Supplementary Information

REST/NRSF-MEDIATED INTRINSIC HOMEOSTASIS PROTECTS NEURONAL NETWORKS FROM HYPEREXCITABILITY

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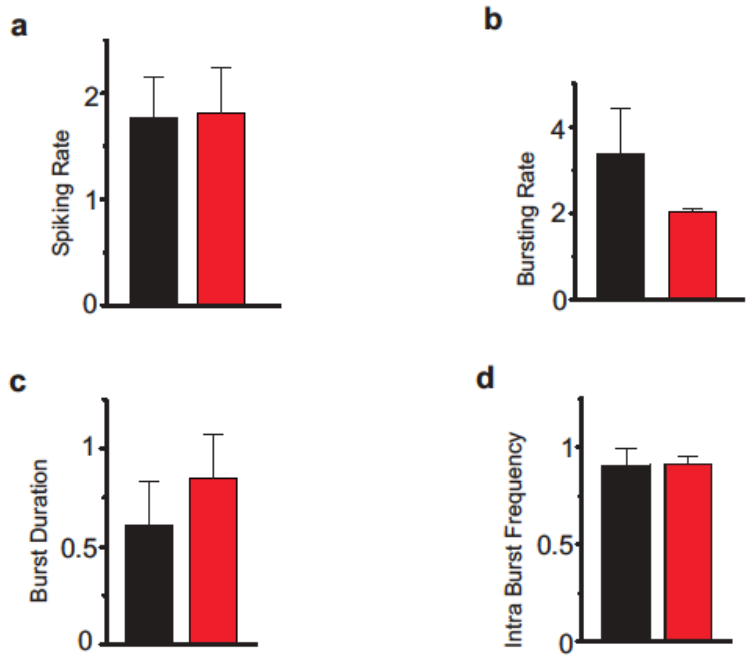
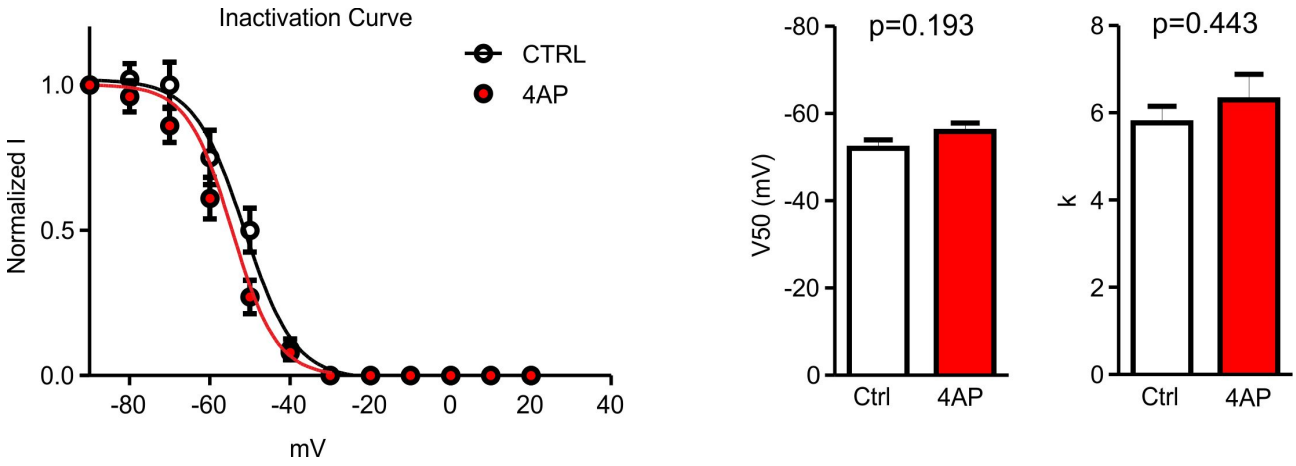
** **equal contribution***

Abbreviated title: Hyperexcitability induces REST-mediated intrinsic homeostasis

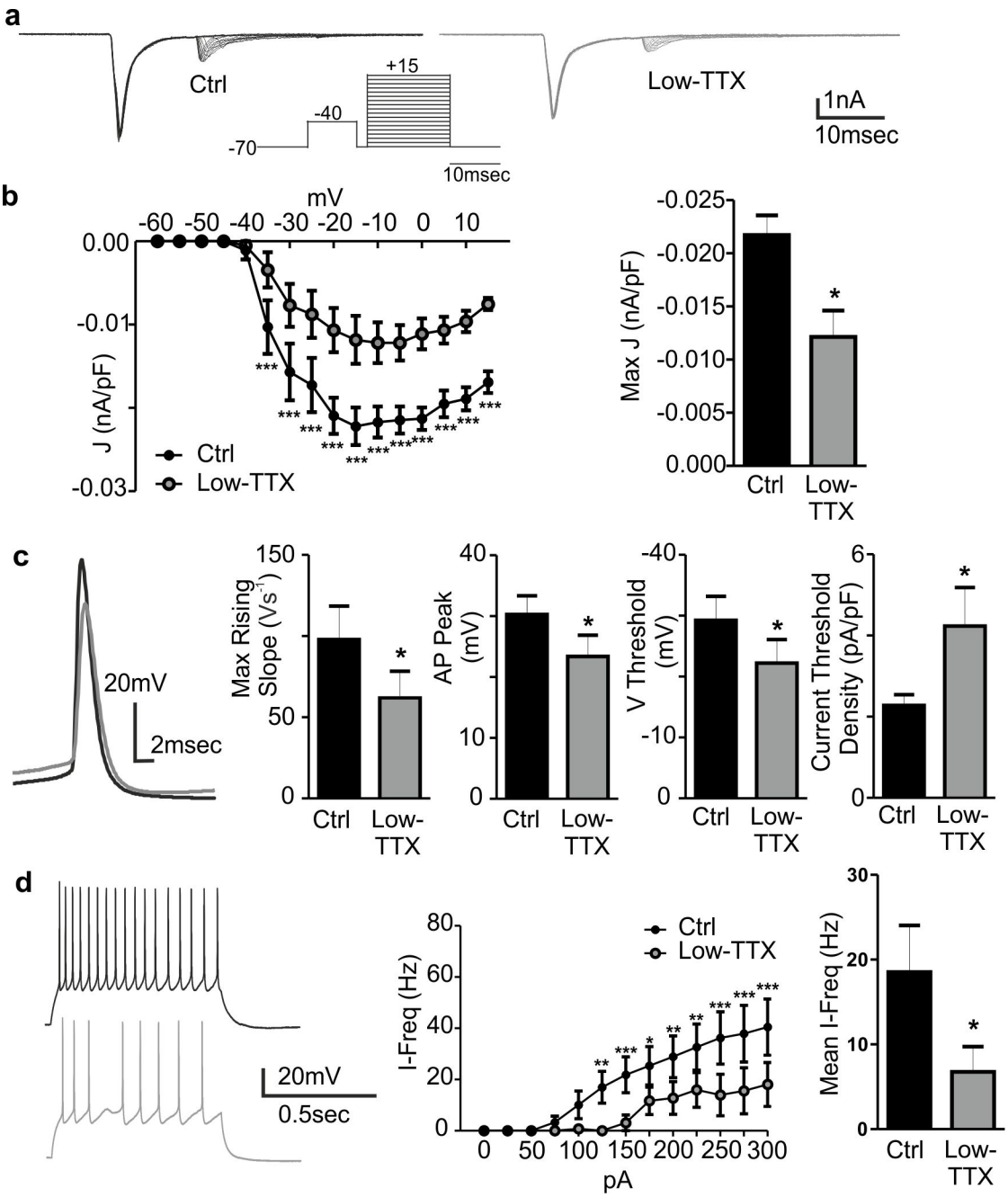
Key Words: excitability; 4-aminopyridine; Na^+ channels; transcriptional repression; intrinsic homeostasis



Supplementary Figure 1. *4AP maintains its activity after 96 h of incubation with cultured neurons.* Neuronal cultures (24 div) were incubated with either fresh 4AP (black bars, n = 3) or 4AP previously used to treat other neuronal cultures for 96 h (red bars, n = 3) and the increase in the firing activity was analyzed by MEA electrophysiology. Cultures were measured for a period of 20 min under control conditions and for further 20 min in the presence of 4AP. Then, the increases in firing rate **(a),** bursting rate **(b)**, burst duration **(c)** and intra-burst frequency **(d)** were calculated by normalizing the values observed in 4AP to the values measured under control conditions. Data are shown as means ± sem ($p>0.05$ for all the parameters, Mann-Whitney's U-test).

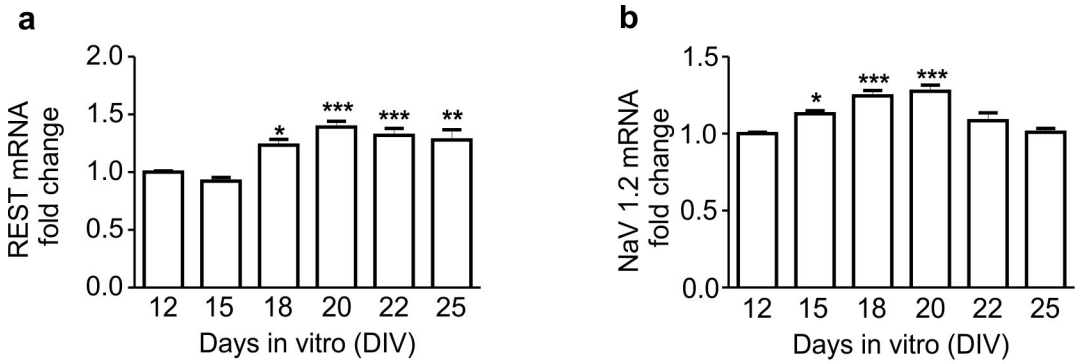


Supplementary Figure 2. *Inactivation curve analysis.* The I/I_{max} *vs* Voltage relationship were fitted for individual cells (n=34 and n=20 for control and 4AP treated, respectively) by using a Boltzmann equation $(I/I_{max}=[1+\exp(V_{50}-V_c)/k)]^{-1}$ (where V_{50} is the potential of half-maximal inactivation, V_c is the command voltage and k is the steepness constant). The calculated mean (± sem) V_{50} and k were not significantly different ($p>0.05$, Student's unpaired two-tailed *t*-test).

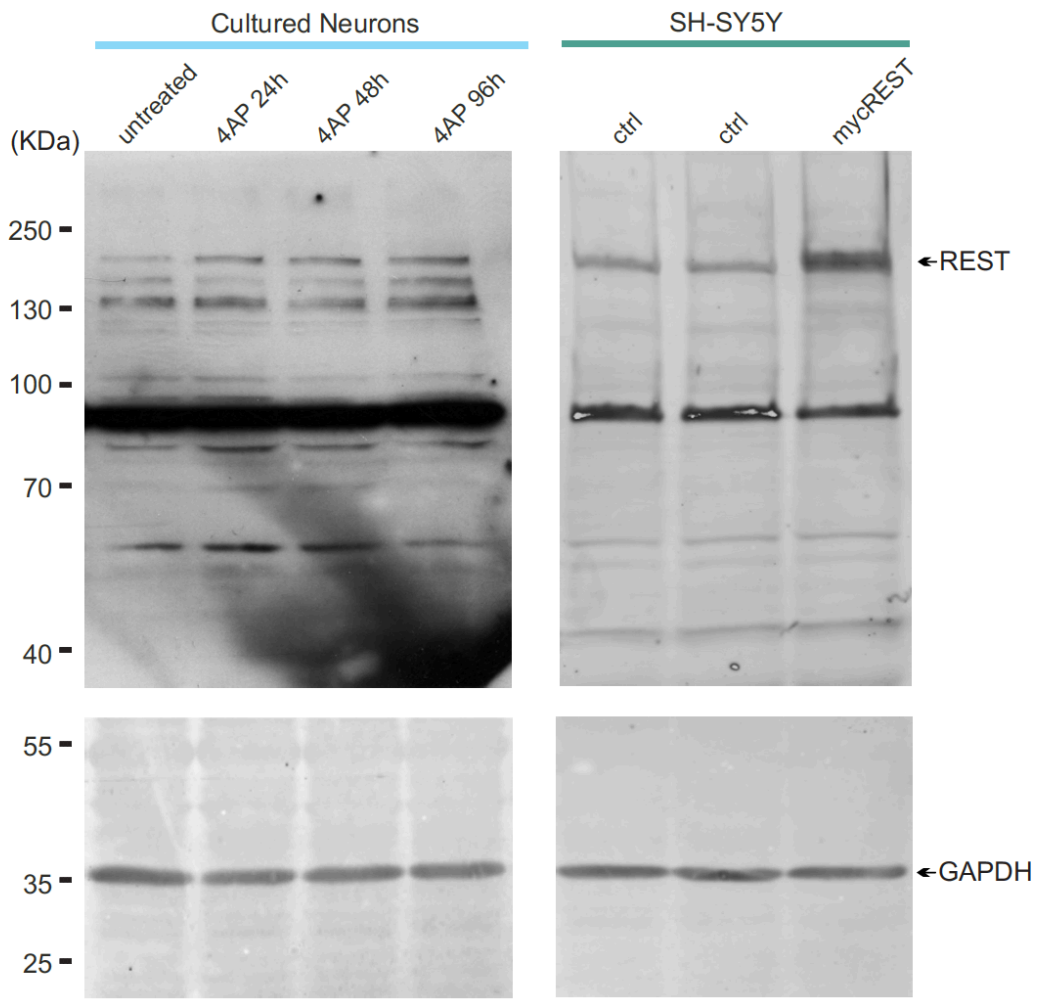


Supplementary Figure 3. *Sub-saturating concentrations of TTX decrease neuronal firing activity.* To verify whether a partial inactivation of Na^+ channels can reduce neuronal firing activity, a sub-saturating concentration of TTX between 90 and 120 nM (Low-TTX) was used in paired experiments after recording the control condition (Ctrl). **a**. To circumvent space-clamp problems, we used a voltage step protocol preceded by a brief pre-pulse at -40 mV that inactivated axonal I_{Na} current, thus allowing to isolate the pure somatic component (Milescu et al, 2010). Representative traces and pre-pulse protocol used to quantify somatic I_{Na} current before (left trace) and after (right trace) TTX application. Measurements were performed by using modified internal (100 mM cesium methanesulfonate, 20 mM CsCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 4 mM ATP, 15

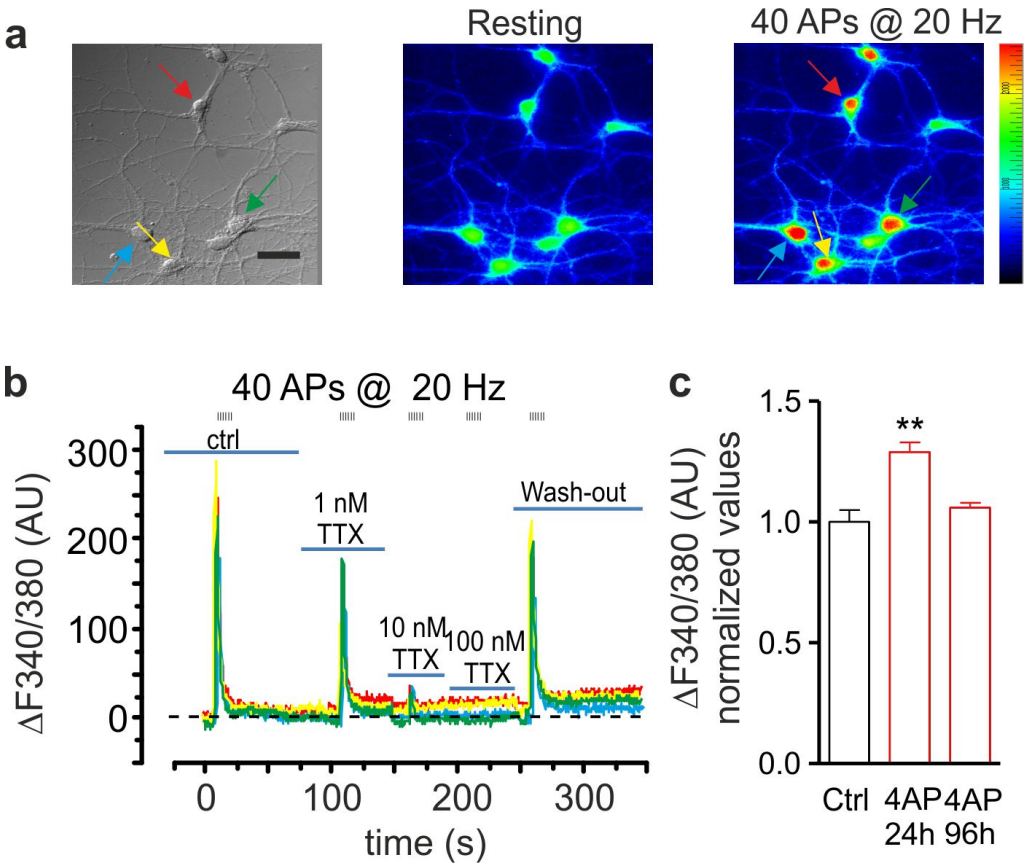
mM phosphocreatine, pH 7.3 CsOH 2M) and external (120 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 20 mM TEA, 100 µM cadmium, pH 7.3 with NaOH 2M) solutions at an holding potential of –70 mV. **b.** Na^+ current density (J) *vs* voltage relationship (left panel) and maximum Na^+ current density (Max J, right panel) were reduced after low-TTX application as compared to control conditions (*p<0.05, **p<0.01, ***p<0.001 Student's paired *t*-test. J *vs* Voltage: Two-way ANOVA for repeated measurements followed by the Bonferroni's multiple comparison test; n=6). **c.** Current clamp action potential shape (left panel) and analysis. The maximum rising slope, the action potential peak and the voltage threshold decrease with low-TTX application, while current threshold density increases (*p<0.05, Student's paired *t*-test; n=6). **d.** Representative traces (left panel) and analysis of neuronal firing activity. Instantaneous frequency *vs* injected current (middle panel) and mean instantaneous frequency (right panel) are reduced upon low-TTX application (*p<0.05, **p<0.01, ***p<0.001, Student's paired *t*-test. I-freq *vs* current: Two-way ANOVA for repeated measurements followed by the Bonferroni's multiple comparison test; n=6). Note that the decrease in both current threshold density and voltage threshold were observed upon low-TTX application, but not during chronic 4AP treatment. This discrepancy might be due to the fact that TTX does not discriminate any specific channel subtype, while the chronic 4AP stimulation could differentially affect the expression of specific Na^+ channel isoforms, thus making the two conditions not completely overlapping.



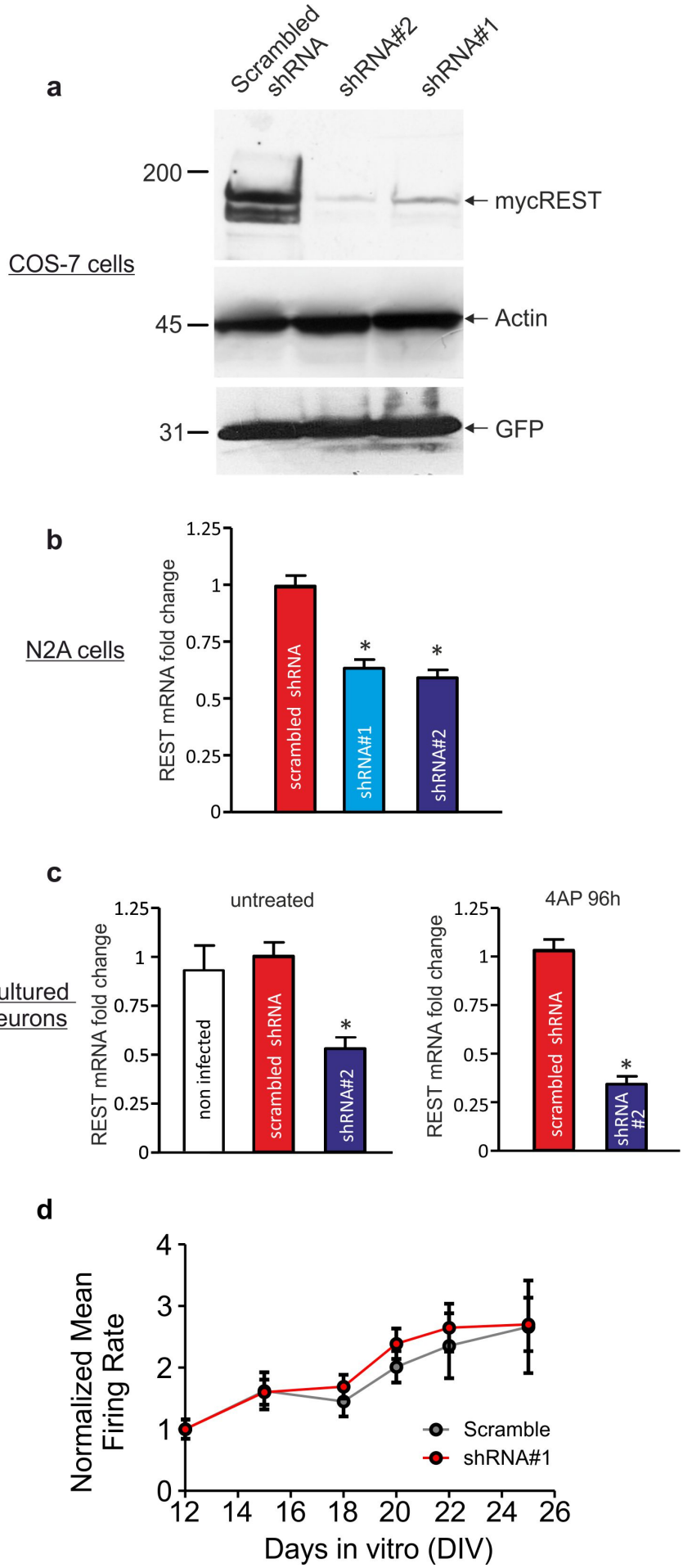
Supplementary Figure 4. qRT-PCR analysis (means ± sem) of REST/NRSF (**a**) and Nav1.2 mRNA (**b**) during culture development in vitro, normalized to the respective values at 12 DIV (*p<0.05, **p<0.01, ***p<0.001, one-way ANOVA followed by Dunnett`s multiple comparisons test; n=6 for each time point from two independent neuronal preparations).



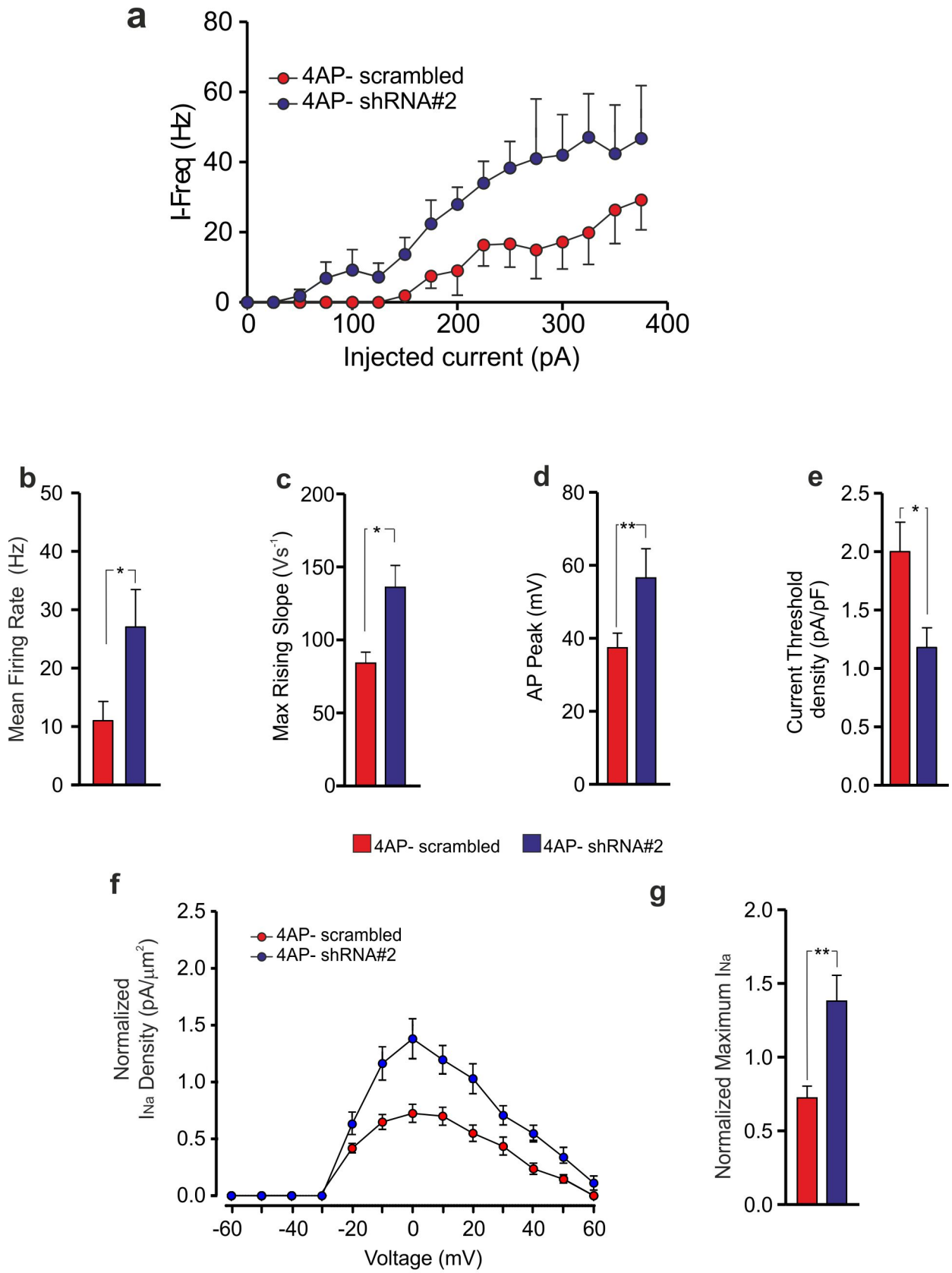
Supplementary Figure 5. *Specificity of the REST antibody.* Representative western blots of either untreated or 4AP-treated cortical neurons, showing the expression of REST/NRSF (upper panels) and GAPDH (lower panels) at the indicated times. The specificity of the REST/NRSF immunoreactive bands was verified by comparison with lysates obtained from SH-SY5Y neuroblastoma cells (right panels) transfected with either the empty (ctrl) or the REST/NRSF expression vector (mycREST). The REST/NRSF mRNA in neurons translates an endogenous protein that migrates at approximately 195 kDa, a molecular mass comparable to that observed upon expression of the recombinant REST/NRSF in SH-SY5Y cells. The corresponding band is clearly upregulated by 4AP treatment in cultured cortical neurons.



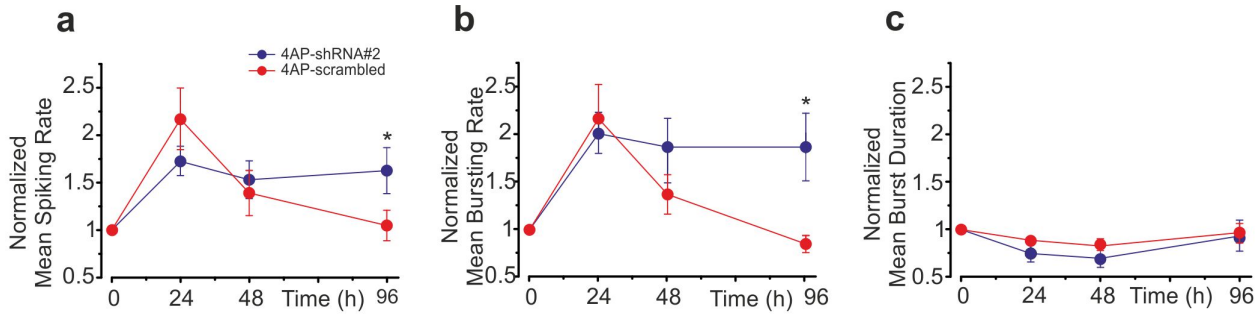
Supplementary Figure 6. *Evoked intracellular Ca^{2+} transients induced by field extracellular stimulation*. (**a**) (*Left*) Representative DIC-bright field image of cultured hippocampal neurons (16 div). Pseudocolor ratio images of Ca^{2+} changes under resting conditions (*middle*) and upon electrical stimulation at 20 Hz for 2 s (*right*) in hippocampal neurons loaded with Fura-2 AM (5 μM). Colored arrows identify neurons analyzed in panel b. Scale bar: 30 μm. (**b**) Time-course of electrically evoked Ca^{2+} transients recorded at the cell body of hippocampal neurons under control conditions (ctrl) and after application of TTX. Color traces refer to arrowed neurons in panel a. (**c**) Homeostatic response of evoked intracellular Ca^{2+} transients induced upon 4AP-mediated elevated activity. Quantitative analysis of intracellular Ca^{2+} transients induced by electrical field stimulation at 20 Hz for 2 s recorded in control neurons or in neurons treated with 4-AP for 24 and 96 hr. (Ctrl n=384 cells; 4AP 24h n=130 cells; 96h n=179 cells; from 3 independent cell preparations and 2-3 coverslips for each cell preparation and each condition). Data were normalized on untreated neurons recorded at the same time point (**p<0.01; One-way ANOVA followed by the Bonferroni's multiple comparison test).


a
Scrambled shRNA
shRNA#2
shRNA#1
200
mycREST
COS-7 cells
45
Actin
31
GFP
b
N2A cells
REST mRNA fold change
1.25
1
0.75
0.5
0.25
0
scrambled shRNA
shRNA#1
shRNA#2
*
*
c
Cultured neurons
untreated
4AP 96h
REST mRNA fold change
non infected
scrambled shRNA
shRNA#2
shRNA #2
d
Normalized Mean Firing Rate
4
3
2
1
0
12
14
16
18
20
22
24
26
Days in vitro (DIV)
Scramble
shRNA#1

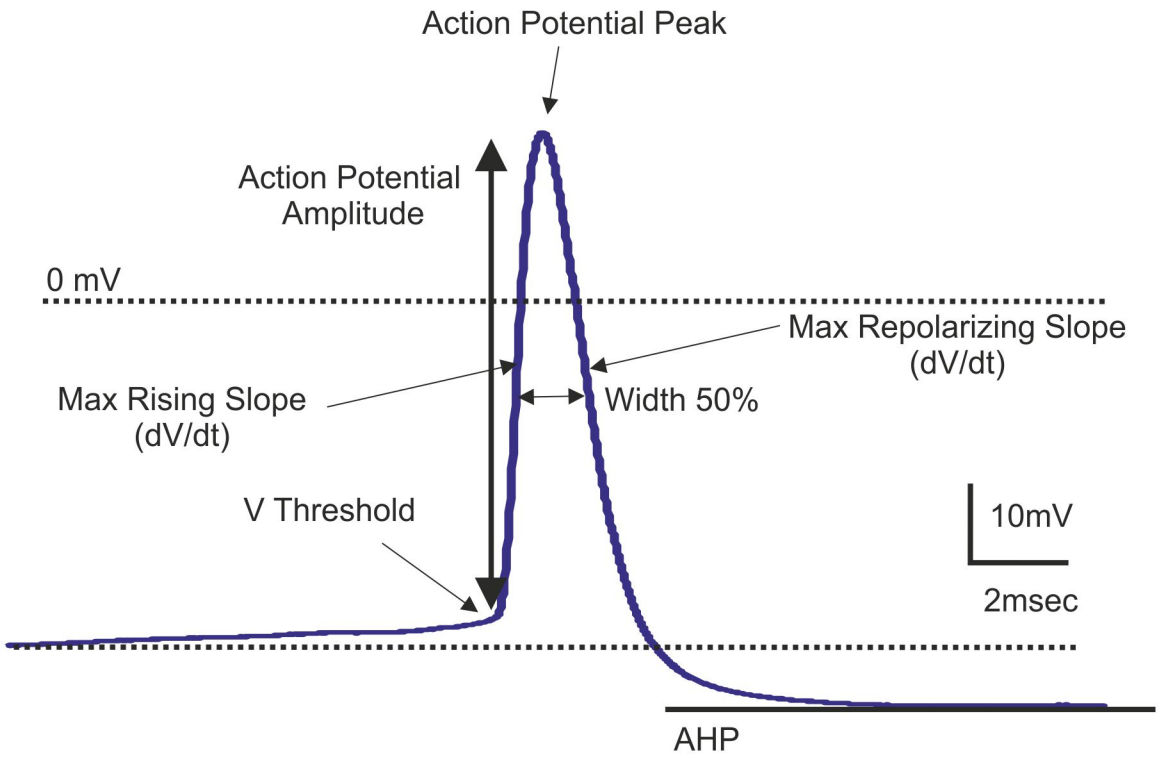
Supplementary Figure 7. *Efficacy of the shRNAs in knocking down REST/NRSF expression in various cell types.* (**a**) COS-7 cells were co-transfected with the vector encoding for mycREST/NRSF together with either shRNA#1, shRNA#2 or scrambled shRNA. Three days later, cells were lysed and analyzed by immunoblotting. The Myc signal, reporting the REST/NRSF expression level, was strongly reduced in both shRNA#1- and shRNA#2-transfected cells as compared to the scrambled shRNA-transfected sample. The actin signal, indicating the total protein, and the GFP signal, reflecting the level of efficiency of shRNA transfection, were comparable. (**b**) The endogenous levels of REST/NRSF mRNA were evaluated by quantitative qRT-PCR on total RNA extracted from the murine neuronal cell line N2A infected with either shRNA#1, shRNA#2 or scrambled shRNA lentiviruses. Both shRNA#1 and shRNA#2 were effective in reducing endogenous REST/NRSF mRNA levels. *$p<0.05$, One-way ANOVA followed by the Bonferroni's multiple comparison test; n=3 experiments. (**c**) The efficacy of shRNA#2 in down-regulating REST mRNA was also evaluated in cultured cortical neurons that were untreated or treated for 96 h with 4AP. The scrambled shRNA did not alter the endogenous REST/NRSF mRNA levels if compared to non-infected neurons (left). shRNA#2 significantly reduced REST/NRSF mRNA levels in both untreated (left) and treated (right) neurons. *$p<0.05$, One-way ANOVA followed by the Bonferroni's multiple comparison test; n=3 independent cultures. **(d)** Firing rate measured in neurons infected with either scrambled shRNA (gray symbols; n=20) or shRNA#1 (red symbols; n=23) as a function of time in vitro (DIV). Data (means ± sem) were normalized to the respective values at 12 DIV.


a
I-Freq (Hz)
Injected current (pA)
4AP- scrambled
4AP- shRNA#2
b
Mean Firing Rate (Hz)
c
Max Rising Slope (Vs-1)
d
AP Peak (mV)
e
Current Threshold density (pA/pF)
4AP- scrambled
4AP- shRNA#2
f
Normalized INa Density (pA/µm2)
Voltage (mV)
g
Normalized Maximum INa

Supplementary Figure 8. Electrophysiological parameters of visually identified excitatory pyramidal neurons transfected with either scrambled shRNA (red symbols/bar) or shRNA#2 (blue symbols/bar). After 96 h treatment with 4AP, the mean instantaneous frequency *vs* injected current curve (**a**), the mean firing rate (**b**), the maximum rising slope (**c**) and the action potential peak (**d**) were significantly higher, while, the current threshold density (**e**) was significantly lower in neurons transfected with shRNA#2 (n=12) with respect to neurons transfected with scrambled shRNA (n=11). After 96 h of 4AP treatment, the normalized I_{Na} density *vs* voltage plot (**f**) and the normalized maximum amplitude measured at 0 mV (**g**) were also markedly higher in neurons transfected with shRNA#2 with respect to neurons transfected with scrambled shRNA (*p<0.05, **p<0.01, Student's unpaired *t*-test).



Supplementary Figure 9. Mean (± sem) spiking rate (**a**), bursting rate (**b**) and burst duration (**c**) as a function of time during 4AP treatment in neuronal cultures infected with either scrambled shRNA (red symbols, n=13) or shRNA#2 (blue symbols, n=13) and normalized with respect to the firing activity before the treatment. All parameters were unchanged between the two experimental groups, except for the spiking rate and burst rate at 96 h (*$p<0.05$, Two-way ANOVA for repeated measures followed by the Bonferroni's multiple comparison test).



	Ctrl (n=15)	4AP (n=19)	p Value	Significance
Current Threshold Density (pA/pF)	3.11 ± 0.57	3.24 ± 0.42	0.85	**ns**
V Threshold (mV)	-31.01 ± 0.92	-30.92 ± 1.09	0.95	**ns**
Max Rising Slope (dV/dt)	123.8 ± 12.66	97.58 ± 7.527	0.039	*
Max Repolarizing Slope (dV/dt)	-38.88 ± 3.85	-32.37 ± 2.28	0.13	**ns**
AP Peak (mV)	31.79 ± 2.57	21.56 ± 1.74	0.001	***
AP amplitude (mV)	63.47 ± 2.82	52.48 ± 1.97	0.002	**
AHP (mV)	-40.91 ± 0.98	-42.41 ± 1.13	0.33	**ns**
Width at 50% of AP amplitude (msec)	1.66 ± 0.10	1.98± 0.16	0.16	**ns**
Capacitance (pF)	57.25 ± 6.16	47.89 ± 2.53	0.18	**ns**
R input (MΩ)	208.6 ± 17.87	209.2 ± 26.19	0.98	**ns**
Membrane potential (mV)	-61.16 ± 1.32	-56.00 ± 0.96	0.003	**

Supplementary Table I. Electrophysiological parameters of the passive membrane properties and the shape of the 1st elicited action potential (upper panel) measured in control and 4AP-treated neurons for 96 hours. The analysis refers to the data reported in Fig. 2. AP, action potential; ns, non significant.

Figure	Panel	p Value	Groups
1	c	0.00047	Ctrl vs 4Ap
	c	0.0000011	Ctrl vs 4AP
	f	0.0009	Ctrl vs 4AP
2	b	0.0005<p<0.036	Ctrl vs 4Ap
	c	0.0017	Ctrl vs 4Ap
	f	0.034	Ctrl vs 4Ap
	g	0.0018	Ctrl vs 4Ap
3	c	0.025	Ctrl vs 4Ap -10mV
	c	0.027	Ctrl vs 4Ap 0mV
	f	0.000002	Ctrl vs 4Ap
4	a	0.024	Ctrl vs 4Ap 8h
	a	0.001	Ctrl vs 4Ap 24h
	a	0.046	Ctrl vs 4Ap 48h
	b	0.001	Ctrl vs 4Ap 8h
	b	0.001	Ctrl vs 4Ap 24h
	b	0.029	Ctrl vs 4Ap 48h
	d	0.029	Ctrl vs 4Ap 48h
	d	0.029	Ctrl vs 4Ap 96h
	f	0.0009	Ctrl vs Glut 96h
5	b	0.016	Ctrl vs REST
	d	0.033	Ctrl vs REST firing rate
	d	0.012	Ctrl vs REST AP peak
	d	0.021	Ctrl vs REST Current density
	e	0.001<p<0.047	Ctrl vs REST
	f	0.002	Ctrl vs REST
6	b	0.014	Ctrl vs REST
	e	0.043	Ctrl vs REST 20div
6	e	0.006	Ctrl vs REST 22div
	e	0.026	Ctrl vs REST 25div
	f	0.045	Ctrl vs REST 20div
	f	0.0004	Ctrl vs REST 22div
	f	0.03	Ctrl vs REST 25div
7	a	0.02	Scramble vs. 4AP-shRNA#1
	a	0.0004	shRNA#1 vs. 4AP-shRNA#1
	a	0.046	4AP-Scramble vs. 4AP- shRNA#1
	c	0.02	Scramble vs. 4AP-Scramble
	c	0.004	4AP-Scramble vs. 4AP- shRNA#1
	d	0.0016	Scramble vs. 4AP-Scramble
	d	0.004	4AP-Scramble vs. 4AP- shRNA#1
	e	0.0056	Scramble vs. 4AP-Scramble
	e	0.0003	4AP-Scramble vs. 4AP- shRNA#1
	f	0.002	Scramble vs. 4AP-shRNA#1
	f	0.039	4AP-Scramble vs. 4AP- shRNA#1
	h	0.026	Scramble vs. 4AP-shRNA#1
	h	0.0005	4AP-Scramble vs. 4AP- shRNA#1
	h	0.046	Scramble vs. 4AP-Scramble
8	e	0.009	4AP-Scramble vs. 4AP- shRNA#1 96h
	e	0.00007	Scramble vs. 4AP Scramble 24h
	e	0.0003	Scramble vs. 4AP-shRNA#1 24h
	e	0.022	Scramble vs. 4AP-shRNA#1 48h
	f	0.0038	4AP-Scramble vs. 4AP- shRNA#1 96h
	f	0.034	Scramble vs. 4AP-shRNA#1 96h
	f	0.009	Scramble vs. 4AP-shRNA#1 24h
	f	0.0003	Scramble vs. 4AP Scramble 24h

Supplementary Table II. Exact values of statistical significance for all experimental data reported in the manuscript**.**

SUPPLEMENTARY METHODS

Cell cultures, transfection and 4AP treatments

Hippocampal and cortical cultures were prepared from mouse C57BL/6J (E17-E18) embryos. All experiments were carried out in accordance with the guidelines of the European Community Council (Directive 2010/63/EU of September 22nd, 2010) and were approved by the Italian Ministry of Health. Dissociated hippocampal and cortical neurons were plated on coverslips coated with (0.1 mg/ml) Poly-L-Lysine at 200 cells/mm^2 and maintained in Neurobasal medium containing B27 Supplement and Glutamax (Invitrogen). Neurons were transfected at 12-14 div with either Lipofectamine 2000 (Invitrogen) or Effectene (Qiagen) *according to* the *manufacturers'* instructions. At this age *in vitro,* our primary cultures contained ≈70% of excitatory of glutamatergic neurons and only ≈30% of GABAergic inhibitory interneurons, as previously observed (Ivenshitz & Segal, 2010). To maximize the probability of co-transfecting neurons, the ratio between the amount of cDNA coding for GFP and either myc-REST/NRSF or empty vector was 1:2. 4AP (100 μM) was dissolved in complete Neurobasal medium applied to neuronal cultures and maintained for the indicated times. Except where indicated, 4AP was then washed out, and biochemical analysis, Ca^{2+} imaging, patch-clamp or MEA recordings were carried on "sister" cultures treated with 4AP or vehicle only. *COS-7 and N2a cells grown in* DMEM supplemented with 10% fetal bovine serum (Invitrogen) were transfected with Lipofectamine 2000 (Invitrogen).

Protein extraction and western blotting

Total protein extracts were obtained from cortical cultures. Cells were washed twice with PBS and lysed in modified RIPA buffer (0.1% SDS; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP40; 0.5% Na Deoxycholate; 1 mM PMSF; Protease Inhibitor Cocktail [Complete Protease Inhibitor Cocktail

Tablets, Roche). The suspension was maintained on ice for 20 min and then centrifuged at 14000 x g for 15 min at 4 °C. Proteins were quantified using DC Protein Assay (BioRad), and 80 μg were separated by 10% SDS-PAGE. The quantification was made by normalizing the optical density of the proteins of interest (PanNav, Na/K pump, REST) to that of the loading control (β-III tubulin, GAPDH) within the same experiment. The analysis was performed by averaging the results of three independent preparations. Each lane corresponds to a single culture coverslip from a single experiment. The immunoreactive bands were analyzed by using the ImageJ software. The following primary antibodies were used: anti-REST (17-641; Millipore, Billerica, MA, USA), anti-GAPDH (SAB3500247; Sigma-Aldrich), anti-β-III Tubulin (G7121; Promega, Madison, WI, USA), anti-panNav (S8809; Sigma-Aldrich) and anti-Na^+/K^+ ATPase α-1 (05-359; Millipore).

Real-time quantitative PCR (RT-qPCR)

RNA was extracted with QIAzol reagent, purified on RNeasy spin columns (Qiagen) and quantified with an ND1000 Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was performed with a QuantiTect Reverse Transcription Kit (Qiagen), which includes a genomic DNA removal step. RT-qPCR was performed in triplicate with QuantiTect SYBR green master mix (Qiagen) on a 7900-HT Fast Real-time System (Applied Biosystems). PCR reaction efficiency was calculated for each primer set and used for subsequent analyses. Gene expression data were normalized by the multiple internal control gene method with the GeNorm algorithm available in the qBasePlus software (Biogazelle, Ghent, Belgium). The expression stability for all tested control genes (PPIA, GAPDH, ACTB, bIII-Tub, HPRT1) was not affected by the 4AP treatment. Gene expression data were normalized to PPIA and GAPDH, based on ranked expression stability of the tested control genes (Vandesompele et al, 2002). Data were normalized to untreated control cultures recorded at the same developmental stage. Primers sequences (5'-3') were: ACTB-F:

AAGTGGTTACAGGAAGTCC; ACTB-R: ATAATTTACACAGAAGCAATGC; GAPDH-F: GAACATCATCCCTGCATCCA; GAPDH-R: CCAGTGAGCTTCCCGTTCA; PPIA-F: CACTGTCGCTTTTCGCCGCTTG; PPIA-R: TTTCTGCTGTCTTTGGAACTTTGTCTGC; HPRT1-F: TGAGGCGGCGAGGGAGAG; HPRT1-R:AAGCGGTCTGAGGAGGAAGC; bIII-Tub-F: GCCTTTGGACACCTATTCAGG; bIII-Tub-R:TTCTTTCCTCAGGACATCCAG; REST-F: GAACCACCTCCCAGTATG; REST-R: CTTCTGACAATCCTCCATAG; NAV1.2-F: GGCTCTGCTGTCATTGTTGGTA; NAV1.2-R: GAAGGCTAGGTGAGTACATCCC.

RNA interference, lentivirus production and infection procedures

The REST/NRSF target sequences used to design shRNA (5'-ACATGCAAGACAGGTTCACAA-3', shRNA#1; 5'-TGCAATTATGTGGCCTCTAAT-3', shRNA#2) were identified by using an RNAi designer algorithm (BLOCK-iT; Invitrogen). The shRNA construct was obtained by cloning the two sequences into pcDNA 6.2-GW/EmGFP-miR using an RNAi expression vector kit (BLOCK-iT Pol II miR; Invitrogen) according to the manufacturer's instructions, thereby creating an expression cassette consisting of the 5'miR flanking region, the miRNA sequence and the 3'miR flanking region. These cassettes were then re-cloned into lentivirus (LV). pCCL.sin.cPPT.PGK.GFP.WPRE bidirectional expression vector and packaging plasmids were a kind gift from M. Amendola and L. Naldini (TIGET, San Raffaele Sci. Institute, Milan, Italy). For the coordinated expression of REST and GFP, the low-affinity NGF receptor in the MA1 construct (Amendola et al, 2005) was replaced with REST cDNA, leading to GFP expression from the minCMV promoter and REST expression from the PGK promoter. As a negative control, the pcDNA 6.2-GW/EmGFP-miR-neg plasmid from a kit containing a sequence targeting any known vertebrate gene (Invitrogen) was used. Third-generation LVs were produced by transient four-plasmid cotransfection into HEK293T cells using the calcium phosphate transfection method. Supernatants were collected, passed through a 0.45

μm filter and purified by ultracentrifugation as previously described. Viral vectors were titrated at concentrations ranging from 1 x 10^8 to 5 x 10^9 transducing units (TU)/ml and used at a multiplicity of infection (MOI) of 1-10. Cultures were infected at 7 div by using 2-5 MOI, and neurons were checked for infection at 12-14 div. The efficiency of infection was estimated to range between 70 and 90% by counting neurons expressing GFP protein with respect to the total number of cells stained with DAPI.

SUPPLEMENTARY REFERENCES

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