functional ablation of sIPSCs in TRN neurons. In a separate group of mice, we verified a functional ablation of GABA_A receptors in whole-cell patch-clamp recordings from TRN PV neurons in vitro. Recordings were performed from adult mice (>2.5 months). In contrast to earlier in development41, at this age in
**Fig. 3** α3KD caused the largest delta (δ, 1.5–4 Hz) effect in the longest NREM bouts before REM. **a** Analysis of non-rapid eye movement sleep (NREM) to rapid eye movement sleep (REM) transitions reveals larger delta power increases in longer NREM episodes, baseline (BL; blue) vs α3KD (red). Periods with stable delta (black bars), were tested for significance (one-tailed paired t-tests, *p < 0.05, n.s. not significant; p-values: 0.023; 0.028; 0.041; 0.039; 0.082). The 24 s prior to NREM-REM transitions, when delta decays, were not included in statistical analyses. **b** Percent change in NREM delta power was positively correlated with NREM bout length prior to REM. P values from t-tests (BL vs α3KD) were negatively correlated with NREM bout length prior to REM as determined using Pearson’s linear correlation, two-tailed, ρ is Pearson’s linear correlation coefficient. Data from 12 h light period. N = 6. Color scales represent normalized power (power at time/power from wakefulness).
mice there is little evidence for functional intra-TRN chemical synapses. Thus, the GABA<sub>A</sub> receptor mediated events we record in TRN PV neurons likely arise from extra-TRN inputs arising in the basal forebrain, lateral hypothalamus and globus pallidus.

In control voltage-clamp recordings from TRN PV neurons held at −70 mV in PV-tdTomato mice (which serve as wild type controls with a visual marker of the correct cell phenotype), spontaneous inhibitory postsynaptic currents (sIPSCs) were observed in the presence of glutamate receptor antagonists (20 µM 6-cyano-7-nitroquinoxaline-2,3-dione +50 µM D-(2R)-amino-5-phosphonopentanoic acid) and were abolished by a GABA<sub>A</sub> receptor antagonist, GABA<sub>zine</sub> (10 µM) (Fig. 4c1). To enhance the driving force for chloride, recordings were made using a patch solution with a high chloride concentration. Thus, sIPSCs were detected as inward currents (Fig. 4c1, 4c2). In PV-Cas9 mice, the frequency of sIPSCs were significantly reduced in recordings from green (PV-Cas9/GFP) and red (transduced with AAV-α<sub>3</sub>-sgRNA-mCherry) fluorescent TRN neurons one month post-injection, whereas the amplitude was unaltered.

Fig. 4 α<sub>3</sub>KD in PV: TRN neurons was validated by histology and in vitro electrophysiology. a GFP indicates rich Cas9 expression within the TRN region (green outline), TRN projections to thalamocortical nuclei and sparse distal expression in the globus pallidus (GP). mCherry reveals widespread transduction of the TRN region by the AAV vector delivering sgNRAs (red) with many of the cells in the area co-expressing both markers (merged; yellow). Percentages of target cells and target area that co-express markers reveal widespread delivery of sgNRAs to target TRN PV neurons in the mice used for in vivo studies. Further quantification of transduction is given in Supplementary Tables 2 and 3. Box plots: center lines represent median values, 75th percentiles are box tops and 25th percentiles are box bottoms, bars represent maximum and minimum. (n = 6). b High magnification (60x) confocal images show triple co-localization of PV (immunohistochemical stain; red), Cas9 (GFP, green), and sgRNA (viral transduction indicated by mCherry; magenta), demonstrating successful targeting of PV+ neurons within the TRN. Micrographs are representative of n = 2. c Inward-going spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from TRN PV neurons using a high chloride intracellular solution in the presence of ionotropic glutamate receptor antagonists are blocked by the selective GABA<sub>A</sub> receptor antagonist, GABA<sub>zine</sub> (10 µM). c<sub>2</sub> Compared with PV+ TRN neurons without KD (control), sIPSCs in α<sub>3</sub>KD PV+ TRN neurons were reduced (α<sub>3</sub>KD). c<sub>3</sub> Compared with control recordings from PV+ TRN neurons without KD (left), the frequency of sIPSCs in α<sub>3</sub>KD PV+ TRN neurons was significantly reduced (right), p = 0.031, unpaired two-tailed t-test. c<sub>4</sub> In control recordings from PV+ TRN neurons without KD, residual sIPSCs in α<sub>3</sub>KD PV+ TRN neurons (right) had unchanged amplitude (c<sub>4</sub>) or decay time constants (c<sub>5</sub>). c<sub>3</sub>–c<sub>5</sub> broad lines indicate means, error bars represent SEM control: n = 6; α<sub>3</sub>KD n = 5.

**Discussion**

In this study, we used cell-type and region-specific CRISPR-Cas9 gene editing in vivo to test the functional role of GABA<sub>A</sub>ergic inhibition onto TRN neurons in controlling sleep physiology. We found that knockdown of α<sub>3</sub>-containing GABA<sub>A</sub> receptors, confirmed using in vitro recording of sIPSCs, selectively enhances the power of NREM delta oscillations during the sleep period of mice. Further analyses identified long NREM episodes prior to NREM-REM transitions as being particularly strongly affected by α<sub>3</sub>KD. The selectivity of our manipulations was confirmed by control experiments with absent or low transduction and experiments targeting a closely related subunit, α<sub>1</sub>, which is not expressed by TRN neurons.

There is an emerging consensus that depolarization of TRN neurons during NREM is an effective way to promote deep sleep. In vitro recordings of sIPSCs, selectively enhances the power of NREM delta oscillations during the sleep period of mice. Further analyses identified long NREM episodes prior to NREM-REM transitions as being particularly strongly affected by α<sub>3</sub>KD. The selectivity of our manipulations was confirmed by control experiments with absent or low transduction and experiments targeting a closely related subunit, α<sub>1</sub>, which is not expressed by TRN neurons.

**No effect by control α1KD.** In another group of mice, we performed the same in vivo experimental protocol with a control AAV vector targeting the GABA<sub>B</sub><sub>R</sub> α1 subunit. The TRN is devoid of α1 subunits, so this experiment controls for non-specific genetic cutting. In these mice (n = 7), we found no change in the amount of NREM delta power (Fig. 5a, b), or other bands in NREM (Fig. 5c) following the α1KD. No further bands of wakefulness or REM sleep were altered either. The time-frequency analysis at NREM-REM transitions (Fig. 5d–f) and at NREM-Wake transitions (Fig. 5g–i) showed no changes. There were also no changes to the duration or frequency of NREM bouts (Fig. 2b), or the proportions of bout durations (Fig. 2d). Our histologic protocol confirmed a similar degree of targeting success (Supplementary Fig. 6) as in the α<sub>3</sub>KD.
the functional importance of slow oscillations and sleep spindles, manipulations which increase TRN activity during sleep may be more beneficial than those which reduce burst discharge or cause strong hyperpolarization.

Our findings differ from previous work which examined constitutive global α3 subunit knockout (KO) mice\textsuperscript{25,45}. In the constitutive KO there was no reduction in sIPSC frequency compared to controls in TRN neurons; in fact, there was a modest increase in frequency, plus a significant increase in amplitude and alterations in kinetics and pharmacology, suggesting developmental compensation. Conversely, we show a significant reduction in sIPSC frequency. Here, the ablation was performed in the adult brain, so developmental compensation was circumvented, which is evident by the lack of change in the amplitude of residual IPSCs or their decay time constant, as well as the fact that residual sIPSCs retained sensitivity to an α3 selective positive allosteric
modulator. Therefore, functional ablation of α3 subunits in adults was feasible with the CRISPR-Cas9 approach and, importantly, allowed us to unravel the role of the α3 subunits in sleep-wake patterns and EEG profiles. Despite marked changes in GABAergic transmission in the TRN in the constitutive global α3 knockout, there was no change in the intrinsic membrane properties of TRN neurons or in the properties of glutamatergic inputs from thalamocortical relay neurons. Similarly, we found no change in the holding current of TRN PV neurons in vitro. Thus, alterations in GABAergic transmission in TRN do not appear to lead to compensatory alterations in the thalamocortical circuitry which generates delta oscillations.

In conclusion, CRISPR-Cas9 cell and region-specific gene editing of α3 subunits in adult mice identified a functional role of GABAB1 receptors on TRN PV neurons in regulating deep NREM sleep. Pharmacological agents which allosterically increase the activity of GABAB1 receptors containing the α1 or α3 subunits are widely used hypnotics. Unfortunately, they promote light sleep with reduced delta power. Compared with baseline (blue), α1KD mice had unchanged non-rapid eye movement (NREM) delta power in the NREM before a transition to REM with a one-tailed paired t-test. Thick lines indicate mean; envelopes indicate SEM. α1KD did not increase the delta in NREM before a transition to wake. Compared with baseline (blue), α1KD (red) mice had unchanged delta power in the NREM before a transition to wake with a one-tailed paired t-test. Thick lines indicate mean; envelopes indicate SEM. n.s. indicates not significant. Data show results from whole 12 period of light.

Methods

Mice. To target our Clustered Regularly Interspersed Short Palindromic Repeats Knock Down (CRISPR KD) selectively to the major subset of Thalamic Reticular Nucleus (TRN) neurons which express Parvalbumin (PV), we crossed male Rosa26lox-stop-lox-Cas9/GFP (Jackson Labs stock # 026175) mice with female PV-Cre (Jackson Labs stock # 017320) mice, generating mice with the key CRISPR enzyme Cas9 and a green fluorescent protein (GFP) reporter expressed selectively in PV neurons, PV-Cas9/GFP mice. For one control group used for in vitro sIPSCs recordings, we used PV-tDTomato mice generated by crossing male Rosa26-lox-stop-lox-tDTomato (Jackson Labs stock # 007914) mice with female PV-Cre mice. Three–eight month-old mice of both sexes were used for in vivo and in vitro experiments. No obvious sex differences were observed so data were pooled. Mice were housed with a 12 h:12 h light-dark cycle with lights on at 7 am, at ambient temperatures 26–34 °C and 30–70% humidity. Food and water were available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of VA Boston Healthcare System and conformed to National Institute of Health, Veterinary Administration and Harvard Medical School guidelines. The work was carried out under protocols 236-B-042916, 359-B-041618 & 400-W-110419.
transgenic mice. We achieved this combination by stereotaxic injection of the AAVs expressing sgRNAs into the TRN of the PV-Cas9/GFP mice. Using a Kopf stereotaxic machine, micro-injectors were then stereotactically implanted into the TRN (Plastics One; Connecticut, United States), Part # C315CG, SCP total, length 12 mm), with retainers inserted (Plastics One, Part # C151/SPC, at AP − 0.7 mm, ML ± 1.5, DV − 1.3). The coordinates for cannula location were selected to be 2 mm above the dura to allow subsequent microinjection into TRN without extensive damage to TRN. We targeted anterior TRN since anterior TRN neurons project to thalamocortical regions which innervate anterior cortex where our EEG recording electrode was located. To record cortical electrical activity, bilateral frontal neocortical EEG screw electrodes (Pinnacle Technology Inc.; Kansas, United States; Part # 8200-K1-SL), using its acquisition software (Sirenia Acquisition). Mice were given immediately after surgery and again 22 days later, given 5 mg/kg of meloxicam (intraperitoneal) loaded with viral solutions was lowered through the cannula, cemented needle (Pinnacle Technology Inc.; Part # 8200-SS). EMG electrodes were placed in the nuchal muscle. All the chronically implanted components were secured with dental cement (Keystone industries, Bosworth Fastray; Part # 18340).

Following one full week of recovery from cannula/electrode implantation, and collection of baseline EEG/EMG records (see next section), AAV microinjections were made into the TRN. Mice were anesthetized by isoflurane (1.5–4% in O2) and depth of anesthesia was monitored by breathing rate, pedal withdrawal and tail pinch reflexes. A 5 μl Hamilton syringe (Part # 87908, Model 75 SN SYR with 33 g cemented needle) loaded with viral solutions was lowered through the cannula, 2 mm beyond the cannula tip, into the TRN (DV − 3.5). 1 μl microinjections were delivered at 0.05 μl/min using a micropump (KD Scientific Legato 130, Massachusetts, United States). Doses of Meloxicam (5 mg/kg; intraperitoneal) were given immediately after surgery and again 24–24 h later, to mitigate any pain associated with the surgery. One month following AAV injection, EEG/EMG signals were again recorded and compared to baseline recordings.

Electroencephalogram (EEG)/Electromyogram (EMG) recordings. To study sleep-wake states and thalamocortical oscillations, we recorded EEG and EMG using Pinnacle Technology Inc. 3 channel (2 EEG/1EMG) systems for mice (Part # 8200-K1-SL), using its acquisition software (Sirenia Acquisition). Mice were tethered to the system via micro contacted EMG electrodes (Pinnacle Technology Inc. Part # 8202-SL) and 24-hour recordings were collected between zeitgeber time 0–24 following a 48-hour period of habituation to the recording apparatus. EEG/EMG data was sampled at 2 kHz, amplified 100x and low pass filtered at 600 Hz. In one mouse from the eSKO cohort and one mouse from the control cohort which lacked transduction of PV neurons, we recorded EMG signals from the trunk and were able to detect EMG activity associated with the surgery. One month following AAV injection, EEG/EMG signals were again recorded and compared to baseline recordings.

Sleep-wake scoring and EEG analysis. We manually scored sleep-wake states from EEG and EMG recordings using four second epochs as follows: Wake was scored when the EEG showed a large EMG signal and synchronized low amplitude EMG signal with muscle tone evident by a large EMG signal; the large EMG signal did not need to be phasic in nature. Wake was defined by EMG activity, not EEG, as we observed sleep spindles and slow waves distinct from wake EEGs in our transgenic mice. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches. REM sleep was scored when the EEG showed large amplitude, slow synchronized waves, and a low EMG signal, except for very brief bursts which were considered twitches.

Behavioral analyses. Scoring was performed in Sirenia Sleep, and EEG signals were again recorded and compared to baseline recordings. Artifact-free data for analyses of EEG signals, such as power spectral density or filtering, was achieved by averaging the data from the two recording chambers, which was used for statistical analysis. Only well-resolved events with amplitudes >10 pA were used for statistical analysis.

Histology. Mice were transcardially perfused with 10 ml phosphate buffered saline (PBS) followed by 10 ml of 10% formalin for fixation. Brains were extracted and embedded in 10% formalin for 1–2 days. After fixation, brains were decased in 10% sucrose solution in PBS before tissue was sliced at a thickness of 40 μm on a freezing microtome (Leica Biosystems, Illinois, United States).

We first confirmed previous findings that both Ca9 and GFP were selectively expressed in TRN PV neurons, using immunohistochemistry for PV in coronal sections containing TRN from PV-Cas9/GFP mice. Free-floating sections in wells were treated with PV primary antibodies sheep anti-PV (1:150; AS-50584; R & D Systems, Minneapolis, MN) and -secondary antibodies (goat anti-sheep IgG conjugated to AlexaFlour 647: 1:250; ab150179; Abcam, Inc. Waltham, MA). GFP signal was enhanced using mouse anti-GFP antibody (1:300; MAB3580; EMD Millipore, Burlington MA) and goat anti-rabbit IgG secondary antibodies. Donkey anti-mouse IgG conjugated to AlexaFlour 488 (1:500; #A-21202; ThermoFisher Scientific, Waltham, MA). Confocal imaging was performed for triple fluorescence (mCherry, AF488, AF670) using 60x oil objective of Leica Dmls confocal microscope and LAS X software.

A histological verification of transduction efficiency, sections were mounted on microscope slides and coverslipped using Vectashield Hard Set mounting medium (Part # H-1400, Vector Labor). Images were collected on a Zeiss Image2 microscope, outfitted with a Hamamatsu Orca R2 camera (C10600) and Stereo Investigator software (MBF Bioscience). For our in vivo sleep-wake experiments, we chose to test confirmed the presence of GFP and Ca9 within TRN by identifying fluorescent markers of sgRNAs (mCherry and Ca9 (GFP)). GFP+/− neurons were identified by green fluorescence in the cytoplasm (excitation/emission 488:509) and Ca9+/− neurons were identified by red fluorescence in the cytoplasm (excitation/emission 590:617). Within that region, we calculated the percentage of targets based on an average of two sections per brain. mCherry signals marking AAV transduction were consistently found within the anterior-posterior border, Bregma −0.8 ± 0.2 mm. Target areas were measured using the Fiji free-hand tool (https://imagej.net/Fiji) which reports a manually drawn area in pixels. We then drew an area around the GFP+/− TRN region and measured all cells within that region. GFP+− neurons were then counted as per the Fiji manual. We first counted all the GFP+− cells within the TRN region, then we counted all the cells that were both mCherry+− and GFP+− within that region. Percentages represent the number of double labeled (mCherry+−GFP+−) cells divided by total number of GFP+− cells within the TRN. Mice were considered successful cases if any TRN region was found to be positive for both mCherry and GFP. All successful cases were used to quantify transduction, as described above. In three cases, no signal for mCherry was found within the GFP+/− TRN region and were excluded from the cohort, in one mouse transduction efficiency was 63.5% (area) and 66.4% (cells). The in vivo data from these four mice were used only in the serendipitous control of Supplementary Fig. S4.

In vitro slice electrophysiology. Adult (3–8 Month) mice were deeply anesthetized with isoflurane and decapitated at 24 h. To knockdown c3 subunits in TRN, mice were injected at 2.5 Months and slices were prepared 4–5 weeks later. Coronal brain sections containing TRN (Bregma −0.46 to −0.94 mm) were cut at 300 μm thickness with a Leica VT1200S vibratome (Leica Biosystems Inc., Buffalo Grove, IL, USA) at 4 °C. After slicing, the slices were placed into ACSF containing the following (in mM): 124 NaCl, 1.8 KCl, 25.6 NaHCO3, 1.2 KH2PO4, 2 CaCl2, 1.3 MgSO4, and 10 glucose (300 mM), saturated with 95% O2/5% CO2 for at least 1 h at room temperature before being transferred to the recording chamber and superfused with warm ACSF (32 °C) at 2–3 ml/min. Power spectral density (PSD) and power spectrum density (PSD) recorded powers of the glutamate receptor antagonists (20 μm 6-cyano-7-nitroquinoxaline-2,3-dione +50 μM D-2-(2-amino-phosphonopentanoic acid) using a Multiclamp 700B amplifier and pClamp 10.0 software ( Molecular devices; California, United States.) A 1 min period after 5 min application of the glutamate receptor antagonists was used for statistical analysis. Only well resolved events with amplitudes >10 pA were analyzed (Igor Pro6.2A, WaveMetrics, Inc., Portland, OR, USA). To analyze the decay of sIPSCs, in clampfit 10.2 software (Molecular Devices, LLC, San Jose, CA, USA), each sIPSCs event was detected and normalized according to its negative peak (range of 0.3 ms around the peak). An averaged trace for each cell was computed and the averaged sIPSCs trace was fitted with a two-term exponential function in GraphPad prism 5 (GraphPad Software, San Diego, CA, USA). Series resistance was 6–20 MΩ.
and was not compensated. Sampling rate was 20 kHz. Records were low-pass (Bessel) filtered at 1 kHz.

**Statistics.** We used a Jarque–Bera test to evaluate data distribution before choosing an appropriate inferential test. Normality was never violated and parametric tests were used uniformly. Multiple two-tailed paired t-tests were used to compare BL vs KD areas under delta power or other delta metrics. Two-tailed paired t-test in bout analyses and spindle analyses, and an unpaired t-test to compare control vs KD sIPSCs. All statistics were performed in MATLAB, GraphPad Prism5 or Microsoft Excel.

**Data availability**

Raw in vivo electrophysiologic data used in this study are available from the Open Science Framework repository at https://osf.io/uf2ca/. Raw in vitro electrophysiologic data used in this study are available from the Open Science Framework repository at https://osf.io/2k8v6/. A Source Data file is provided with this paper.

**Code availability**

Custom code central and original to this manuscript are available from the Open Science Framework repository at https://osf.io/uf2ca/.

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Competing interests

D.S.U., J.T.M., J.M.M., R.E.S. and R.B. are Research Health Scientists at VA Boston Healthcare System, West Roxbury, MA. The contents of this work do not represent the views of the US Department of Veterans Affairs or the United States Government. J.T.M. received partial salary compensation and funding from Merck MISP (Merck Investigator Sponsored Programs) but has no conflict of interest with this work. Other authors declare no competing interests.

Additional information

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