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Supplemental Information

An Intrinsic Transcriptional Program Underlying Synaptic Scaling during Activity Suppression

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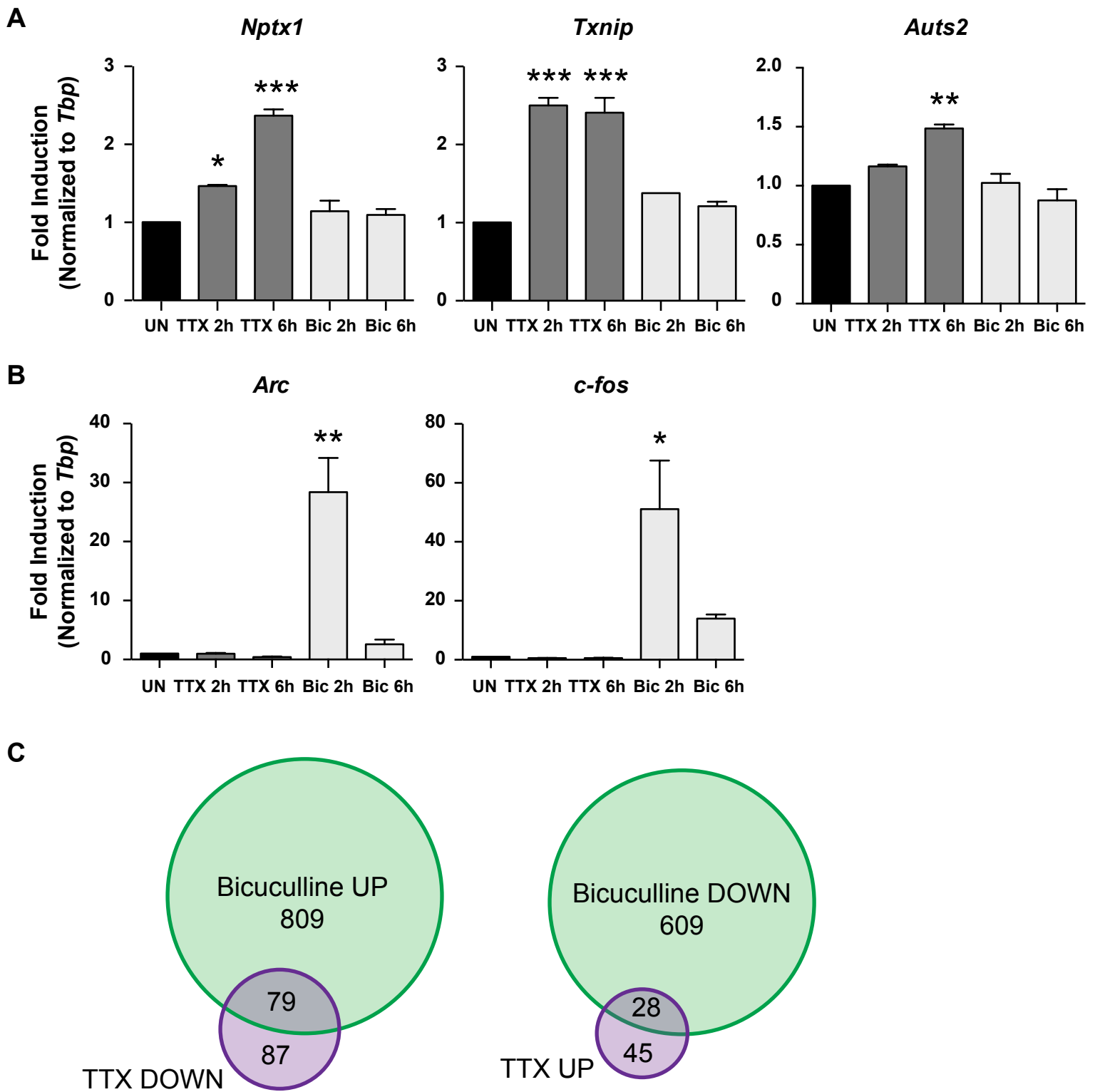


Figure S1. Confirmation of genome-wide data with RT-qPCR, Related to Figure 1. qRT-PCR results of TTX induced genes (**A**) and Bic induced genes (**B**) from cells that were treated with TTX or Bic for the indicated time points. $n = 2$ biological replicates. (**C**) Venn diagrams of bidirectional genes, comparing genes upregulated by bicuculline and downregulated by TTX (left) or genes downregulated by bicuculline and upregulated by TTX. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. p values determined by a one-way ANOVA using Dunnett's multiple comparisons test.

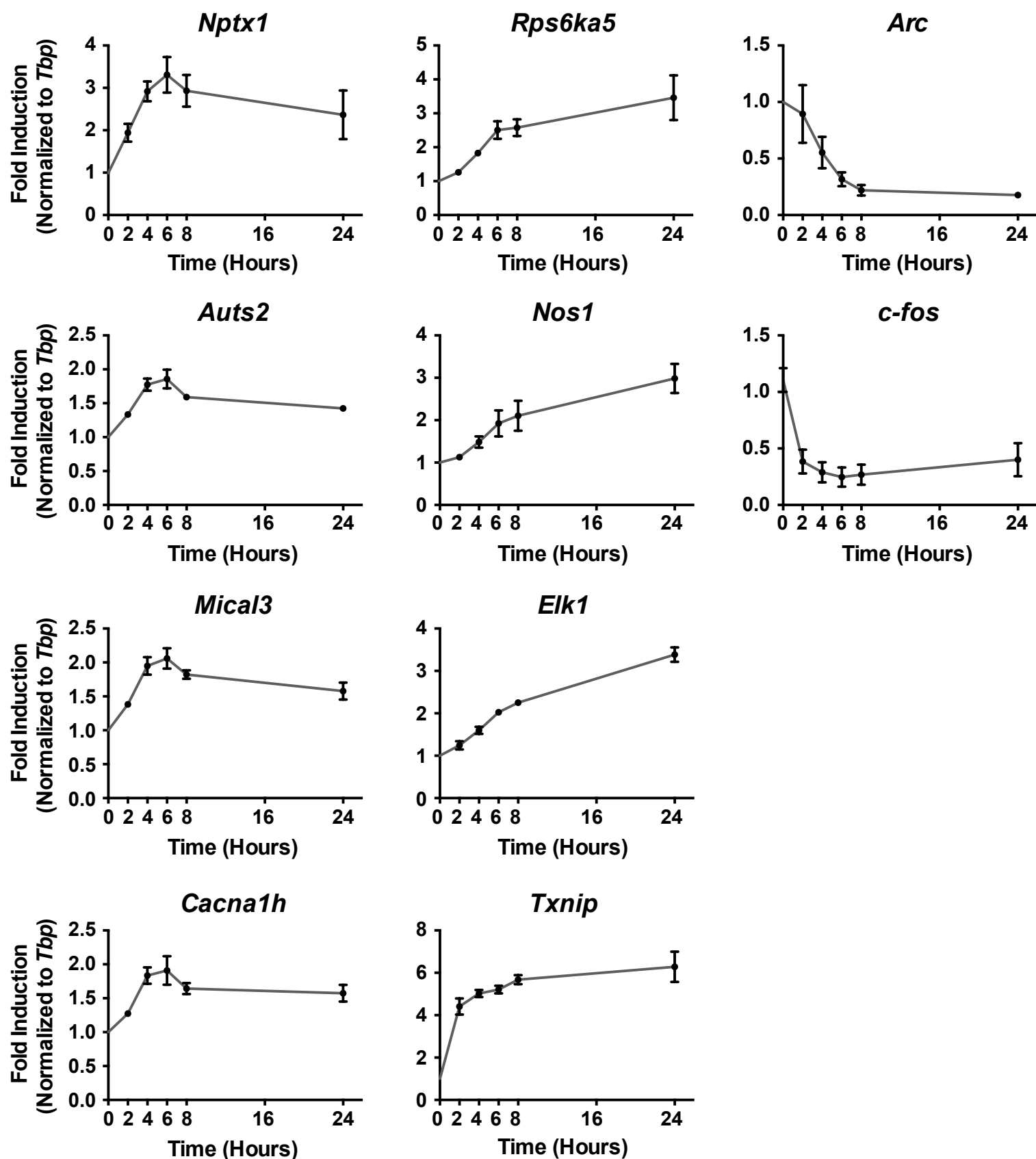


Figure S2. There are two waves of TTX-dependent gene induction, Related to Figure 1. qRT-PCR results from cells that were treated with TTX for the indicated time points. RNA levels were measured for the indicated genes. The columns represent distinct classes of genes based on their induction patterns. The left column contains genes that peak around 6 h of TTX treatment, while the middle column contains genes that continue to rise until 24 h. The right column contains immediate early genes that are induced in response to Bic, and decrease with TTX treatment. $n = 3$ biological replicates. Error bars represent SEM.

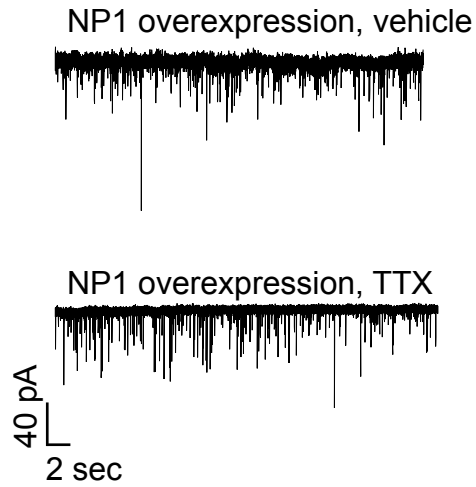
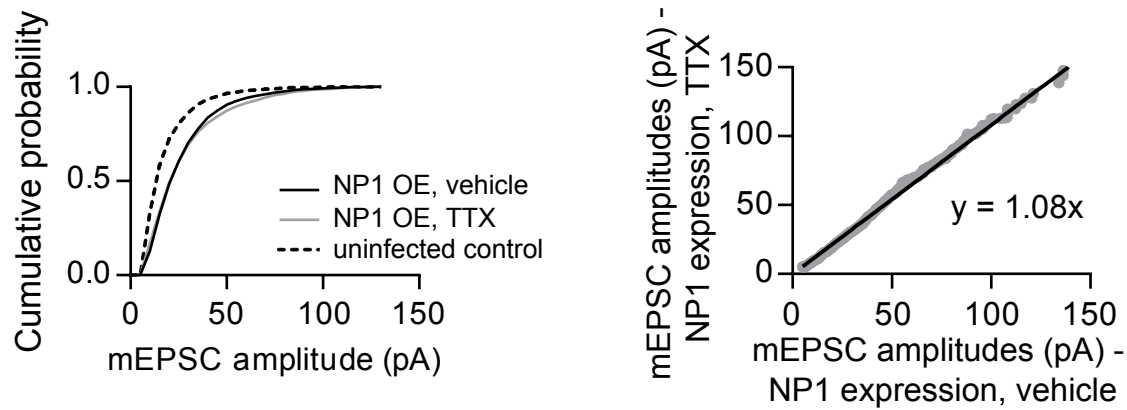
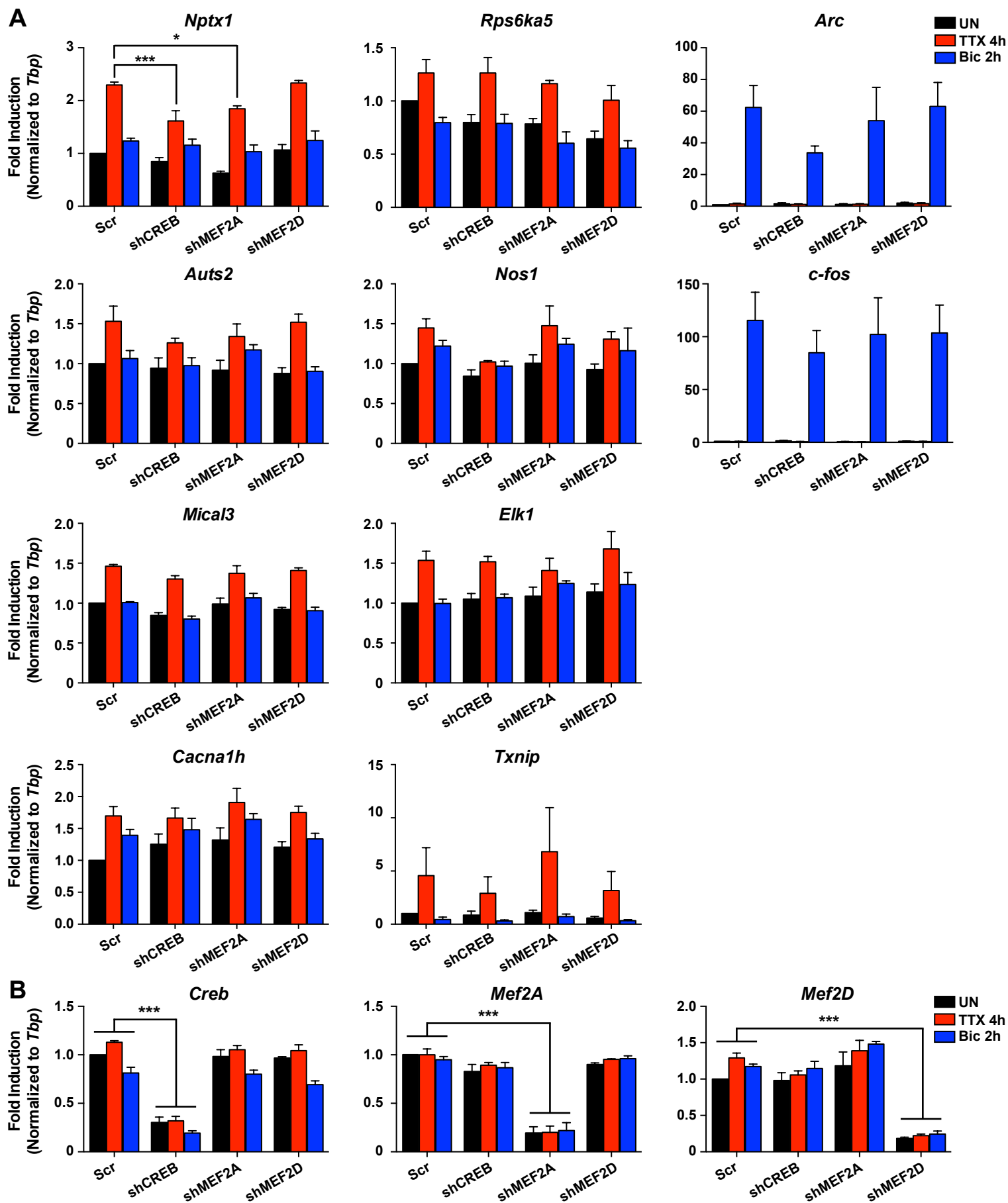
A**B**

Figure S3. Overexpression of *Nptx1* occludes TTX-induced scaling, Related to Figure 3. (A) Example traces of AMPA-mediated mEPSCs from either vehicle or TTX treated cells. (B) Cumulative probability histograms of AMPA-mediated mEPSC amplitudes from cells overexpressing NP1 in either vehicle or TTX treated conditions indicate that TTX-induced scaling is occluded by high NP1 levels. NP1/TTX (n = 11) vs NP1/veh (n = 10), $p = 0.005$, $D = 0.055$.



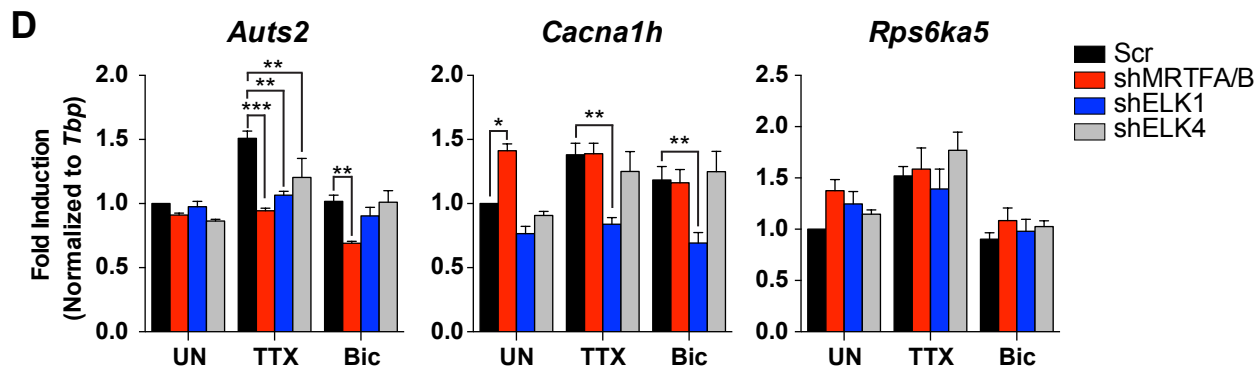
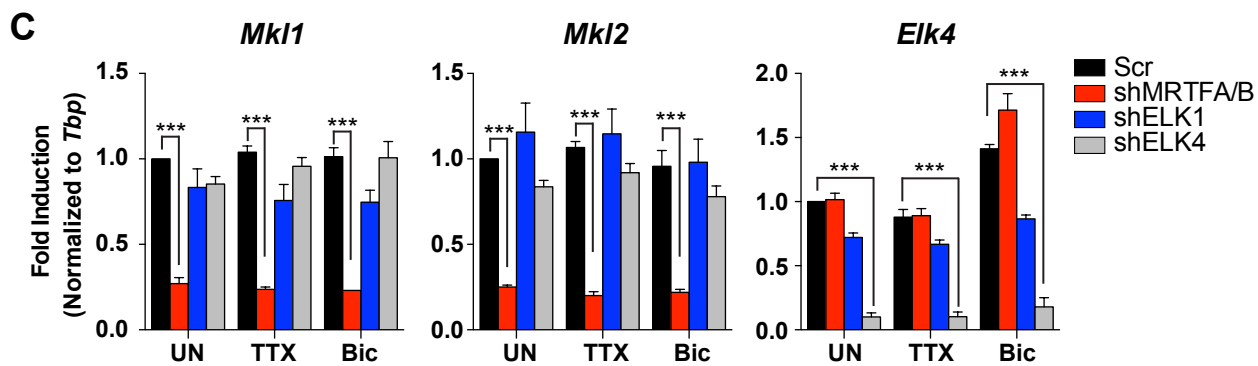
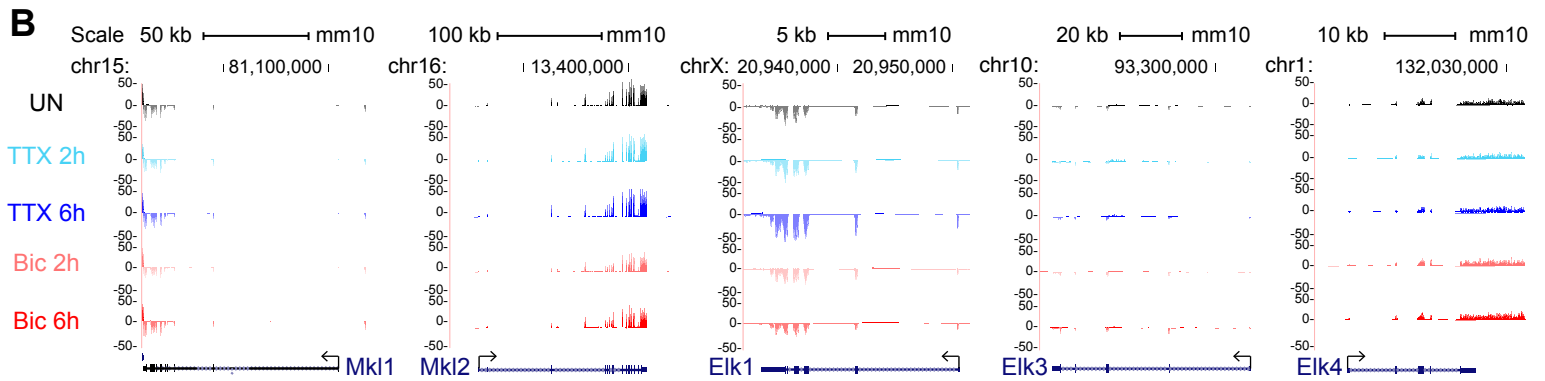
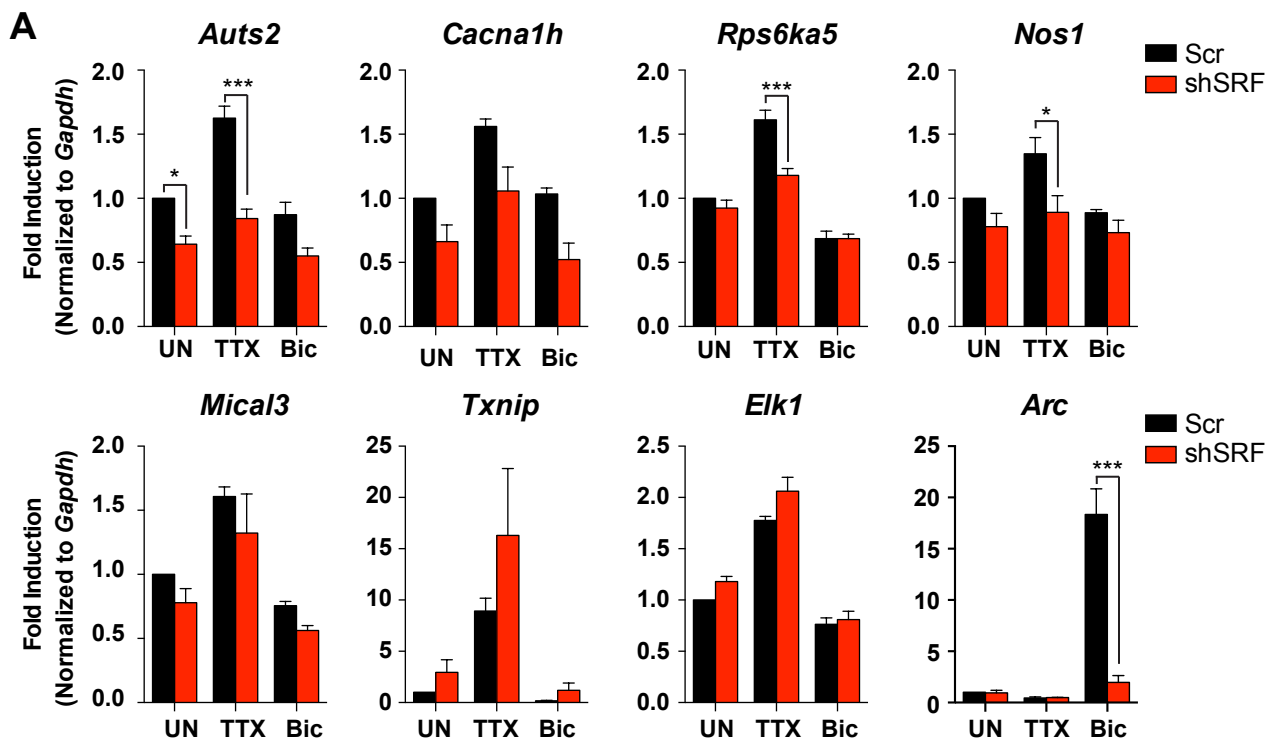


Figure S5. SRF and its cofactor ELK1 regulate the expression of TTX-induced genes, related to Figure 4. (A) qRT-PCR results of TTX-induced genes from cells that were infected with the scrambled control or shSRF knockdown. Cells were left untreated (UN) or treated with TTX or Bic. n = 3 biological replicates. The Bic induced gene *Arc* was tested as a control of knockdown efficiency. (B) UCSC genome browser views of the SRF cofactor family members: *Mkl1*, *Mkl2*, *Elk1*, *Elk3*, and *Elk4* (left to right). (C) qRT-PCR results of the other cofactor family members after knockdown of the indicated family member (shMRTFA/B or shElk4) compared to a scrambled control. Cells were either left untreated or treated with TTX or Bic. (D) qRT-PCR results of TTX-induced genes after the indicated cofactor knockdown. n = 3 biological replicates. *, p < 0.05; **, p < 0.01; ***, p < 0.001. p values determined by a two-way ANOVA with Tukey's multiple comparisons test. Error bars represent SEM.

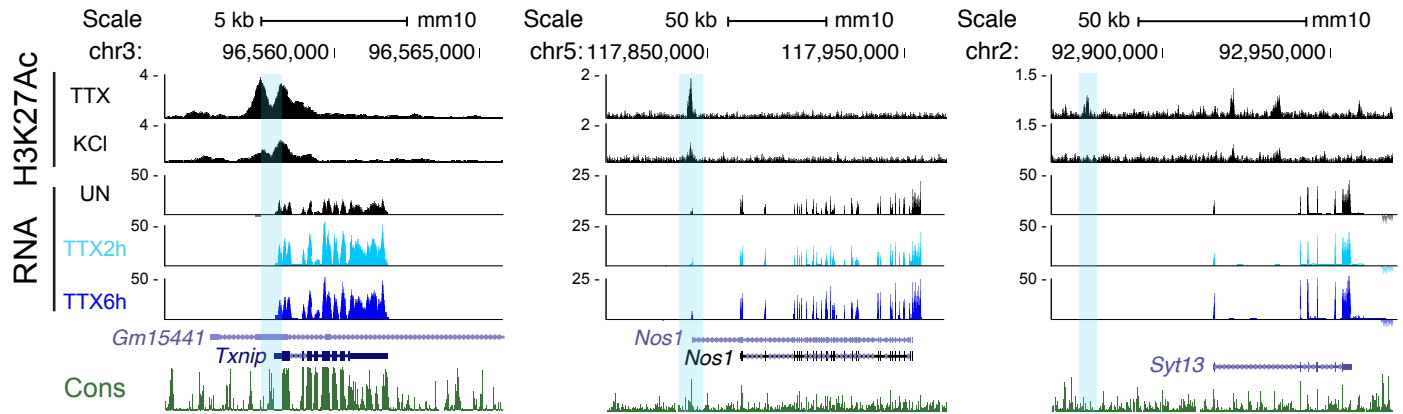
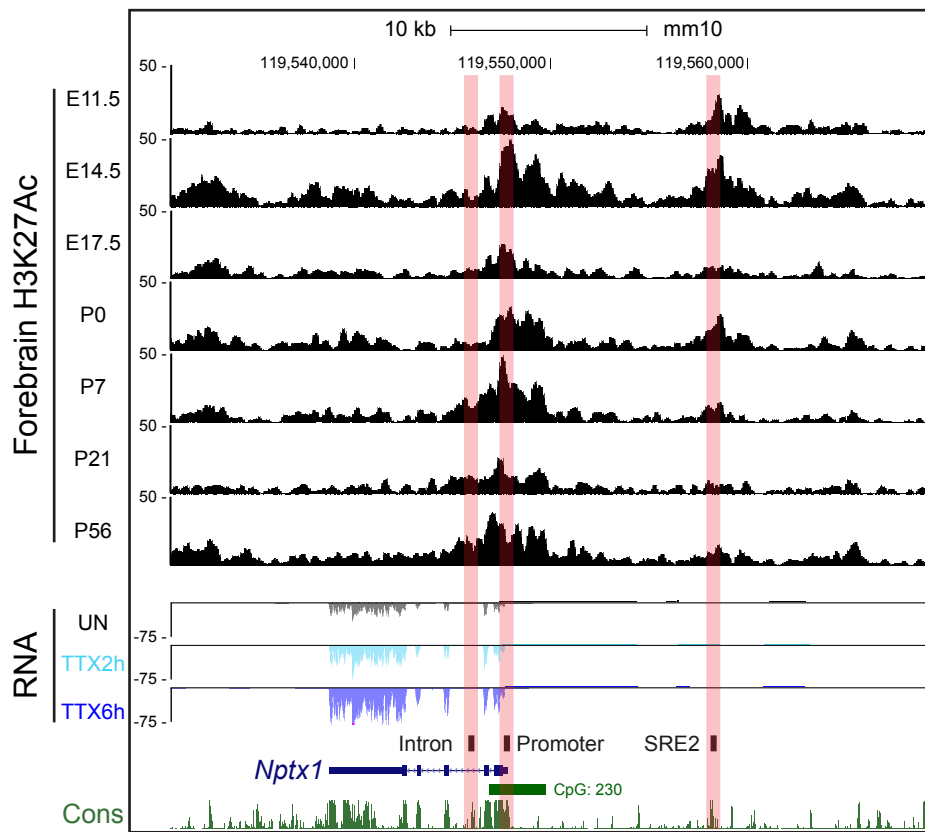
A**B**

Figure S6. H3K27Ac peaks surround TTX up regulated genes, Related to Figure 4. (A) UCSC genome browser views of genes with a decreasing H3K27Ac peak nearby: *Txnip*, *Nos1*, and *Syt13*. The H3K27Ac data from Malik et al., 2014 is aligned with the RNA-seq data for the indicated conditions. Blue bars represent the decreasing H3K27Ac peaks. **(B)** A UCSC genome browser view of the *Nptx1* gene aligned with RNA-seq data from the indicated conditions and H3K27Ac ChIP data from Nord et al., 2013. The H3K27Ac ChIP was performed with forebrain tissue at the indicated time points throughout development. A potential SRE (SRE2) is located within a peak that decreases throughout development, while the peak around the promoter expands into the intronic region (Intron) as development progresses. Red bars highlight the regions amplified in ChIP experiments.

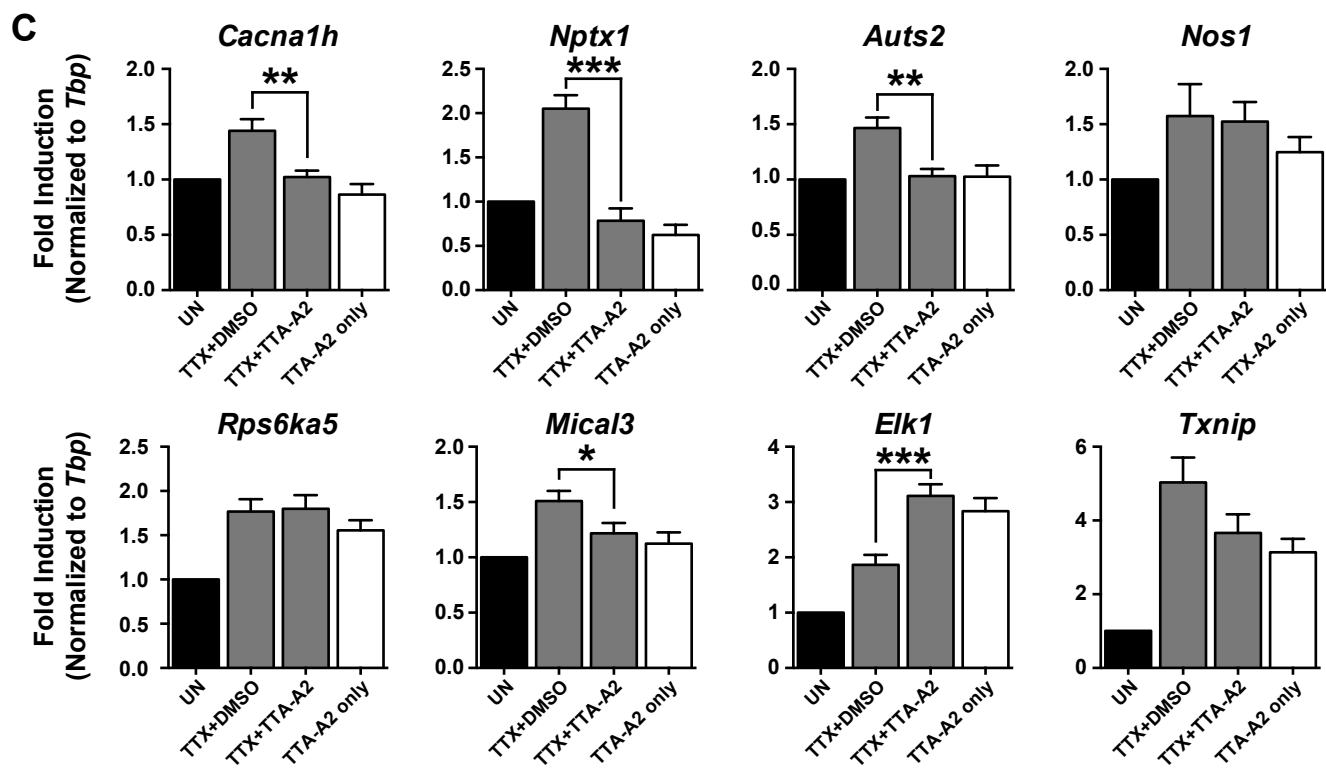
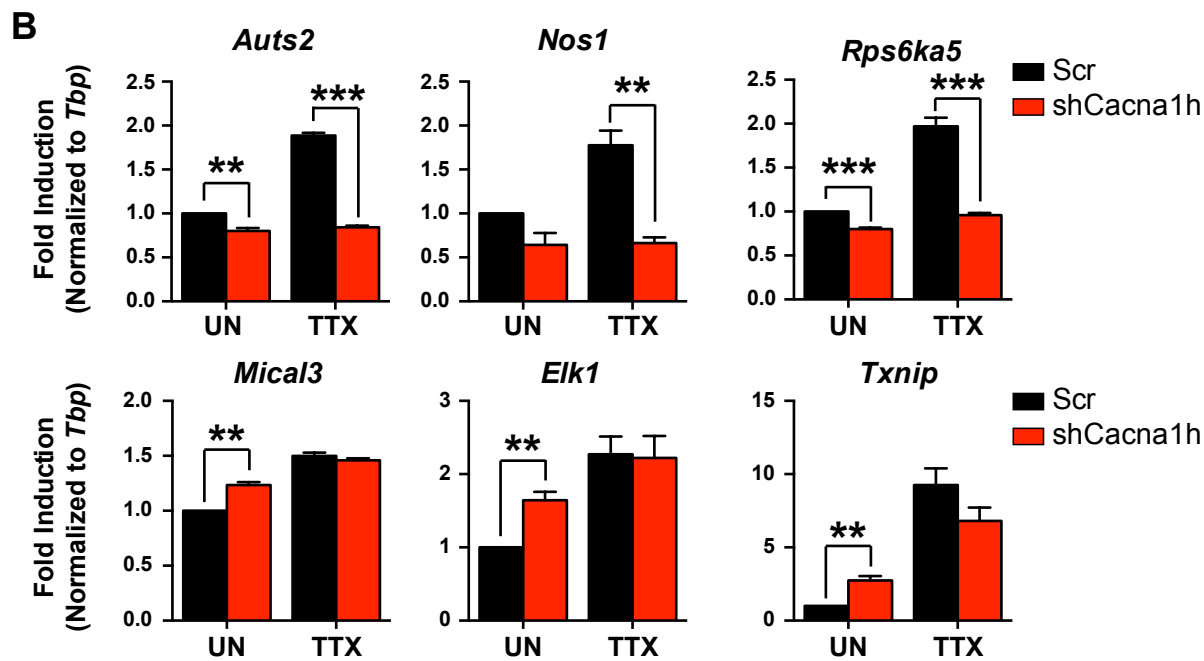
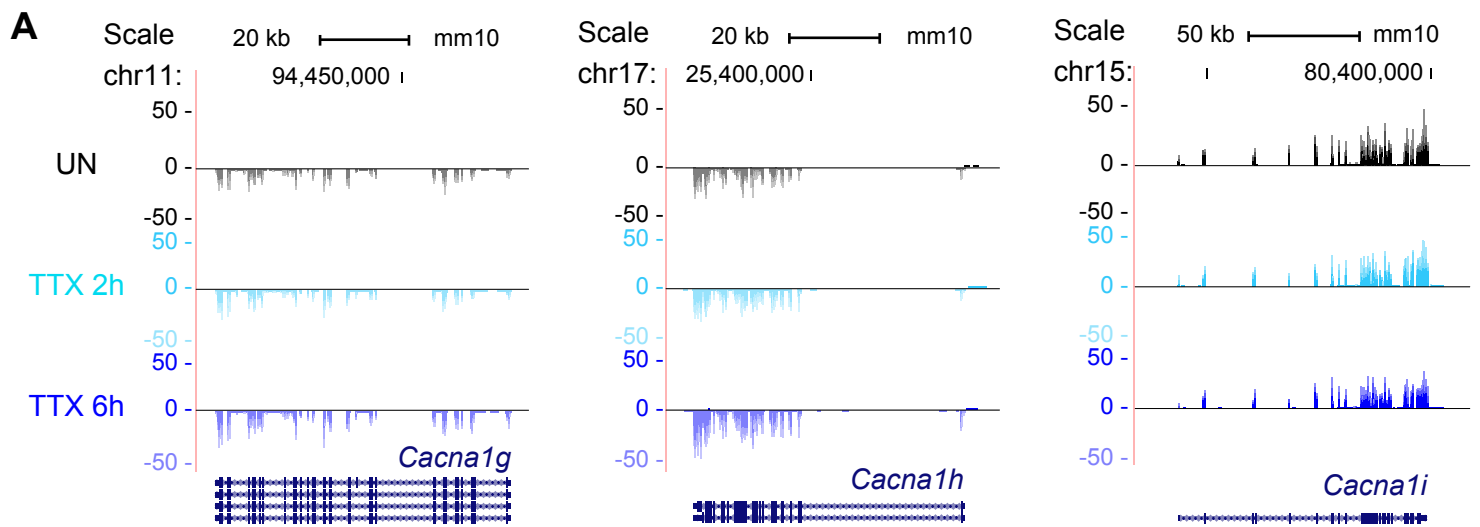
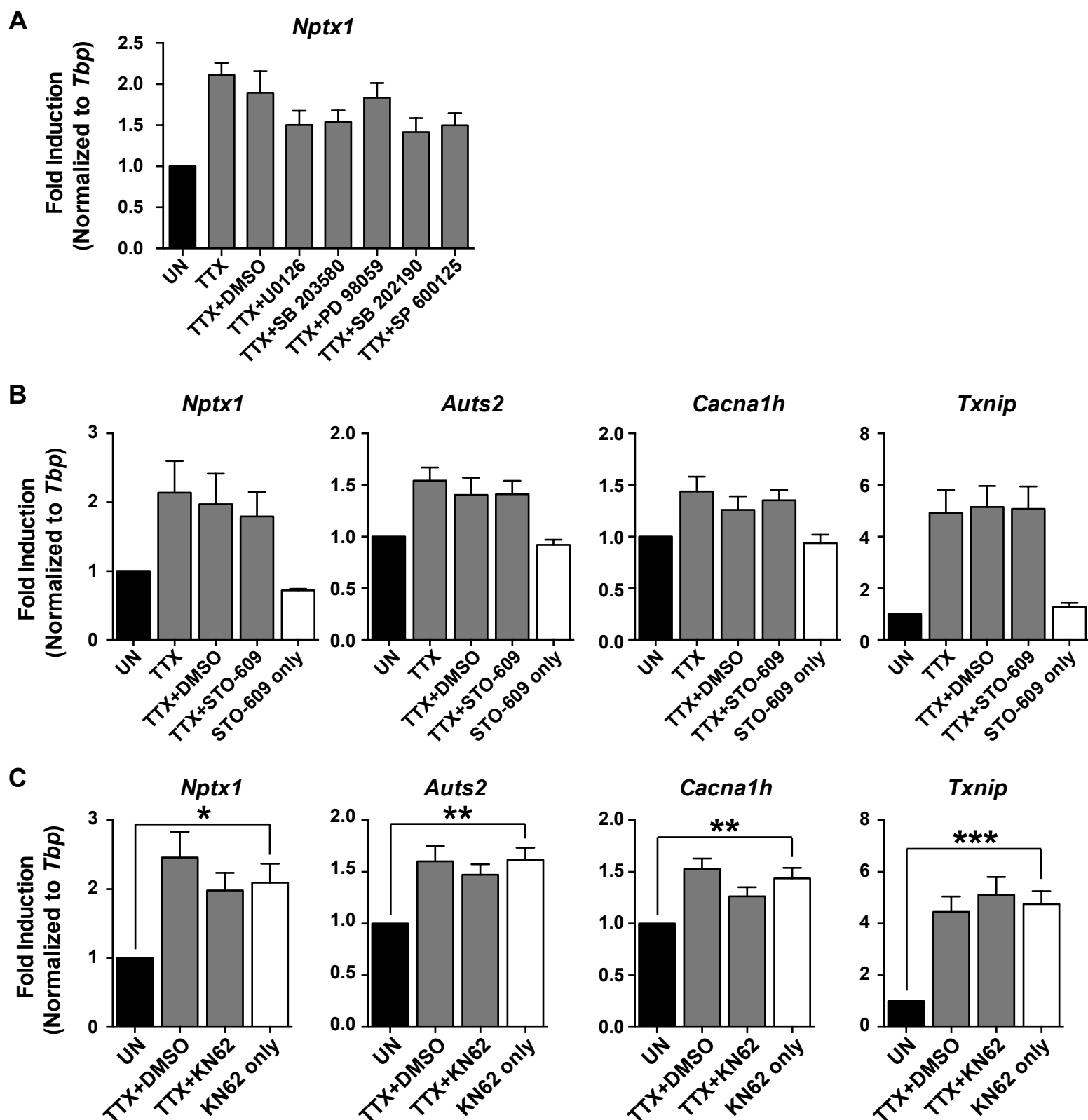


Figure S7. Cortical neurons express all three isoforms of the T-VGCC, related to Figure 5. (A) UCSC genome browser views of the RNA-seq data from cells treated with TTX for 2 or 6 h, aligned with the genomic loci of the three T-VGCC genes: *Cacna1g*, *Cacna1h*, and *Cacna1i*. These genes encode the CaV3.1, CaV3.2, and CaV3.3 subtypes, respectively. **(B)** RNA expression levels of the indicated genes after knockdown of *Cacna1h* (CaV3.2) or infection with a scrambled control, in either untreated or TTX-treated conditions. n = 3 biological replicates. **(C)** RNA expression levels of the indicated genes in response to TTA-A2 treatment. Cells were either untreated (UN), treated with TTX for 6 h pretreated with DMSO or TTA-A2, or treated with TTA-A2 alone. n = 7 biological replicates. *, p < 0.05; **, p < 0.01; ***, p < 0.001. p values for **(B)** were determined by multiple t tests, as performed, where statistical significance was determined using the Holm-Sidak method. p values for **(C)** were determined using a one-way ANOVA using Dunnet's multiple comparisons test. Error bars represent SEM.



Supplemental Table 1, related to Figure 1: Genes that are differentially regulated by TTX and Blicuculline, attached as an excel file.

Supplemental Table 2, related to Figure 1: TTX-induced genes that have been associated with neurodevelopmental disorders (Gene information from SFARI and other published sources)

Gene ID	Gene Name	Associated Disorder	Reference
<i>Auts2</i>	<i>Autism susceptibility candidate 2</i>	ASD, ADHD, epilepsy	Sultana et al., 2002; Kalscheuer et al., 2007
<i>Cacna1h</i>	<i>Calcium channel, voltage-dependent, alpha 1H subunit</i>	ASD	Splawski et al., 2006
<i>Bbs4</i>	<i>Bardet-Biedl syndrome 4</i>	Bardet-Biedl syndrome, ASD	Girirajan et al., 2013
<i>Fan1</i>	<i>FANCD2/FANCI-associated nuclease 1</i>	ASD and schizophrenia	Ionita-Laza et al., 2013
<i>Ogt</i>	<i>O-linked N-acetylglucosamine (GlcNAc) transferase</i>	Maternal stress marker	Howerton et al., 2013
<i>Smg6</i>	<i>Smg-6 homolog, nonsense mediated mRNA decay factor (C. elegans)</i>	ASD, neurodevelopmental disorders	Nguyen et al., 2013
<i>Upf2</i>	<i>UPF2 regulator of nonsense transcripts homolog (yeast)</i>	ASD, neurodevelopmental disorders	Nguyen et al., 2013
<i>Nos1</i>	<i>Nitric Oxide Synthase I</i>	Schizophrenia	Shinkai et al., 2002
<i>Elk1</i>	<i>Elk1, member of ETS oncogene category</i>	Schizophrenia	Sanders et al., 2013
<i>Nptx1</i>	<i>Neuronal pentraxin 1</i>	Bipolar Disorder	Rajkumar et al., 2015
<i>Myt1</i>	<i>Myelin transcription factor 1</i>	Schizophrenia	Fernandez-Enright et al., 2013
<i>Dbp</i>	<i>D site of albumin promoter binding protein 1</i>	Schizophrenia	Sanders et al., 2013

Supplemental Experimental Methods

Lentiviral infections

Lentiviral constructs containing the indicated shRNAs in the pLLX vector were transfected into HEK293T cells with the helper plasmids Δ 8.9 and VsVg using Fugene HD (Promega) before incubating for 48-72 h. Viral supernatant was then added directly to the cultures to infect the neurons. shRNA sequences were designed using GE Dharmacon siDesign Center or as previously described (Abad et al., 2006; Joo et al., 2015; Lee et al., 2010; Rodriguez-Gomez et al., 2012). The shRNA sequences used are: *Scrambled*, 5'-GCCCTGCCACCGTAATTTA-3'; *shNPI*, 5'-GTACAGCCGCCTCAATTCT-3'; *shSRF*, 5'-AAGATGGAGTTCATCGACAAC-3'; *shCREB*, 5'-GGAGTCTGTGGATAGTGTA-3'; *shMEF2A*, 5'-GTTATCTCAGGGTTCAAAT-3'; *shMEF2D*, 5'-GTAGCTCTCTGGTCACTCC-3'; *shMRTFA/B*, 5'-CATGGAGCTGGTGGAGAAGAA-3'; *shELK1*, 5'-GGGATGGTGGTGAAGTTCAAGT-3'; *shELK4*, 5'-GCAACGAGCCCTAGTCTTTCT-3'; *shCaen1h*, 5'-GGGCTTCCTTTAGTAGCAA-3'.

Pharmacological Inhibitors

To induce scaling, 1 μ M TTX (Tocris) or 50 μ M bicuculline (Sigma) was used. Cells were also pretreated with the following inhibitors at the indicated concentrations: 5 μ M NBQX (Tocris), 100 μ M AP-5 (Tocris), 10 μ M Nimodipine (Tocris), 5 mM EGTA (Bio-world), 10 μ M EGTA-AM (Life Technologies), 2 μ M ω -Conotoxin GVIA (Alomone labs), 2 μ M ω -Agatoxin IVA (Alomone labs), 2 μ M SNX-482 (Alomone labs), 10 μ M NNC 55-0396 dihydrochloride (Alomone labs), 1 μ M Mibefradil (Alomone labs), 50 μ M TTA-A2 (Alomone labs), 10 μ M KN-62 (Tocris), 3 μ M STO-609 (Tocris), and 10 μ M each of the MAPK inhibitors: U0126, SB 203580, PD 98059, SB 202190, SP 600125 (Tocris). 5 μ g/mL Actinomycin D was added to block transcription. EGTA-AM, Nimodipine, TTA-A2, STO-609, KN-62, Actinomycin D, and the MAPK inhibitors were dissolved in DMSO to make stock solutions. Cells were pretreated with EGTA-AM for 30 min before exchanging the media and being stimulated with TTX. Cells were pretreated with EGTA for 5 min before stimulation, and for all other inhibitors, cells were pretreated for 15 min before stimulation by either TTX or bicuculline.

Whole cell voltage clamp recordings

At DIV14-18, dissociated hippocampal cultures were voltage-clamped at -70 mV, as previously described (Reese and Kavalali, 2015), using an Axon Instruments Axopatch 200B amplifier. Access resistance was less than 25 M Ω for each recording. The internal pipette solution contained (in mM): 120 K-Gluconate, 20 KCl, 10 NaCl, 10 HEPES, 0.6 EGTA, 4 Mg-ATP and 0.3 Na-GTP at pH 7.3. In order to isolate AMPA-mEPSCs, the extracellular solution contained 1 μ M TTX, 50 μ M picrotoxin (to block mIPSCs), 50 μ M (2R)-amino-5-phosphonovaleric acid (AP5), 2 mM Ca²⁺ and 1.25 mM Mg²⁺. All recordings were performed under continuous perfusion, and cells were perfused for 3 min before recording to achieve stable baselines. The AMPA-mEPSCs were quantified with Synaptosoft MiniAnalysis software. 4 minutes were analyzed per recording. To ensure that high frequency cells did not skew the amplitude comparisons by being overrepresented, 200 mEPSC amplitudes were randomly selected from each recording to generate the cumulative probability histograms and rank order plots. Significance was determined using the Kolmogorov-Smirnov test, which was performed using PAST 3.1 (<http://folk.uio.no/ohammer/past/>).

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (Joo et al., 2015; Kim et al., 2010; Schaukowitch et al., 2014). Briefly, neurons were crosslinked with 1% formaldehyde for 10 min before quenching with 2M glycine (125 mM final concentration). Cells were washed with cold PBS and harvested in PBS with proteinase inhibitors (Roche). Cells were lysed with Buffer I (50 mM Hepes KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 10 % Glycerol, 0.5% NP-40, protease inhibitors) and nuclei were resuspended in Buffer II (300 mM NaCl, 1 % Triton-X 100, 0.1% Sodium deoxycholate, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, pH 8.0, 10 mM Tris, pH 8.0, protease inhibitors) before being sonicated to shear the DNA on a Misonix sonicator. Lysates were incubated with 4 μ g of the following antibodies overnight at 4°C: anti-SRF (H-300, Santa Cruz Biotechnology Cat# sc-13029, RRID:AB_2302440), anti-ELK1 (I-20 X, Santa Cruz Biotechnology, Cat# sc-355, RRID:AB_631429). The next day, Protein A/G beads (Santa Cruz) were added for 2 h at 4°C, and then the beads were washed with Low, High, and LiCl buffers. DNA was eluted with Elution buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 1 % SDS) at 65°C for 10 min, and then reverse crosslinked at 65°C for 5-8 h. DNA was incubated with RNase A (Qiagen), followed by Proteinase K (NEB), and purified by Phenol:Chloroform extraction, followed by a commercial DNA purification kit (Qiagen). Regions were amplified using qPCR with the primers listed below.

Immunostaining

Hippocampal neurons from P3 rats were cultured until DIV14-17. Cells were treated with 1 μ M TTX overnight. Cells were washed once with PBS before fixation with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed three times with PBS. To stain for surface NP1, neurons were left unpermeabilized, and blocked with 10% Normal Donkey Serum (NDS; Jackson ImmunoResearch Laboratories) in PBS for 1 h at room temperature. Primary antibody incubation was performed for 2 h at room temperature in 2% NDS in PBS at the following dilution: anti-NP1 (BD Biosciences Cat# 610369, RRID:AB_397754, 1:300). Cells were washed three times with PBS before secondary incubation for 1 h at room temperature. The secondary antibody Alexa-Fluor anti-Mouse 555 was diluted 1:1000 in 2% NDS in PBS. Cells were washed three times in PBS, and mounted using hard-set mounting solution (VectaShield). Images were taken on a Zeiss LSM 150 using a 63x objective, and were quantified using ImageJ. For immunohistochemistry, mice were perfused with 4% formaldehyde and brains were incubated in 4% paraformaldehyde overnight. After three washes with PBS, the brains were incubated with 30% sucrose and then mounted in Tissue-tek (Sakura). The brains were sliced at 40 μ m, and the slices were incubated with blocking solution (10% NDS in PBS) for 1 h at room temperature. Primary antibody incubation was performed overnight at 4°C in 0.1% TX-100 in PBS at a dilution of 1:50. Cells were washed four times with 0.1% TX-100 in PBS, and incubated with secondary antibody Alexa-Fluor anti-Mouse 555 at a dilution of 1:200 in 0.1% TX-100 in PBS for 1 h at room temperature. Slices were again washed four times with 0.1% TX-100 and coverslips were applied with hard-set mounting solution (VectaShield).

RNA-seq analysis

The sequencing reads were mapped to the mm10 UCSC annotation using TOPHAT (Trapnell et al., 2009) with the parameters (-a 8 -m 0 -I 500000 -p 8 -g 20 --library-type fr-firststrand --no-novel-indels --segment-mismatches 2). RPKM calculations were done using the HOMER package by normalizing to 10 million reads. Tracks for visualization on UCSC browser are generated using HOMER. Heatmaps were generated in JavaTreeView from log transformed fold change values, normalized to the unstimulated condition, using hierarchical clustering in Cluster. Venn diagrams were created using BioVenn (Hulsen et al., 2008).

ChIP-seq analysis

H3K27Ac ChIP-seq (GSE60192) data was downloaded from GEO. Bowtie2 (Langmead et al., 2009) aligner was used to align the fastq reads to mm10 UCSC annotation. Duplicates were removed using Picard MarkDuplicates (<http://broadinstitute.github.io/picard/>) and visualization tracks were generated using HOMER. Using MACS, the KCl-K27ac peaks were identified by using the Unstimulated-K27ac samples as control at a p-value of 1E-5. Closest genes were identified near the negative peaks generated for KCl-K27ac through MACS by using in-house downstream scripts.

GRO-seq analysis

10 million nuclei per sample were extracted and used for global run-on, and base hydrolysis was performed as previously described (Core et al., 2008). Nascent RNAs were immunoprecipitated with anti-BrdU antibody-conjugated beads (Santa Cruz Biotech) twice. Between two immunoprecipitations, purified run-on RNAs were subjected to polyA tailing by using Poly(A)-polymerase (NEB). Subsequently, RNAs are subjected to first-strand cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen) with the oNTI223 primer. Extra oNTI223 primers were removed by Exonuclease I (NEB) and cDNAs were size-selected in an 8 % polyacrylamide TBE-urea gel. Purified cDNAs were subsequently circularized using CircLigase (Epicentre) and relinearized with ApeI (NEB). The relinearized DNA template were subjected to PCR amplification by using Phusion High-Fidelity DNA Polymerase (NEB) and Illumina TrueSeq small-RNA sample barcoded primers. Subsequently, PCR products were isolated by 6 % polyacrylamide TBE gel and purified. The final libraries were sequenced using an Illumina HiSeq per the manufacturer's instructions. The raw fastq reads were trimmed using CUTADAPT with parameters -a AAAAAAAAAAAAAAAAAAAAAA -z -e 0.10 -f fastq -m 32 (Chae et al., 2015). Then the surviving reads were submitted to BWA aligner for mapping to the mm10 UCSC annotation. Samtools and the HOMER package were used to make visualization tracks and RPKM calculations. RPKM was calculated by normalizing to 10 million reads.

Western blotting

Protein was extracted from neurons using sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Before loading, samples were mixed with loading buffer (5% 2-mercaptoethanol, 0.1% bromophenol blue) and denatured at 95°C. Proteins were run on a 12% SDS-PAGE gel, and analyzed by western blotting with the following antibodies:

anti-NP1 (BD Biosciences Cat# 610369, RRID:AB_397754, 1:1000) or anti- β -actin (Sigma-Aldrich Cat#A5441, RRID:AB_476744, 1:5000).

Visual Cortex experiments

For dark rearing experiments, male C57Bl6/J mice were placed into completely dark chambers at P21-25 at the end of their light cycle. The visual cortices were removed, and RNA was extracted in Trizol after 24 h in the dark. For measuring protein levels, mice were taken out of the dark after 3 d. Intracocular injections of TTX were performed as in (Frenkel and Bear, 2004). Briefly, 1 μ L of 1 mM TTX or PBS was injected into each eye of the mouse using a glass pipette at a rate of 1 μ L/1 min (Joo et al., 2015). RNA was then extracted from visual cortex of the opposite hemisphere with Trizol.

Primer Sequences

Primers for RT-qPCR

<i>Nptx1</i> -forward	GAAGAAGAGCCTGCCAGAGAT
<i>Nptx1</i> -reverse	CAGGCACAGCATAAGAGAAGG
<i>Rps6ka5</i> -forward	CTTGACGCACAATGGCTACG
<i>Rps6ka5</i> -reverse	CCTGCCCTGACAGCATTGTA
<i>Auts2</i> -forward	TGAGGGATCCTTACCGTGAC
<i>Auts2</i> -reverse	ATCGGTTCGGCTTCATACAAC
<i>Cacna1h</i> -forward	GCCAGAAATGCTACCTGGGT
<i>Cacna1h</i> -reverse	CAGAGAGGCTCACGTTGTGT
<i>Nos1</i> -forward	GGTTTAAGGACCTGGGGCTC
<i>Nos1</i> -reverse	CCAGCCACTAAAGGGACAGG
<i>Txnip</i> -forward	TGCAGGAAATGAAGCATCTG
<i>Txnip</i> -reverse	GTTCCCCGCTGTAGAGACTG
<i>Elk1</i> -forward	CTCTGCATCCACCAACTTGA
<i>Elk1</i> -reverse	CTGCTGCAGCTTCTGAGAGA
<i>Mical3</i> -forward	TTCTCACCTTGCGCAAAAGC
<i>Mical3</i> -reverse	CGATGCCGCCATATCAGAGT
<i>Arc</i> -forward	GTGAAGACAAGCCAGCATGA
<i>Arc</i> -reverse	CCAAGAGGACCAAGGGTACA
<i>c-fos</i> -forward	ATCCTTGGAGCCAGTCAAGA
<i>c-fos</i> -reverse	ATGATGCCGGAACAAGAAG
<i>Nptx1</i> -pre-mRNA-forward	AACACGCCTTAAGATGGCAC
<i>Nptx1</i> -pre-mRNA-reverse	CCCTGCTCGGACTGCAAAAT
<i>Auts2</i> -pre-mRNA-forward	CCATTTTCCCAGGGCTTTGC
<i>Auts2</i> -pre-mRNA-reverse	GCCTCTTGGGTGTGTTCTCA
<i>Txnip</i> -pre-mRNA-forward	TGCTATTCTCACAGGGTCAACA
<i>Txnip</i> -pre-mRNA-reverse	GGGGAATAGAGAGGAAATTTTCAG
<i>Arc</i> -pre-mRNA-forward	GTGCCAGTGCCCACTACAAT
<i>Arc</i> -pre-mRNA-reverse	CCAAGTCCACTTTCCAGGAG
<i>Auts2</i> -short-forward	CGGCTAGGTCCACAAGTCTG
<i>Auts2</i> -short-reverse	CTGCCGTTTAGGATCCGGTT
<i>Srf</i> -forward	GGTGCCAGGTAGTTGGTGAT
<i>Srf</i> -reverse	TGAAGCCAGCATTACAGTC
<i>Mkl1</i> -forward	CTGTCTAAGCCCTGGTGCTC
<i>Mkl1</i> -reverse	GCTCCTCAATCTGCTTGTC
<i>Mkl2</i> -forward	TCATGCAACTTTTCGCTCAC
<i>Mkl2</i> -reverse	GCATAGCACGTGGTCAAAGA
<i>Elk4</i> -forward	TGCAACGAGCCCTAGTCTTT
<i>Elk4</i> -reverse	TTCCAGGGAAGGTAGTGTGG
<i>Creb</i> -forward	AAACATACCAGATCCGCACA
<i>Creb</i> -reverse	CTCTCTCTCCGTGCTGCTT

<i>Mef2a</i> -forward	AGGGCTGCTTGTCTTAGATG
<i>Mef2a</i> -reverse	ACCAGCGCTGACCTGTCT
<i>Mef2d</i> -forward	CTGGCACTAGGCAATGTCAC
<i>Mef2d</i> -reverse	AGTGGGGCTGTTGCTGAG
<i>Tbp</i> -forward	TGACTCCTGGAATCCCCATC
<i>Tbp</i> -reverse	TTGCTGCTGCTGTCTTTGTT
<i>Gapdh</i> -forward	AGGTCGGTGTGAACGGATTTG
<i>Gapdh</i> -reverse	TGTAGACCATGTAGTTGAGGTCA
eNptx1-forward (eRNA-F)	ACATGCTCACGGTTGTGGAT
eNptx1-reverse (eRNA-R)	TTCTCTAGCCAGCAGTTGCC

Primers for ChIP qPCR

<i>Nptx1</i> -SRE1-forward	CAGGCTGTGTGAGGTAGTGG
<i>Nptx1</i> -SRE1-reverse	CACCAGGGTTTCCACGATCA
<i>Nptx1</i> -E1-forward	AGCAAAACCAGGAGCCAAGA
<i>Nptx1</i> -E1-reverse	CCACGCTGTGAACTCGGTAG
<i>Nptx1</i> -Intron-forward	AACACGCCTTAAGATGGCAC
<i>Nptx1</i> -Intron-reverse	CCCTGCTCGGACTGCAAAAT
<i>Nptx1</i> -Promoter-forward	TGAGACCGGGATCGGGC
<i>Nptx1</i> -Promoter-reverse	GAAGACTGTGCGGCGTAGAA
<i>Nptx1</i> -Neg1-forward	CACAGCGGTGAGCTCTAACT
<i>Nptx1</i> -Neg1-reverse	AAGAGGAATGTGGGCTGGTG
<i>Nptx1</i> -SRE2-forward	AGGGACAGCTTTTCGGATGG
<i>Nptx1</i> -SRE2-reverse	CTTGCCAATGAGATGGGGGT
Neg2-forward	GCAGAGCGGACCATAAACAT
Neg2-reverse	CCGTGAAATGAGTTGCCTTT
<i>c-fos</i> -Promoter-forward	CCTCCCTCCTTTACACAGGA
<i>c-fos</i> -Promoter-reverse	ACCTTCCGCGTGTAGGATTT

References for Supplemental information

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