Title: CRISPR knockdown of $GABA_A$ alpha3 subunits on thalamic reticular neurons enhances deep sleep

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Abstract:

Identification of mechanisms which increase deep sleep could lead to novel treatments which promote the restorative effects of sleep. Here, knockdown of the α3 $GABA_A$-receptor subunit from parvalbumin neurons in the thalamic reticular nucleus using CRISPR-Cas9 gene editing increased non-rapid-eye-movement (NREM) sleep and the thalamocortical delta oscillations implicated in many health-promoting effects of sleep. Inhibitory synaptic currents were strongly reduced *in vitro*. Effects were selective to the mouse sleep (light) period. Further analysis identified a novel deep-sleep state in mice prior to NREM-REM transitions which was preferentially affected by deletion of α3 subunits. Our results identify a functional role for $GABA_A$ receptors on TRN neurons and suggest antagonism of α3 subunits as a strategy to enhance deep sleep.

One Sentence Summary:

Selective genetic knockdown of the major α subunit of $GABA_A$ receptors present in the thalamic reticular nucleus enhanced deep sleep in mice.

Main Text:

Sleep is vital for maintaining physical and mental well-being. In particular, thalamocortical delta (0.5-4 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 (*1–4*). 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 (*5–8*). Although hypnotic agents which potentiate the activity of $GABA_A$ receptors promote sleep induction, they also reduce delta oscillations, suggesting that a subset of $GABA_A$ receptors prevents deep restorative sleep (*9*, *10*). Thus, identification and elimination of this confounding effect of the most widely used hypnotics, which target $GABA_A$ receptors, could be beneficial in developing sleep drugs which boost the positive effects of sleep. Recent work showed the thalamic reticular nucleus (TRN) plays a role in modulating delta power (*11*). Here we used state-of-the-art CRISPR-Cas9 gene editing to test the hypothesis that $GABA_A$ receptors on TRN neurons suppress NREM sleep delta oscillations.

In the adult brain, most $GABA_ARs$ consist of two α subunits (α1–6), two β subunits (β1–3), and one γ subunit (γ1–3)(*12*) (**Fig. 1A**). In the mouse thalamus, all synaptic $GABA_ARs$ in thalamocortical relay nuclei contain α1, whereas $GABA_ARs$ in the TRN contain α3 (*13*). 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**). We then constructed an adeno-associated viral (AAV) vector to target the obligatory 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**).

α3KD in TRN PV neurons resulted in a significant increase in NREM sleep time (n=7 histologically confirmed cases; one-tailed paired t-test, t (6) = -2.25, p = 0.03) [means (standard error (SEM): BL = 53.02 (1.68), α3KD = 56.77 (1.92)] and a decrease in wakefulness during the light period when compared with baseline (BL) recordings (**Fig. 1C, D**). REM sleep was unchanged. Power spectral analysis of NREM sleep oscillations revealed a marked change in the quality of NREM sleep, whereby delta power was significantly higher in the α3KD condition (**Fig. 1E**; one-tailed paired t-test, t (6) = -2.4, p = 0.027; means (SEM): BL = 38.9(4.8), α3KD = 47.06(2.2)]). We found no change in any other frequency bands in NREM, and no changes in any frequency bands of wakefulness or REM sleep (**Fig. S1**). We observed a significant reduction in the proportion of shortest bout durations (**Fig. S2**), suggesting more consolidated NREM sleep following α3KD. Analysis of sleep spindles using a recently validated algorithm (*14*) did not identify any difference in spindle density, frequency or duration (**Fig. S3**). No significant changes in NREM sleep time were found during the mouse active (dark) period (**Fig. S4**). No NREM or delta effects were observed in three mice that showed no AAV-α3-sgRNA-mCherry transduction in TRN (**Fig. S5**).

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 sleep; humans are more likely to awaken from the lighter stages N1 or N2 (*15*). Thus, to separate lighter from deeper NREM sleep in mice, we analyzed delta oscillations at NREM→REM and NREM→wakefulness transitions. The heightened delta power associated with α3KD was only evident during deeper NREM sleep immediately preceding transitions to REM sleep (**Fig. 1F-H**) [means (standard error (SEM): BL = 2.31 (0.16), α3KD = 2.77 (0.16); 22.3(10.04)% increase; t (6) = 2.18, 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 (**Fig. S6**).

Selective deletion of α3 subunits in TRN PV neurons requires the combination of selective expression of Cas9 in PV neurons and sgRNA targeting α3 subunits in the same cells.

Furthermore, mCherry (red; marker of sgRNA) was expressed in the majority of TRN PV neurons (green and blue) within the core of the injection site (**Fig. 2A**). In the seven α3KD-confirmed mice, we found a high percentage of PV+ TRN neurons (GFP+: 90±4%) were transduced by AAV-α3-sgRNA-mCherry, and a large proportion of TRN (90±4%) area was covered (**Fig. 2B**). The change in NREM time strongly correlated (Pearson correlation; $\rho = 0.74$, $p = 0.03$) to the extent of viral transduction of PV+ TRN neurons (**Fig. 2C**). In 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 with PV antibodies (blue secondary antibodies) (**Fig. 2E**), consistent with the previously published validation of Cas9 selective expression in PV+ neurons in this mouse model (*16*).

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*. 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). To enhance the driving force for chloride, recordings were made using a patch solution with a high chloride concentration. Thus, IPSCs were detected as inward currents. In PV-Cas9 mice, sIPSCs were significantly reduced in recordings from green (PV-Cas9/GFP) and red (transduced with AAV-α3-sgRNA-mCherry) fluorescent TRN neurons [Frequency: PV-tdTomato: 3.47±0.75Hz (N=6 from four animals); PV-Cas9+AAV-α3-sgRNA: 1.01±0.53 (N=5 from four animals); $t(9)=2.560$, $p=0.031$, t-test. Amplitude: PV-tdTomato: -38.4±4.6 pA (N=6); PV-Cas9+AAV- α3-sgRNA : -42.1±7.7 pA (N=3 from two animals, in the other recordings sIPSCs were not detectable), $t(7)=0.4426$, $p=0.67$, t-test] (**Fig. 2D**). Residual sIPSCs in PV+ TRN neurons in PV-Cas9 mice with α3KD retained sensitivity to an α3 selective positive allosteric modulator, TP003 [1µM TP003 increased sIPSC decay time by 13.9±2.1%, $t(2)=7.228$, $p=0.02$, N=3, paired t-test], suggesting that other α subunits were not upregulated in response to the α3KD.

In another group of mice, we performed the same *in vivo* experimental protocol with a control AAV vector targeting the $GABA_AR$ 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 sleep, wakefulness or REM sleep following the α1KD (**Fig. 3A, B**). Moreover, there was no change in the quality of NREM sleep, as measured by delta wave power (**Fig. 3C**). Indeed, no frequency bands of NREM sleep, wakefulness or REM sleep were altered. The time-frequency analysis at NREM-REM transitions (**Fig. 3D-F**) and at NREM-Wake transitions (**Fig. 3G-I**) showed no changes. Our histologic protocol confirmed a similar degree of targeting success (**Fig. S7**).

In this study, we used cell-type and region-specific CRISPR-Cas9 gene editing *in vivo* to test the functional role of GABAergic inhibition onto TRN neurons in controlling sleep physiology. To the best of our knowledge this is the first time that CRISPR-Cas9 technology has been used *in vivo* to alter thalamocortical oscillations. We found that knockdown of α3-containing GABA receptors, confirmed using *in vitro* recording of sIPSCs, increases NREM sleep time and the power of NREM delta oscillations during the sleep period of mice. Our novel analyses identified a deep sleep state prior to NREM-REM transitions which was particularly

strongly affected by α3KD. The selectivity of our manipulations was confirmed by our control experiments targeting a closely related subunit, α1, 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 (*11*). TRN neurons receive GABAergic inputs from basal forebrain, lateral hypothalamus and globus pallidus, and several of these neuronal groups maintain a high discharge rate during NREM sleep (*17–19*). Thus, withdrawal of these inputs by removing their postsynaptic targets will lead to a higher discharge rate of TRN neurons during NREM sleep, particularly during deeper stages of NREM prior to REM sleep when excitatory inputs from brainstem aminergic cell groups wane. In turn, increased activity of TRN GABAergic neurons will lead to hyperpolarization of thalamocortical relay neurons, bringing their membrane potential into the correct range to generate delta oscillations. This interpretation of our results is consistent with previous work which suggested that modulating the polarization level and discharge rate of TRN neurons affects delta oscillations and NREM sleep (*11*, *17*, *18*, *20–22*).

Our findings differ from previous work which examined constitutive global α3 subunit KO mice (*23*). However, in the constitutive knockout 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, suggesting a 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 residual sIPSCs retaining 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.

In conclusion, CRISPR-Cas9 cell and region-specific gene editing of α3 subunits in adult mice identified a functional role of $GABA_A$ receptors on TRN PV neurons in regulating deep NREM sleep. Pharmacological agents which allosterically increase the activity of $GABA_A$ receptors containing the α1 or α3 subunits are widely used hypnotics. Unfortunately, they promote light sleep with reduced delta power (*9*, *10*, *24*). Our results suggest that the delta suppressing effect of z-drugs comes from potentiating the α3 containing $GABA_A$Rs of the TRN (i.e. the opposite effect that we report here; α3KD leads to increased delta power). Clinically, this is a problem because high NREM delta waves of deep sleep are restorative, important for memory consolidation (*25*) and clearance of toxic metabolites (*3*). Here knockdown of α3 subunits on TRN neurons enhanced deep sleep while not negatively affecting other sleep oscillations or wake power spectra. Thus, our results suggest that a pharmacological agent which antagonizes α3 subunits, either on its own or in combination with positive allosteric agonism of α1 subunits would be an ideal hypnotic to promote deep, restorative sleep.

References and Notes:

1. G. Tononi, C. Cirelli, Sleep function and synaptic homeostasis. *Sleep Med. Rev.* **10**, 49–62 (2006).

2. M. Dworak, R. W. McCarley, T. Kim, A. V Kalinchuk, R. Basheer, Sleep and brain energy levels: ATP changes during sleep. *J. Neurosci.* **30**, 9007–9016 (2010).

3. L. Xie, H. Kang, Q. Xu, M. J. Chen, Y. Liao, M. Thiyagarajan, J. O'Donnell, D. J. Christensen, C. Nicholson, J. J. Iliff, T. Takano, R. Deane, M. Nedergaard, Sleep Drives Metabolite Clearance from the Adult Brain. *Science.* **342**, 373–377 (2013).

4. M. R. Dolsen, P. Cheng, J. T. Arnedt, L. Swanson, M. D. Casement, H. S. Kim, J. R. Goldschmied, R. F. Hoffmann, R. Armitage, P. J. Deldin, Neurophysiological correlates of suicidal ideation in major depressive disorder: Hyperarousal during sleep. *J. Affect. Disord.* **212**, 160–166 (2017).

5. E. Stepanski, J. Lamphere, P. Badia, F. Zorick, T. Roth, Sleep fragmentation and daytime sleepiness. *Sleep*. **7**, 18–26 (1984).

6. J. G. Ellis, M. L. Perlis, L. F. Neale, C. A. Espie, C. H. Bastien, The natural history of insomnia: focus on prevalence and incidence of acute insomnia. *J. Psychiatr. Res.* **46**, 1278–1285 (2012).

7. G. P. Luz, T. M. Guimarães, T. E. Weaver, L. E. Nery, L. O. E Silva, L. Badke, G. Coelho, A. Millani-Carneiro, S. Tufik, L. Bittencourt, Impaired sustained attention and lapses are present in patients with mild obstructive sleep apnea. *Sleep Breath.* **20**, 681–687 (2016).

8. S. A. Sands, P. I. Terrill, B. A. Edwards, L. Taranto Montemurro, A. Azarbarzin, M. Marques, C. M. de Melo, S. H. Loring, J. P. Butler, D. P. White, A. Wellman, Quantifying the Arousal Threshold Using Polysomnography in Obstructive Sleep Apnea. *Sleep*. **41**, 1–9 (2018).

9. D. S. Uygun, Z. Ye, A. Y. Zecharia, E. C. Harding, X. Yu, R. Yustos, A. L. Vyssotski, S. G. Brickley, N. P. Franks, W. Wisden, Bottom-Up versus Top-Down Induction of Sleep by Zolpidem Acting on Histaminergic and Neocortex Neurons. *J. Neurosci.* **36**, 11171–11184 (2016).

10. C. Alexandre, A. Dordal, R. Aixendri, A. Guzman, M. Hamon, J. Adrien, Sleep-Stabilizing Effects of E-6199, Compared to Zopiclone, Zolpidem and THIP in Mice. *Sleep*. **31**, 259–270 (2008).

11. L. D. Lewis, J. Voigts, F. J. Flores, L. I. Schmitt, M. A. Wilson, M. M. Halassa, E. N. Brown, Thalamic reticular nucleus induces fast and local modulation of arousal state. *Elife*. **4**, 1–23 (2015).

12. R. W. Olsen, W. Sieghart, GABA A receptors: subtypes provide diversity of function and

pharmacology. *Neuropharmacology*. **56**, 141–148 (2009).

13. H. Hörtnagl, R. O. Tasan, A. Wieselthaler, E. Kirchmair, W. Sieghart, G. Sperk, Patterns of mRNA and protein expression for 12 GABAA receptor subunits in the mouse brain. *Neuroscience*. **236**, 345–372 (2013).

14. D. S. Uygun, F. Katsuki, Y. Bolortuya, D. D. Aguilar, J. T. McKenna, S. Thankachan, R. W. McCarley, R. Basheer, R. E. Brown, R. E. Strecker, J. M. McNally, Validation of an automated sleep spindle detection method for mouse electroencephalography. *Sleep*. **42**, 1–13 (2019).

15. M. H. Kryger, T. Roth, W. C. Dement, *Principles and Practice of Sleep Medicine* (Elsevier, 2005; https://linkinghub.elsevier.com/retrieve/pii/B0721607977X50010).

16. R. J. Platt, S. Chen, Y. Zhou, M. J. Yim, L. Swiech, H. R. Kempton, J. E. Dahlman, O. Parnas, T. M. Eisenhaure, M. Jovanovic, D. B. Graham, S. Jhunjhunwala, M. Heidenreich, R. J. Xavier, R. Langer, D. G. Anderson, N. Hacohen, A. Regev, G. Feng, P. A. Sharp, F. Zhang, CRISPR-Cas9 knockin mice for genome editing and cancer modeling. *Cell*. **159**, 440–455 (2014).

17. S. Thankachan, F. Katsuki, J. T. McKenna, C. Yang, C. Shukla, K. Deisseroth, D. S. Uygun, R. E. Strecker, R. E. Brown, J. M. McNally, R. Basheer, Thalamic Reticular Nucleus Parvalbumin Neurons Regulate Sleep Spindles and Electrophysiological Aspects of Schizophrenia in Mice. *Sci. Rep.* **9**, 1–16 (2019).

18. C. G. Herrera, M. C. Cadavieco, S. Jego, A. Ponomarenko, T. Korotkova, A. Adamantidis, Hypothalamic feedforward inhibition of thalamocortical network controls arousal and consciousness. *Nat. Neurosci.* **19**, 290–298 (2016).

19. A. Clemente-Perez, S. R. Makinson, B. Higashikubo, S. Brovarney, F. S. Cho, A. Urry, S. S. Holden, M. Wimer, C. Dávid, L. E. Fenno, L. Acsády, K. Deisseroth, J. T. Paz, Distinct Thalamic Reticular Cell Types Differentially Modulate Normal and Pathological Cortical Rhythms. *Cell Rep.* **19**, 2130–2142 (2017).

20. F. Espinosa, M. A. Torres-Vega, G. A. Marks, R. H. Joho, Ablation of Kv3.1 and Kv3.3 Potassium Channels Disrupts Thalamocortical Oscillations In Vitro and In Vivo. *J. Neurosci.* **28**, 5570–5581 (2008).

21. R. H. Joho, C. S. Ho, G. A. Marks, Increased gamma- and decreased delta-oscillations in a mouse deficient for a potassium channel expressed in fast-spiking interneurons. *J. Neurophysiol.* **82**, 1855–1864 (1999).

22. R. Ochoa-Sanchez, S. Comai, B. Lacoste, F. R. Bambico, S. Dominguez-Lopez, G. Spadoni, S. Rivara, A. Bedini, D. Angeloni, F. Fraschini, M. Mor, G. Tarzia, L. Descarries, G. Gobbi, Promotion of Non-Rapid Eye Movement Sleep and Activation of Reticular Thalamic Neurons by a Novel MT2 Melatonin Receptor Ligand. *J. Neurosci.* **31**, 18439–18452 (2011).

23. R. Winsky-Sommerer, A. Knapman, D. E. Fedele, C. M. Schofield, V. V. Vyazovskiy, U. Rudolph, J. R. Huguenard, J.-M. Fritschy, I. Tobler, Normal sleep homeostasis and lack of epilepsy phenotype in GABAA receptor α3 subunit-knockout mice. *Neuroscience*. **154**, 595–605 (2008).

24. H. P. Landolt, L. A. Finelli, C. Roth, A. Buck, P. Achermann, A. A. Borbély, Zolpidem and sleep deprivation: different effect on EEG power spectra. *J. Sleep Res.* **9**, 175–183 (2000).

25. D. B. Headley, D. Paré, Common oscillatory mechanisms across multiple memory systems. *NPJ Sci. Learn.* **2**, 1–8 (2017).

26. L. Swiech, M. Heidenreich, A. Banerjee, N. Habib, Y. Li, J. Trombetta, M. Sur, F. Zhang, In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nat. Biotechnol.* **33**, 102–106 (2015).

27. S. Bae, J. Park, J.-S. Kim, Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. *Bioinformatics*. **30**, 1473–5 (2014).

28. K. Labun, T. G. Montague, M. Krause, Y. N. Torres Cleuren, H. Tjeldnes, E. Valen, CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* **47**, W171–W174 (2019).

29. M. J. Prerau, R. E. Brown, M. T. Bianchi, J. M. Ellenbogen, P. L. Purdon, Sleep Neurophysiological Dynamics Through the Lens of Multitaper Spectral Analysis. *Physiology (Bethesda).* **32**, 60–92 (2017).

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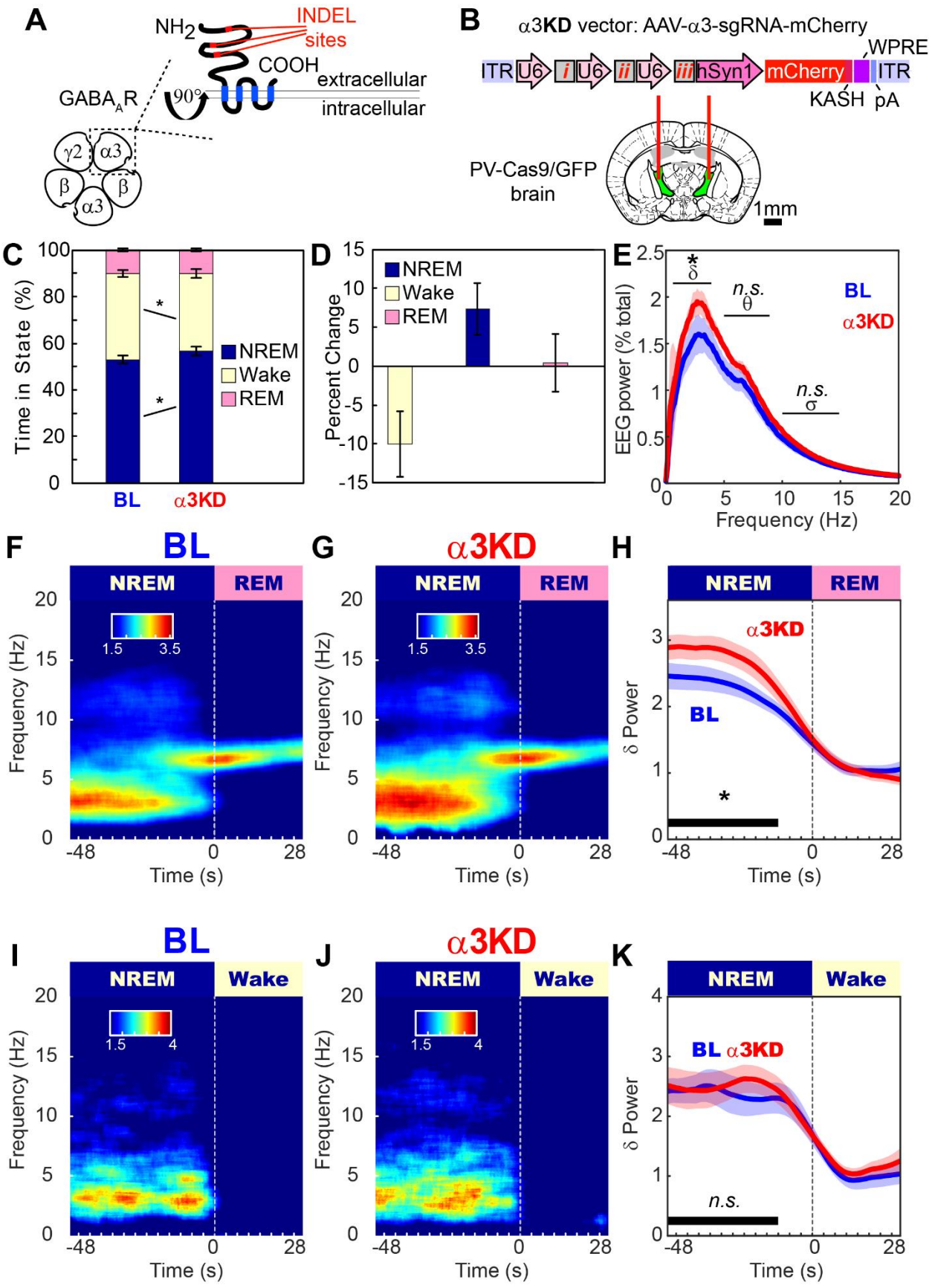

Figure 1. α3KD in PV+TRN neurons increased NREM time, decreased wake time and increased NREM delta power, especially at NREM to REM transitions. A. The $GABA_AR$ is a pentameric heteromeric ion channel. CRISPR-Cas9 abscission was directed to three locations (INDEL sites) of the gene which correspond to the large extracellular region of the α3 subunit, a necessary structural component in $GABA_ARs$ of the TRN. B. Adeno-Associated Viral 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 promoter, were injected into the TRN region of PV-Cas9/GFP mice *in vivo* via guide cannulas. C. Compared with their baseline (BL) levels from before the α3KD was initiated, the mice spent more time in NREM and less time in Wake in the light inactive period. D. Percent change from baseline in wake and NREM shows that wake was reduced and NREM was increased by α3KD but REM sleep was unchanged. E. Compared with their baseline (BL) recordings, α3KD mice had higher NREM delta power, but unchanged sigma or theta power. F. Baseline time-frequency power dynamics presents a surge in delta power in NREM leading to a transition to REM. G. After α3KD, the delta power surge in NREM before a transition to REM was increased. H. Compared with their BL levels (blue), α3KD mice had higher delta power in the NREM before a transition to REM (red). I. BL time-frequency power dynamics presents a surge in delta power in NREM leading to a transition to wake as well. J. α3KD did not increase this delta power surge that occurs during NREM before a transition to wake. K. Compared with BL (blue), α3KD (red) did not correspond to a change in delta power in the NREM before a transition to wake.

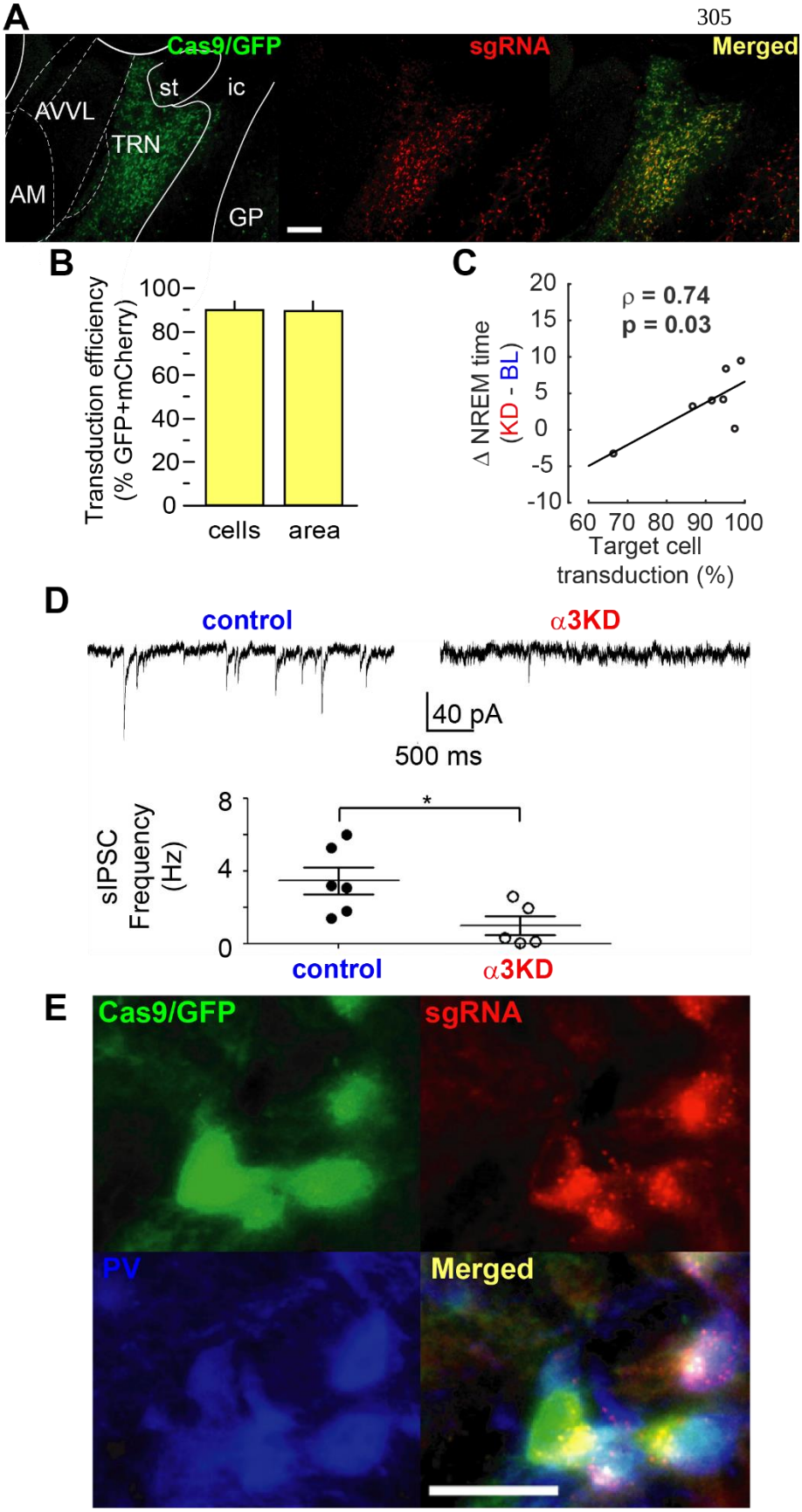

Figure 2. α3KD in PV+ TRN neurons was validated by histology and *in vitro* electrophysiology. A. GFP indicates rich Cas9 expression within the TRN region (green), mCherry reveals widespread transduction of the TRN region by the AAV vector delivering sgRNAs (red) with many of the cells in the area co-expressing both markers (merged; yellow). Scale bar = 200 μm. B. Percentages of target cells and target area that co-express markers reveal widespread delivery of sgRNAs to target cells in the mice used for *in vivo* studies. C. The increase in NREM sleep time evident in the light period was positively correlated with the percentage of PV+ TRN neurons transduced by the AAV expressing sgRNA targeting α3 subunits (GFP+mCherry). D. Compared with BL PV+ TRN neurons without KD (left), sIPSCs in α3KD PV+ TRN neurons were significantly reduced (right), with significantly reduced sIPSC frequency (bottom). E. High magnification imaging shows triple co-localization of Cas9 (GFP; green), sgRNA (mCherry; red) and PV (immunohistochemical stain; blue), demonstrating successful targeting of PV+ neurons within the TRN. Scale bar = 25 μm.

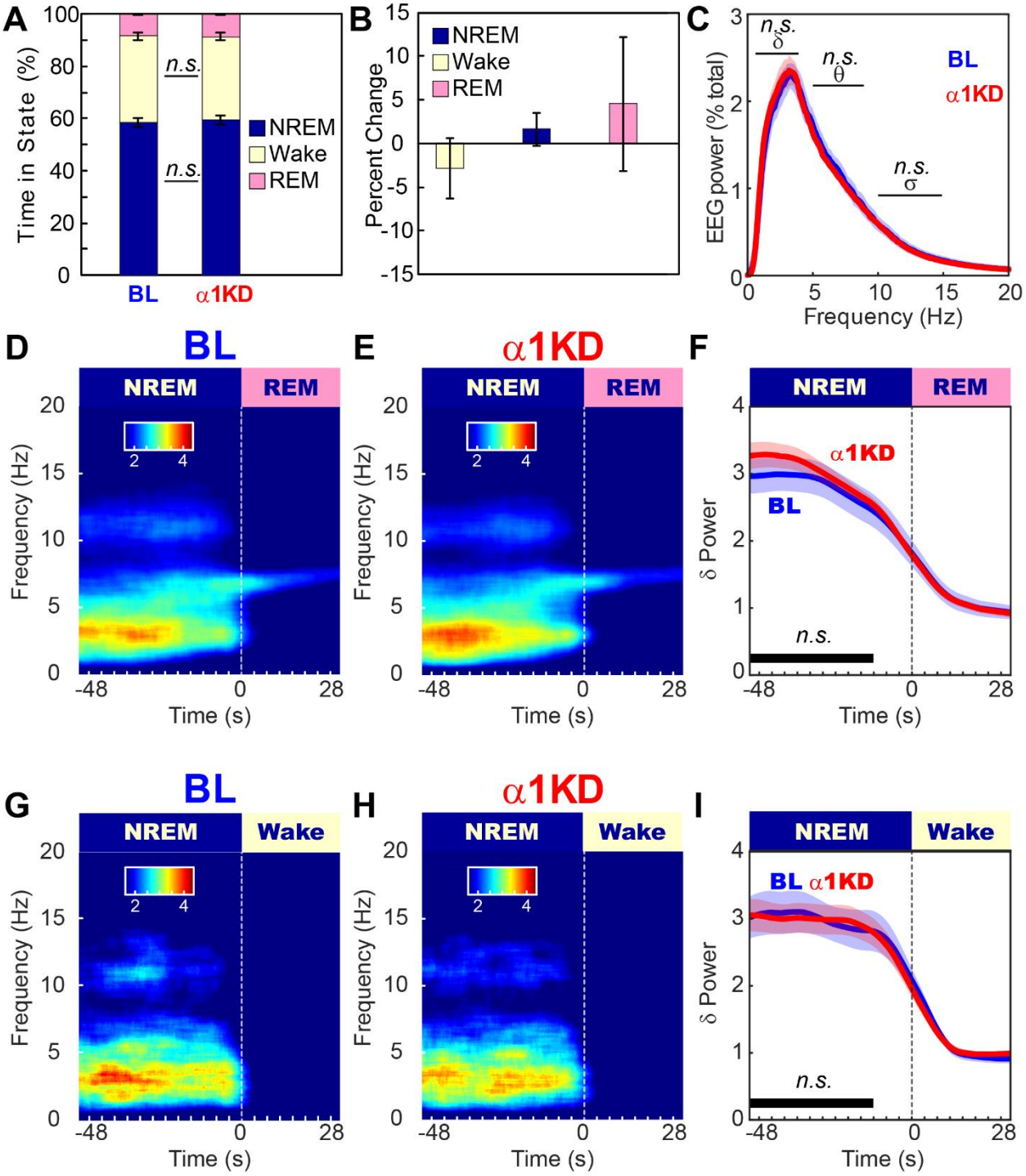

Figure 3. The control cohort with α1KD in PV+TRN neurons displayed no changes to NREM or wake time, or delta power in any states, including transitions from NREM to REM, in the light inactive period. A. Compared with their baseline levels (BL) recorded before the α1KD was initiated, α1KD mice spent the same proportion of time in Wake, NREM and REM sleep. B. Percent change from baseline in wake NREM and REM showed no consistent directional change after the α1KD. C. Compared with their baseline (BL) recordings, α1KD mice had the same amount of NREM delta, sigma and theta power. D. Baseline time-frequency power dynamics reveals a surge in delta power in NREM leading to a transition to REM. E. After α1KD, the delta power surge in NREM before a transition to REM is the same as in baseline records. F. Compared with baseline (blue), α1KD (red) mice had unaltered delta power in the NREM before a transition to REM ($p = 0.27$). G. Baseline time-frequency power dynamics reveals a surge in delta power in NREM leading to a transition to wake. H. α1KD did not increase the delta power surge in NREM before a transition to wake. I. Compared with baseline (blue), α1KD (red) mice had unchanged delta power in the NREM before a transition to wake ($p = 0.49$).