1. Supplementary Discussion

In vivo CRISPR/Cas9 screen reveals key genes regulating electrophysiological diversity in TRN

We sought to identify genes contributing to specific neurophysiological properties of TRN neurons. To screen candidate genes (Supplementary Table S3), we used a pooled AAV-mediated CRISPR/Cas9 *in vivo* knockout approach by combining sgRNA targeting 4 or 5 genes in each pool (Extended Data Fig. 7a). We targeted genes (Extended Data Fig. 7) that are: 1) enriched in either TRN *Spp1*$^+$ or *Ecel1*$^+$ subpopulations, 2) likely important for neurophysiological properties such as ion channels and neurotransmitter receptors, 3) disease risk genes, and/or 4) differentially expressed between TRN and *Pvalb*$^+$ neurons in other brain regions, which were identified from in-house generated single-cell transcriptomic data of hippocampus, motor cortex (M2), somatosensory cortex, striatum as well as from published datasets (mousebrain.org, see Methods)

We tested 33 genes in 7 viral pools and a negative control targeting *Ighe* encoding immunoglobulin epsilon (Extended Data Fig. 7b). Viral pools with AAV U6-sgRNA-Syn1-EGFP were injected into the TRN of transgenic mice that ubiquitously express Cas9 in all cells[64]. Electrophysiological recordings of brain slices were performed 10 days post-injection on virally-infected TRN neurons with high EGFP expression. We classified recorded neurons as *Spp1*$^+$ and *Ecel1*$^+$-like if their electrophysiological parameters fall within the 90% confidence ellipse of *Spp1*$^+$ or *Ecel1*$^+$ neurons from the Patch-Seq data (Extended Data Fig. 8a, b). Two pools – one targeting NMDA receptor and calcium channel-related genes (pool 5), and other one targeting *Ache* and *Hrh3* (pool 7) – caused a significant reduction of hyperpolarization induced rebound bursting compared to the negative control (Extended Data Fig. 8c, d, e). Pool 7 also lowered rebound intra-burst spike frequency (Rb frequency, Supplementary Table S4). Interestingly, pool 3, which included sgRNA for the voltage-gated potassium channels *Kcng1, Kcnc3, Kcng4* and *Kcnd2* as well as the voltage-gated potassium channel interacting protein, *Kcnip1*, caused significant up-regulation of rebound bursting in TRN neurons (Extended Data Fig. 8c, d, e). To determine whether a particular gene in the pool was responsible for the increased rebound bursting, we generated sgRNAs targeting each individual gene in pool 3 for a second round of screening. We found that knockout of *Kcng1* increased bursting firing in *Ecel1*$^+$-like cells. Strikingly, the knockout of *Kcnd2* increased rebound bursting in both *Spp1*$^+$-like and *Ecel1*$^+$-like neurons, without modifying other rebound bursting properties (Extended Data Fig. 9a, b, c, Supplementary Table S5). Single cell sorting and PCR analysis confirmed a high rate of knockout of *Kcnd2* (Extended Data Fig. 9d, e), and deep sequencing of top predicted off-target loci for each of the *Kcnd2* targeting sgRNA showed minimal mutations with the highest site exhibiting indels of 3%. Thus, our results suggest that *Kcnd2* and *Kcng1* may normally limit burst firing in TRN neurons.

By applying pooled loss-of-function CRISPR/Cas9 screens, we were able to identify mediators of the rebound burst firing activity in TRN neurons. We found that knock-out of *Kcnd2*, a voltage gated potassium channel, increased rebound burst firing of TRN neurons. *Kcnd2* gain of function mutations are found in patients with epilepsy, and recently a *de novo* missense variant was identified in twins with autism and severe seizures[65,66]. In addition to *Kcnd2*, our screening has also nominated several other candidates whose perturbation significantly affect TRN function. Therefore, a larger study is warranted to carry out a comprehensive characterization and functional investigations into each key molecule for their

role in TRN circuitry. For example, it will be important to generate humanized animal models carrying *Kcnd2* mutations to gain mechanistic insights into how disrupted TRN function contributes to aberrant TC circuit function in neurological disorders.

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2. Supplementary Methods

CRISPR knockout efficiency and specificity

Primer design for accessing off-target rate

To design primers for quantifying the off-target rate for each sgRNA, the top five genomic off-target sites were identified by Benchling (https://benchling.com). PCR primers were designed (Supplementary Table S8) using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) for each site with the amplicon length constrained to be shorter than 350bp.

Cell isolation, nucleus isolation, and FACS Sorting for bulk cell/nuclei collection

For cell isolation, a protease digestion of slices method[67] was adapted. Briefly, coronal slices of 300µm thickness were obtained from mice injected with AAV:ITR-U6-sgRNA(Kcnd2)-hSyn-EGFP-KASH-WPRE-shortPA-ITR (AAV-Kcnd2-KO) at 30-35 postnatal days with cold saline. The area of interest was dissected and incubated at 32 °C in Hibernate A -Ca^{2+} (Thermo Fisher Scientific, #A1247501) with papain (Worthington Biochemical Corporation, #PDS2) at a final concentration of 34 U/ml for 1 hour. The tissue was then transferred to the complete medium composed of Hibernate A, 1X B27 Supplement (Thermo Fisher Scientific, MA, USA, #17504044), 1X Glutamax Supplement (Thermo Fisher Scientific, #35050061) and mechanically triturated and gently passed through Pasteur pipettes with tip size around 900 µm and 500µm sequentially. After a homogeneous cell mixture was obtained, 2 ml suspension was transferred to an Eppendorf tube and spun down at 200g for 3 minutes at 4 °C. The pellet was re-suspended in 300 µl PBS with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, #A1933). Lastly, cells were filtered through a 30µm MACS SmartStrainers cell strainer (Miltenyi Biotec, #130-098-458) and diluted to 300 µl before cell sorting.

For nuclei isolation, nuclei were purified from tissue using Nuclei EZ Lysis Buffer (Sigma-Aldrich, NUC-101) as described. Vybrant DyeCycle Violet (Thermo Fisher Scientific, #V35003) was used to stain the nuclei for 5 minutes on ice before FACS sorting.

FACS gating on FSC, SSC and on fluorescent channels was set to include only $Violet^{+}$ and GFP^{+} (for AAV injected nuclei tagged by EGFP-KASH through viral injection). A total of 1.6k cells and 72k nuclei were collected into 200 µl DNA/RNA Shield (Zymo Research, #R1100-50) and kept at -80°C until processing.

Genomic DNA extraction from sorted bulk cells/nuclei
The genomic DNA (gDNA) from FACS sorted cells and nuclei was extracted with Quick-DNA Microprep kit (Zymo Research, #D3020) following manufacturer's protocol. The brain tissue of mice without AAV injection was also extracted for genomic DNA to use as the control.

Whole genome amplification, PCR, and gel purification
The genomic DNA purified from the pooled FACS sorted cells or nuclei was evenly divided and whole-genome amplified using REPLI-g single cell kit (Qiagen, #150343) in order to increase the nuclei/cell number for the analysis of each off-target locus. The whole-genome amplified DNA was mixed according to the nuclei/cell number of initial input DNAs, so that every cell and nucleus was equally represented. Next, each of the off-target candidate locus was amplified in separate reactions using Herculase II fusion DNA polymerase (Agilent Technologies, #600675) supplemented with 8% DMSO and primers listed in Supplementary Table S6. PCR protocols are as follows: 95 °C for 2 min, then 30 or 35 cycles of 95 °C for 20 sec, 55 or 60 °C for 20 sec (optimized for each locus), and 72 °C for 30 sec, followed by 72 °C for 5 min. For gDNA extracted from FACS sorted samples, 300ng mixed whole-genome amplified DNA was used in a PCR reaction. For gDNA extracted from brain tissue, 100 ng of purified genomic DNA was used in a PCR reaction. PCR amplicon (150-200 bp) from each reaction was analyzed on 2% agarose gel and purified using Zymoclean gel DNA recovery kit (Zymo Research, #D4007). The concentrations of purified amplicons were measured using NanoDrop (Thermo Fisher Scientific,) and Qubit (Thermo Fisher Scientific).

Off-target amplicon library construction and sequencing
The amplicons from each off-target candidate locus were pooled in equal molar quantity separately for AAV-Kcnd2-KO and the control. A total of 10 ng of each pooled PCR product was used to construct the Illumina sequencing libraries with reagents from the TruSeq Stranded mRNA Sample Preparation kit (Illumina, #20020594) following the manufacturer's protocol with the modifications: 1) the library construction started at the "Adenylate 3' Ends" step. 2) two barcodes were used for each pooled PCR product to increase the index complexity. 3) half of the ligation products were used in the final PCR enrichment reaction. The libraries were sequenced twice on a MiSeq with 150 bp paired-end reads and 6 base Index 1 read to an averaged depth of 55,000 reads per locus per sample (Supplementary Table S8).

Maximum likelihood estimation for editing rate
Target deep sequencing reads were mapped and identified for indels by using CRISPresso with default setting for paired-end reads[68]. The indel rate for each off-target site was estimated by fitting a binomial model which takes the sequencing and PCR amplification error into account as previously described[69]. Briefly, let the number of reads in sample *i* having target locus *j* counted as having an indel be *x*, the total number of reads covering target locus *j* in sample *i* be *n*, the sequencing and PCR amplification error rate be *p*, the true indel rate be *q*, for control samples, we can write

$$prob(n_i, p_j, q_j) = \binom{n_i}{x_i} p_j^{x_i} (1 - p_j)^{n_i - x_i}$$

and for CRISPR targeted samples,

$$prob(n_i, p_j, q_j) = \left(\frac{n_i(1-q_j)}{x_i - n_i q_j}\right) p_j^{x_i - n_i q_j} (1-p_j)^{n_i - x_i}$$

The maximum-likelihood estimate (MLE) for the frequency of true indels q can be found by maximizing the sample log likelihood

$$LL(p_j, q_j) = \sum_i \quad log(prob(n_i, p_j, q_j))$$

The maximization was evaluated numerically in Matlab (MathWorks).
The upper bound *ub* for q for target locus j was calculated using Wilson score intervals as,

$$ub = \left(\sum n_i q_j + \frac{z^2}{2} + z\sqrt{\sum n_i q_j(1-q_j) + \frac{z^2}{4}}\right) / (\sum n_i + z^2)$$

where z was set to 1.96 for a confidence of 95% and the sum over i is taken over only CRISPR targeted samples.

Primer design for assessing on-target knockout efficiency

We used Sanger sequencing to assess the on-target indels, as the length spanned by the five sgRNAs exceeds the maximum read length by Illumina NGS. To design primers (efficiency primers) for quantifying the on-target knockout efficiency, the genomic sequences containing all five sgRNA target sites for each target was obtained from mouse genome (GRCm38). Amplicons and primers were designed (Supplementary Table S6) using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast), with the amplicon length constrained to be shorter than 1600bp, so that the amplicon can be fully sequenced with Sanger sequencing.

Single Cell/Nucleus collection, cDNA Synthesis and Amplification

We set FACS gating on FSC, SSC and on fluorescent channels to include only Violet$^+$ and GFP$^+$ (for *Kcnd2* targeted nuclei tagged by GFP-KASH through viral injection). We sorted single cell or nucleus into a well in the 96-well PCR plates pre-loaded with 5 µl TCL buffer and 1% 2-Mercaptoethanol in each well and stored at -80°C until processing. We synthesized and amplified cDNA from single cell/nucleus the same way as described in the "Single nucleus RNA library construction and sequencing" section. We used four 96-well plates of single nuclei cDNA generated the same way from whole cortex tissue of a wildtype mouse as the control.

Quantification of on-target efficiency

We amplified *Gapdh* and *Kcnd2* in each cDNA library from both the control and *Kcnd2* targeted sample, with the following PCR setup and programs: KAPA HiFi HotStart ReadyMix 2x mix (Kapa Biosystems, #KK2602) 10µl, ddH_2O 9 µl, cDNA 1 µl, forward and reverse primer pairs (1µM each primer) 2.5 µl. PCR was run as 95 °C 5 min, 35 cycles of 98 °C 20 sec, 62 °C 15 sec, 72 °C 60 sec, and 72 °C 5 min extension. The *Kcnd2* amplicon was used as template in a second round nested PCR for high amplification specificity: KAPA HiFi HotStart ReadyMix 2x mix 10µl, ddH_2O 9 µl, *Kcnd2* amplicons from the first round PCR 1 µl, forward and reverse primer pairs (10µM each primer) 0.6 µl. PCR was run as 95 °C 5 min, 35 cycles of 98 °C 20 sec, 62 °C 15 sec, 72 °C 60 sec, and 72 °C 5 min extension. We sent *Kcnd2* amplicons showing the wildtype length for Sanger sequencing to check for small indels. We excluded cells/nuclei that did not have *Gapdh* amplified and counted cells/nuclei as *Kcnd2* knockout if any of the

following conditions held: (1) *Gapdh* amplified, *Kcnd2* not amplified; (2) *Gapdh* amplified, *Kcnd2* amplicon containing indel shown by Sanger sequencing. We calculated the maximum likelihood estimate (MLE) and Wilcoxon score interval for the on-target efficiency as described in the section "Maximum likelihood estimation for indel rate", with the effect of PCR dropout accounted for by using the indel percentage of the control samples in the estimation.

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3. Supplementary Table S1. Summary of Intrinsic membrane properties of TRN neurons with Patch-seq.

	RMP (mV)	Rm (MOhms)	Tau (ms)	Cm (pF)
Spp1	-56.7±7.9	568.1±377.8	37.6±10.5	79.7±8.3
Ecel1	-58.1±11.8	649.9±387.2	34.8±13.9	63.2±6.8
DP	-58.4±12.5	463.4±288.1	33.6±15.5	82.7±16.5
DN	-60.3±10	508.2±171.3	34.6±16.7	70.8±10.8

Note:
$Spp1^+$ n=29, $Ecel1^+$ n=15, DP n=10, DN n=13 neurons collected from 5 mice.
Data presented as mean ± SD.

4. **Supplementary Table S2. Stereotactic coordinates applied for the thalamic relay nuclei**

Target	AP	ML	DV
VPM	-1.5	1.6	-3.7
POm	-1.7	1.6	-3.2
dLGN	-1.8	2.3	-2.7
LP	-1.7	1.3	-2.6
vMGN	-2.7	2.25	-3.2
dMGN	-2.65	2.25	-2.8
VM	-0.8	0.9	-4.2
VL	-0.2	1.4	-3.6

5. **Supplementary Table S3. Selected genes included in the pooled CRISPR/Cas9 *in vivo* screen.**

Pool #	Gene names
Pool #1	Kcnc2, Cacna1i, Scn4b, Erbb4
Pool #2	Kcnq2, Kcnc1, Kcnn1, Kcns3
Pool #3	Kcng1, Kcnc3, Kcng4, Kcnip1, Kcnd2
Pool #4	Slc6a1, Gabrb1, Gabra3, Syt2
Pool #5	Cacna1g, Cacna1h, Arc, Grin2a, Grin2c, Chrm2
Pool #6	Grm3, Grm7, Grm8, Rgs6, Gprc5b
Pool #7	Slc25a37, Hrh3, Gpm6a, Ache, Itpr2, Trpv2
Negative control	IghE

6. **Supplementary Table S4. Summary of rebound burst properties of TRN neurons following the viral mediated pooled CRISPR/Cas9 *in vivo* screening**

Ecel1	Max burst #	Latency (ms)	Burst AHP (mV)	Rb frequency (Hz)	Duration (ms)
Pool #1	0.66±0.5	207.1±130	13.5±9.5	107.9±27.9	37.6±15.7
Pool #2	0.9±0.5	167.9±84.4	9.3±4.6	49.9±15.4****	98.4±45.8
Pool #3	2±0.8**	141.8±55.8	17.8±8.3	101.5±31.8	54.4±26.8
Pool #4	0.75±0.7	131.7±52.8	9.2±5.8	82.7±34.7	114.6±34.9
Pool #5	0.7±0.5	143.8±73.5	13.9±10.4	60.9±38**	74.6±19.4
Pool #6	1.2±0.6	153.3±60.4	12.6±5.8	93.1±42.2	100.2±51.9
Pool #7	0.4±0.5*	126.9±43.6	5.3±4.5	73±24.2*	122.4±75.5
Ighe (Ctrl)	1±5	121.9±74.2	14.4±8.3	128±35.8	59.5±25.1
Spp1	**Max burst #**	**Latency (ms)**	**Burst AHP (mV)**	**Rb frequency (Hz)**	**Duration (ms)**
Pool #1	0.7±0.5	207.1±130	13.5±9.5	107.9±27.9	37.6±15.7
Pool #2	3.8±0.5	157.2±63.7	28.4±8.1	123.9±42.9	51±21.1
Pool #3	8.4±2**	104.2±42.8	27±9.5	165.5±22.4	41.5±12.4
Pool #4	4.3±1	130.9±58.5	18±7.7	139.3±24	56.4±15.5
Pool #5	3±0.9*	143.6±72.8	27.2±7.9	113.9±27	58.4±14.2
Pool #6	5.7±1.7	228.2±86.4	25.8±4.7	126.9±37.4	58.4±20.1
Pool #7	2.8±2.2*	137.3±51.2	21±5.2	115.8±38.8*	80.7±19.9
Ighe (Ctrl)	5.2±2.2	157.2±62.5	25.1±8.4	133.7±21.6	54.2±24.3

Note: 'Spp1' *Ighe*- n=12, Pool1- n=12, Pool2- n=9, Pool3- n=13, Pool4- n=9, Pool5- n=10, Pool6- n=9, and Pool7- n=10. 'Ecel1' Ighe- n=9, Pool1- n=12, Pool2- n=13, Pool3- n=10, Pool4- n=8, Pool5- n=10, Pool6- n=8, and Pool7- n=9 from 24 mice (3 mice per pool). Each experimental group was compared individually against 'Spp' or 'Ecel1' in the *Ighe* group with two-sided unpaired t-test. No adjustment for multiple test were applicable. 'Spp1' Pool1, $p = 4.87\times10^{7}$; Pool3, p=0.0033; Pool5, p=0.0088; Pool7, p=0.0065. 'Ecel1' Pool3, p=0.0081; Pool7, p=0.023. Data presented as mean ± SD.

7. **Supplementary Table S5. Summary of rebound burst properties of TRN neurons following the viral mediated Pool 3 individual gene knockout with CRISPR/Cas9 system**

Ecel1	Max burst #	Latency (ms)	Burst AHP (mV)	Rb frequency (Hz)	Duration (ms)
Kcng1	1.8±0.8	126.3±27.3	15.6±6.5	102.8±27.2	54.5±9.3
Kcnc3	1±0.7	114.1±27.3	9.7±3.4	80.6±14.1	110.7±26
Kcng4	1.2±0.5	109.9±33.1	11.8±1.6	98.3±62.5	57.5±11.7
Kcnip1	1.7±0.5	119.7±17.2	15.3±7.6	102.9±36.3	68.2±26.3
Kcnd2	1.85±0.9*	104.8±47	19.8±6.7	132.2±49.8	44.8±10.3
Ighe (Ctrl)	1±5	121.9±74.2	14.4±8.3	128±35.8	59.5±25.1
Spp1	**Max burst #**	**Latency (ms)**	**Burst AHP (mV)**	**Rb frequency (Hz)**	**Duration (ms)**
Kcng1	4.9±1.9	152.1±54.8	23.05±8.5	131.5±17.4	66.7±34
Kcnc3	4.5±1.6	183.2±62.4	20.8±5.5	121.9±9.9	68.3±19.9
Kcng4	5±2.3	86.9±20.5	19.7±6.7	150.8±19.9	55.9±18.6
Kcnip1	6.3±1.8	121±38	23.05±7	131.6±16	52.7±20.3
Kcnd2	7.6±1.9**	139.5±62.5	21.5±7	151.1±23.6	58.6±15.6
Ighe (Ctrl)	5.2±2.2	157.2±62.5	25.1±5.5	133.7±21.6	54.2±24.3

Note: 'Spp1' *Kcng1*- n=6, *Kcnc3*- n=6, *Kcng4*- n=5, *Kcnip1*- n=7, *Kcnd2*- n=7. 'Ecel1' *Kcng1*- n=10, *Kcnc3*- n=8, *Kcng4*- n=9, *Kcnip1*- n=11, *Kcnd2*- n=11. Each experimental groups were compared individually against 'Spp' or 'Ecel1' in the *Ighe* group with two-sided unpaired t-test. No adjustment for multiple test were applicable. 'Spp1' *Kcnd2*, p=0.0095. 'Ecel1' *Kcng1*, p=0.0088; *Kcnd2*, p=0.019. Data presented as mean ± SD.

8. **Supplementary Table S6. Primer sequences**

9. **Supplementary Table S7. Design of sgRNA targets**

10. **Supplementary Table S8. CRISPR screen efficiency and specificity**