Supplementary Materials for

The human language and epilepsy associated gene SRPX2 regulates synapse formation and vocalization in mice

G.M. Sia, R.L. Clem, R.L. Huganir.

correspondence to: rhuganir@jhmi.edu

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Materials and Methods

Primary neuron culture

Dissociated hippocampal cultures were prepared from E18 Sprague-Dawley rats as described previously (*33*, *34*), and maintained in Neurobasal medium with B27 supplement (Invitrogen). Low efficiency transfections were performed with Lipofectamine 2000 (Invitrogen), and high throughput high efficiency electroporation transfections were performed with the Nucleofector 96 well Shuttle System (Amaxa/Lonza). For the experiment overexpressing rat and human SRPX2 in neurons, cells were transfected at DIV 4, and stained 7 days later. For all other experiments, neurons were transfected at DIV 4 and stained 10-14 days later.

High throughput screening

A subpopulation of secreted and transmembrane genes from the Ultimate ORF Collection (Invitrogen) were assayed for synaptogenic activity in an overexpression screen. Briefly, ORFs were transferred from the donor plasmids into mammalian expression plasmids according to the manufacturer's instructions. Neuronal cultures were transfected at DIV 4 with individual genes and blue fluorescent protein (*35*) as a morphology marker, and processed for immunocytochemistry 7 days later to assess for excitatory and inhibitory synaptic density in transfected neurons. Hits were re-assayed in secondary screens for verification.

Immunocytochemistry

Neuronal cultures were fixed with 4% PFA 4% sucrose in PBS at room temperature for 30 minutes, and then permeabilized with 0.2% Triton X-100 in PBS for 10 minutes on ice. Primary antibodies were diluted in 10% normal goat serum in PBS and incubated with neurons for 2 hrs at room temperature, and secondary antibodies were similarly diluted in 10% normal goat serum and incubated with neurons for 1 hour. For the SRPX2 antibody, cultures were incubated live with primary antibody for surface staining, before being processed as above.

Images were acquired with a Zeiss Axiophot with a 63x 1.4 NA objective. ImageJ was used to apply a fixed threshold to all synaptic images to segment out the relevant synaptic puncta. The BFP channel was similarly thresholded to show the perimeter of the transfected dendrites, and the resulting binary image was applied as a mask onto the synaptic images to delineate the synaptic puncta that colocalized with the transfected dendrites. The number of colocalized synaptic puncta and the dendrite length were determined, and the puncta density per unit dendrite length was calculated. The spine density was similarly determined from the thresholded BFP image. A total of 20-35 cells from 3 or more separate culture batches were examined for each condition. During the above analysis, the investigator was blind to the experimental condition. The data sets for BFP and scrambled shRNA were not significantly different and were pooled. Data was compared by ANOVA with post-hoc Tukey test.

For analysis of dendritic morphology, branch points on the dendritic tree were counted, and Sholl analysis was performed with the Fiji Sholl Analysis plugin (http://fiji.sc/Sholl_Analysis). The center of the soma was used as the center of concentric

circles with a starting radius of 10 μm and an ending radius of 50 μm, with consecutive radii spaced 5 μm apart.

Western blotting

For western blot analysis of dissociated neurons, neurons were electroporated with the Nucleofector System at plating, cultured for 4-7 days, and then solubilized in SDS sample buffer and quantitated for protein concentration. Equal protein amounts were run out on an SDS-PAGE gel, transferred, and then blotted. Comparison of SRPX2 levels in GFP vs FoxP2 electroporated neurons were performed with unpaired t-test.
For western blot analysis of brains, appropriate brain regions were dissected and homogenized, quantitated for protein concentration, and equal protein amounts were run out on an SDS-PAGE gel, transferred, and then blotted.

Coimmunoprecipitation

HEK293 cells were transfected with SRPX2-GFP, and either SRXP2-Flag, SRPX2-DN-Flag, or SRPX-Flag with Lipofectamine 2000 (Invitrogen). After an overnight incubation, culture medium was collected and spun down to remove debris, and the supernatant was incubated with a monovalent GFP antibody covalently coupled to agarose beads (GFP-Trap, Chomotek) for 1 hr. Beads were washed with IP buffer (1% Triton X-100 in PBS) for six times, and then eluted with 2x SDS sample buffer. Eluates as well as inputs were immunoblotted with anti-Flag antibody (rabbit polyclonal, Sigma).

Conditioned medium treatment

HEK293 cells were transfected with GFP, SRPX2-Flag, or SRPX2-DN-Flag for 6 hours with Lipofectamine 2000. Cells were then washed and incubated with Neurobasal medium overnight for 18 hours. Conditioned medium was then harvested, spun to remove debris, supplemented with B27 supplement and glutamine, and added to DIV 4 cultured neurons. After 10 days, cultured neurons were processed for immunocytochemistry as described above.

Chromatin immunoprecipitation (ChIP)

Each ChIP reaction was performed from cortices pooled from two P0 C57BL/6J mouse pups. Briefly, cortices were finely minced in ice-cold PBS with protease inhibitors, fixed at room temperature in 1% paraformaldehyde for 10 minutes, Dounce-homogenized, and the nuclei pelleted by spinning at 1000g. Nuclei were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 7.5, Complete protease inhibitors (Roche)) for 10 minutes, sonicated for 200-350 pulses on a Branson Sonifier 250 at a power setting of 4 and duty cycle of 10%, with cooling on ice every 10 pulses. Sonicated chromatin was spun at 14000g for 10 minutes to pellet debris, diluted to 10 times volume with IP buffer (1.1% Triton X-100, 20 mM Tris pH 8.0, 2 mM EDTA, 150 mM NaCl, Complete protease inhibitors), and used for immunprecipitation with a rabbit antibody against FoxP2 (Abcam) and normal rabbit IgG control antibody (Millipore) overnight in the cold room. Antibodies was immunoprecipitated with Protein A-Sepharose beads (Amersham) preblocked with BSA and salmon sperm DNA, and beads were washed twice each with IP buffer, low salt buffer (0.1% SDS, 0.1% Triton X-100, 150 mM NaCl, 20 mM Tris pH 8.0), high salt buffer ((0.1% SDS, 0.1% Triton X-100, 500 mM NaCl, 20

mM Tris pH 8.0), LiCl buffer (0.5 M LiCl, 1% NP-40, 1% DOC, 1 mM EDTA, 10 mM Tris pH 8.0), and TE buffer (10 mM Tris-Cl pH 7.5 1 mM EDTA). Elution was performed by vortexing beads gently with 1% SDS 0.1 M sodium bicarbonate pH 8.0 thrice for 15 minutes each at room temperature. Semi-quantitative PCR was performed with eluates and 1:50 dilution of input chromatin using Platinum Taq DNA polymerase (Invitrogen) using manufacturer suggested conditions, except that primer concentration was reduced to 0.1 μM for improved specificity. Touchdown PCR cycling conditions were as follows : (1) 95°C for 5 min, (2) 95°C for 30 s, (3) 70°C for 30 s, dropping 1°C per cycle, (4) 72°C for 30 s, (5) repeat steps 2 through 4 for 10 cycles, (6) 95°C for 30 s, (7) 60°C for 30 s, (8) 72°C for 30 s, (9) repeat steps 6 through 8 for 25 cycles, (10) 72°C for 10 min. Reactions were performed in triplicate. Primers were as follows : *β-actin* forward primer 5'-CCCCAAAGGCTGAGAAGTTA-3' reverse primer 5'-AGGGTACCACCGGAAAAGTC-3', *Slc17a3* forward primer 5'-TGGCAAAAATGAGTGAGGTCTGA-3' reverse primer 5'-TCCTTTTTCCGGTTTGAAATGG-3', *SRPX2* forward primer 5'-AACAAACAAGCAGCAACAACAACAAT-3' reverse primer 5'-GGGATATGATCAATATACGCCCCTTT-3'.

In utero electroporation

In utero electroporation of E12.5 mouse pups were performed as described (*36*). Briefly, pregnant females were anesthesized with Avertin via intraperitoneal injection, and surgical procedures were performed to expose the uterus and embryos. A glass microcapillary pipette was used to deliver 1 μl of 2.5 mg/ml DNA in PBS into the lateral ventricles of the embryos. Four electrical pulses at 30V, each with a duration of 50 ms, were delivered at 1 s intervals to both hemispheres of the injected embryos. The uterus with the electroporated embryos were then replaced in the abdominal cavity and the surgical opening was sutured. Embryos were electroporated with DsRed2 as morphology marker, as well as various SRPX2 constructs. All procedures were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

Isolation induced ultrasonic vocalization behavior task

Isolation induced ultrasonic vocalization in infant pups were assessed as previously described (*37*), with hardware and software (Med Associates, Vermont) provided by the Johns Hopkins Behavioral Core facility. Ten minutes before the experiment, the dam was separated from its litter to create a stable pretest baseline. P7 pups were isolated one by one from their home cage and placed in a recording box situated in an anechoic chamber. An ultrasonic microphone was positioned approximately 5 cm above the pup and set to record 50-100 kHz vocalizations with a 40 db cutoff over a 5 minute recording period. After the test, the pup was tattooed on its toes for later identification. Pups were processed for immunohistochemistry at P21 as described below, and vocalization data from pups with untransfected cortical regions were discarded. The total number of pups for each condition ranged from 23-46, and was sourced from 8-16 separate litters. Statistical comparisons were performed by ANOVA, post-hoc Tukey.

Electrophysiology

In utero electroporated mice aged P17-21 were anesthetized by isoflurane inhalation and decapitated. Brains were quickly dissected in ice-cold buffer containing 210.3 mM sucrose, 11 mM glucose, 2.5 mM KCl, 1 mM NaH2PO4, 26.2 mM NaHCO3, 0.5 mM ascorbate, 2 mM myo-inositol, 0.5 mM CaCl2 and 4 mM MgCl2. Brains were vibratome-sectioned in the same solution at 350 μm and transferred to normal ACSF composed of 119 mM NaCl, 2.5 mM KCl, 1 mM NaH2PO4, 26.2 mM NaHCO3, 11 mM glucose, 2 mM CaCl2 and 2 mM MgCl2. Slices were recovered at 35 °C for 40 min and then maintained at room temperature (22–25 °C). Neurons were targeted for whole-cell patch-clamp recording with borosilicate glass electrodes having a resistance of 3–6 MΩ. The electrode internal solution was composed of 130 mM cesium methanesulphonate, 10 mM HEPES, 0.5 mM EGTA, 8 mM CsCl, 5 mM TEA-Cl, 1 mM QX-314, 10 mM Na phosphocreatine, 0.5 mM Na-GTP and 4 mM Na-ATP. Cortical pyramidal neurons were selected from layer V of the posterior medial barrel subfield of primary somatosensory cortex. AMPA receptor-mediated miniature EPSCs were collected in external solution supplemented with the following: 1 μM tetrodotoxin, 100 μM D,L-APV (2-amino-5-phosphonovalerate) and 100 μM picrotoxin. Data were acquired with a Multiclamp 700B and PCLAMP 10 software (Molecular Devices) at 10 kHz. Prior to mEPSC detection and analysis, current traces were low-pass filtered at 1 kHz. Events having amplitude of 2× root mean square noise were detected using Mini Analysis (Synaptosoft). Kinetic measurements were performed on scaled, mean EPSC traces using a monoexponential decay function. Rise times correspond to 20–80% of peak amplitude. All experimental groups were compared against scrambled shRNA control group with the Dunnett Multiple Comparison Test**.**

Immunohistochemistry

Electroporated mice were trancardially perfused with 4% PFA in PBS at P21, and the brains were collected and postfixed with 4% PFA in PBS overnight, then cryoprotected with 30% sucrose in PBS overnight. The brains were then frozen and 120 μm saggital sections were cut. Slices were incubated with blocking buffer (10% NGS and 0.25% Triton X-100 in PBS) for 4 hr and then stained overnight with anti-DsRed antibody diluted in blocking buffer. This was followed by incubation with secondary antibody in blocking buffer overnight and mounting. Transfected layer V/VI cortical neurons were imaged by taking 0.15 μm optical sections of the dendritic tree with a Zeiss LSM 510 confocal microscope with a 63x NA 1.40 objective. The primary apical dendrite was examined in the Imaris software (Bitplane) and the spine density at various distances from the soma was determined. Some SRPX2-dn experiments were controlled with GFP transfection in littermates; the spine densities for GFP and scrambled shRNA were not significantly different and were subsequently pooled.

Antibodies

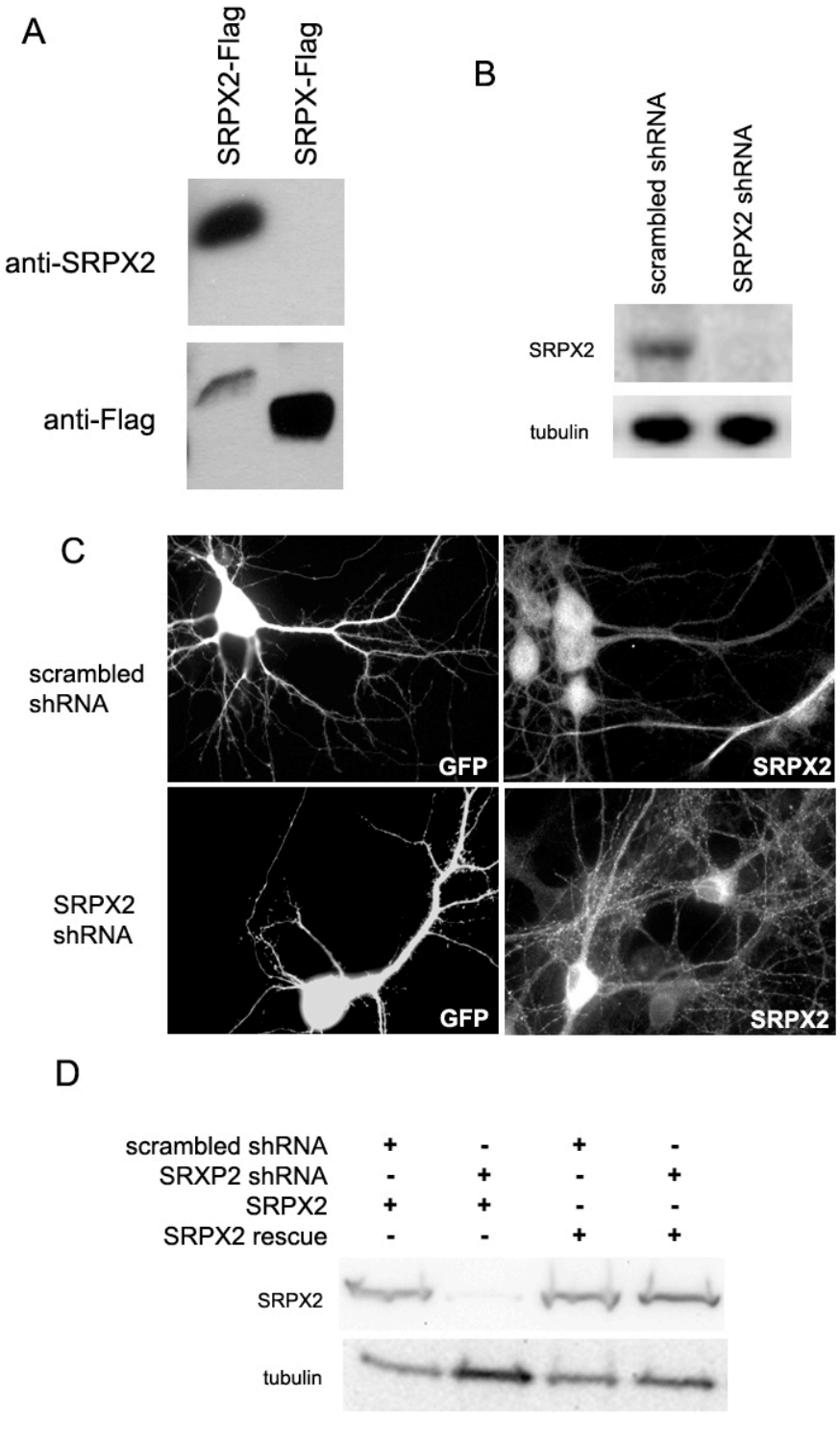
The SRPX2 polyclonal antibody was generated by injecting rabbits with the C-terminal 16 amino acids of rat SRPX2 protein conjugated to keyhole limpet hemocyanin. The antibody was then affinity-purified, and its specificity was verified by lack of reactivity with SRPX and decreased reactivity in SRPX2 knockdown cultures (data not shown). Dilutions and sources of commercial antibodies are as follows : anti-PSD95

(Chemicon 6G6 5 µg/ml), anti-VGlut1 (Chemicon guinea pig serum 1:3000), anti-gephyrin (Synaptic Systems 1 µg/ml), anti-VGAT (Synaptic Systems 1 µg/ml), anti-GFP (Invitrogen chicken IgY 1:300), anti-DsRed (Clontech rabbit polyclonal 1:400).

DNA

Human SRPX2 gene was obtained from the Ultimate ORF Collection. Rat SRPX2 was subcloned by RT-PCR from rat brain cDNA. The SRPX2 knockdown construct was made by subcloning the sequence 5'-GAAACTAACTGCTCGAGTA-3' into the pSUPER vector (OligoEngine) as per manufacturer's instructions. The SRPX2 rescue construct was made by incorporating 3 silent mutations (in lower case) in the targeted region in the rat SRPX2 background as follows : 5'-GAAACTcACcGCcCGAGTA-3'. The SRPX2 dominant negative construct was made by site directed mutagenesis of the rat SRPX2 gene to generate the Y72S mutation found in human patients (*9*). FoxP2 construct was obtained by RT-PCR amplification from rat cDNA. All constructs were verified by DNA sequencing after cloning.

Figure S1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SRPX2-Flag
SRPX-Flag
anti-SRPX2
anti-Flag
B
scrambled shRNA
SRPX2 shRNA
SRPX2
tubulin
C
scrambled shRNA
SRPX2 shRNA
GFP
SRPX2
GFP
SRPX2
D
scrambled shRNA + - + -
SRXP2 shRNA - + - +
SRPX2 + + - -
SRPX2 rescue - - + +
SRPX2
tubulin

Fig. S1. Generation of SRPX2 antibody, knockdown and rescue constructs.

A, Medium conditioned by HEK293 cells transfected with SRPX2-Flag or SRPX-Flag was immunoblotted with anti-SRPX2 antibody, and then stripped and reblotted with anti-Flag antibody. **B,** Western analysis of rat cortical neurons electroporated with SRPX2 shRNA construct. Rat cortical neurons were dissociated and electroporated with scrambled or SRPX2 shRNA before plating for culture. At DIV 3, cultures were lysed and blotted for SRPX2 and tubulin. **C,** Images of DIV 7 rat cortical neurons cultures transfected with SRPX2 shRNA or scrambled shRNA and GFP as a cell marker. Cultures were immunostained 3 days later for SRPX2. **D,** Western analysis for FLAG in HEK cells transfected with scrambled or SRPX2 shRNA, along with wild-type or mutated/rescue FLAG-SRXP2. Cells were lysed 1 day later and processed for immunoblotting with anti-SRPX2 and anti-tubulin antibodies.

Figure S2

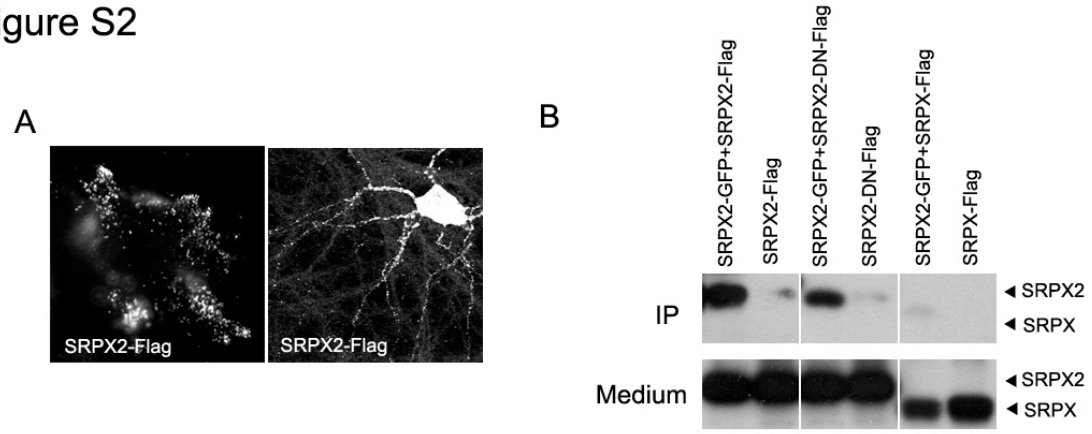

Fig. S2. Secreted SRPX2 oligomerizes with itself and with SRPX2-DN

A, Images of HEK cells and rat cortical neurons transfected with SRPX2-Flag and surface-stained for FLAG one day later. SRXP2 aggregates are found deposited on the transfected cells. **B,** SRPX2-Flag, SRPX2-DN-Flag, and SRPX-Flag were transfected into HEK293 cells with or without SRPX2-GFP. One day later, conditioned media were immunoprecipitated with anti-GFP beads, and eluates were blotted for Flag. SRPX2 coimmunoprecipitates with itself and with SRPX2-DN, but does not coimmunoprecipitate with the closely related family member SRPX.

Figure S3

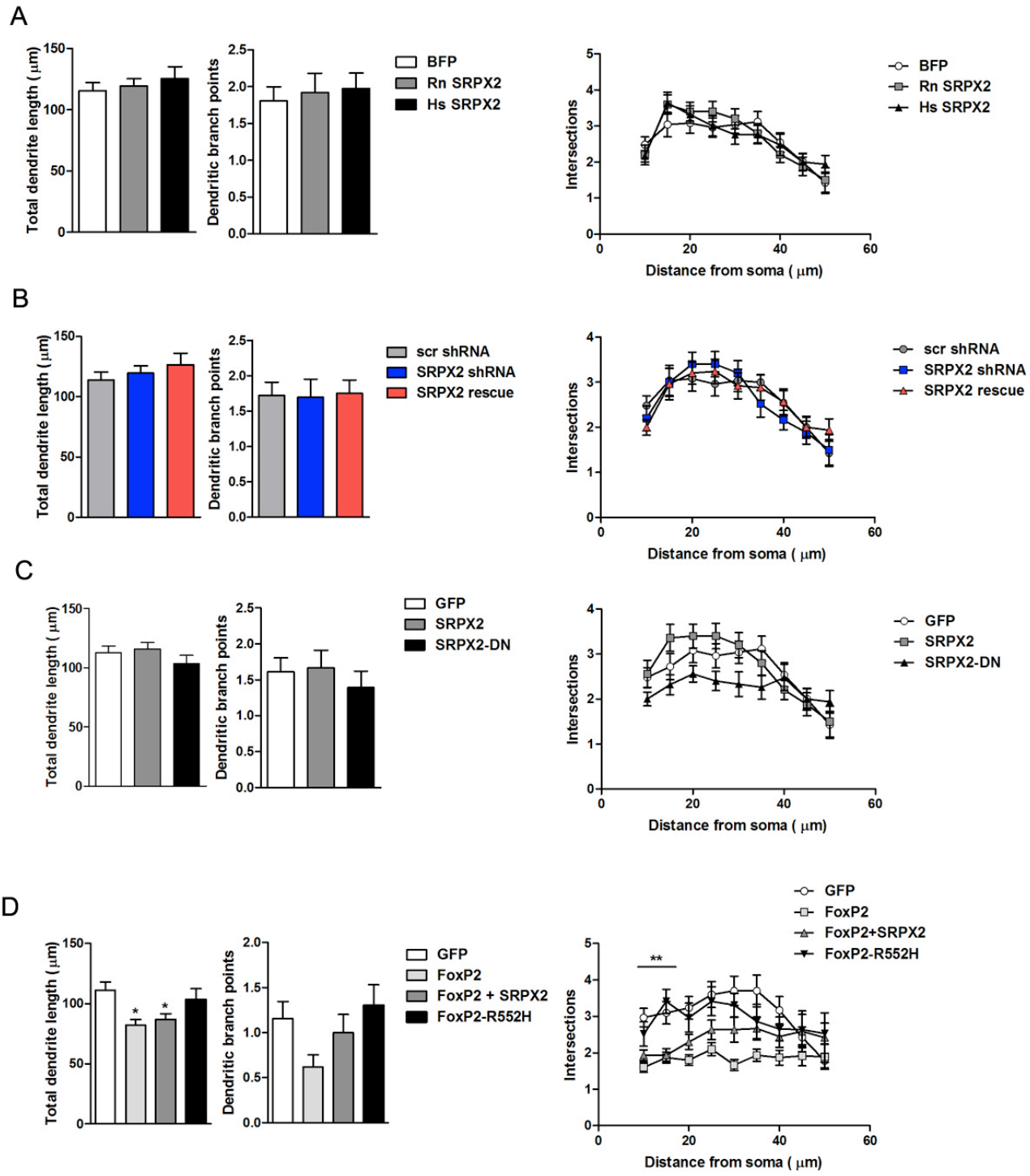
A
Total dendrite length (μm)
Dendritic branch points
BFP
Rn SRPX2
Hs SRPX2
Intersections
Distance from soma (μm)
B
scr shRNA
SRPX2 shRNA
SRPX2 rescue
C
GFP
SRPX2
SRPX2-DN
D
FoxP2
FoxP2 + SRPX2
FoxP2-R552H
FoxP2+SRPX2
*
**

Fig. S3. Analysis of dendritic morphology

A, Dissociated rat cortical neurons were transfected at DIV 4 with BFP, BFP and rat SRPX2, or BFP and human SRPX2, and imaged 3-4 days later. Average dendritic length and number of dendrite branch points was determined, and Sholl analysis was performed, for each experimental group. Comparisons were performed by ANOVA, and no significant differences between experimental groups was found. **B,** Dissociated rat cortical neurons were transfected BFP and scrambled shRNA, BFP and SRPX2 shRNA, or BFP, SRPX2 shRNA and SRPX2 rescue construct, and stained with anti-GFP antibodies to visualize dendritic morphology. Average dendritic length and number of dendrite branch points was determined, and Sholl analysis was performed, for each experimental group. Comparisons were performed by ANOVA, and no significant differences between experimental groups was found. **C,** Dissociated rat cortical neurons were incubated for 10 days with medium conditioned by HEK293 cells overexpressing GFP, SRPX2, or SRPX2-DN, and then immunostained with anti-MAP2 antibody to visualize dendritic morphology. Average dendritic length and number of dendrite branch points was determined, and Sholl analysis was performed, for each experimental group. Comparisons were performed by ANOVA, and no significant differences between experimental groups was found. **D,** Dissociated rat cortical neurons were cotransfected with GFP and FoxP2, FoxP2 + SRPX2, or FoxP2-R552H, and stained with anti-GFP antibodies to visualize dendritic morphology. Average dendritic length and number of dendrite branch points was determined, and Sholl analysis was performed, for each experimental group. Comparisons were performed by ANOVA, post-hoc Tukey, * $p < 0.05$, ** $p < 0.01$. FoxP2 and FoxP2 + SRPX2 neurons were found to have significantly shorter total dendritic length and a reduction in Sholl intersections at a distance of 10-15 μm from the soma, a reflection of the lower number of primary dendrites in these neurons.

Figure S4

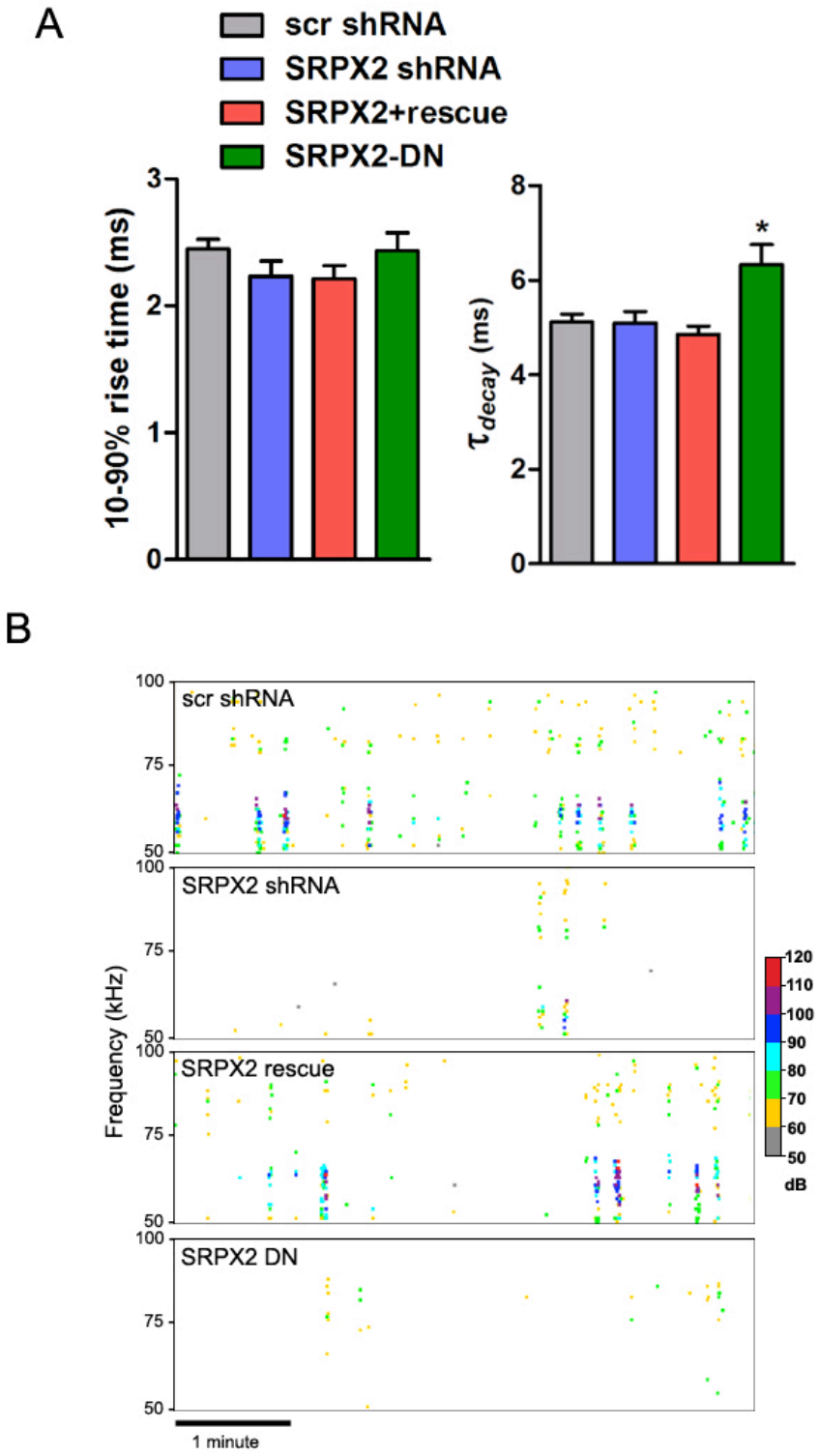
A
scr shRNA
SRPX2 shRNA
SRPX2+rescue
SRPX2-DN
10-90% rise time (ms)
3
2
1
0
τ_{decay} (ms)
8
6
4
2
0
*
B
scr shRNA
SRPX2 shRNA
SRPX2 rescue
SRPX2 DN
Frequency (kHz)
100
75
50
120
110
100
90
80
70
60
50
dB
1 minute

Fig. S4. mEPSC kinetics and USV spectrogram

A, Average 10-90% rise times and decay time constant of mEPSCs from electroporated layer V/VI cortical neurons. Error bars ± sem. All experimental groups compared against scrambled shRNA control group with Dunnett Multiple Comparison Test. * $p < 0.05$. **B,** Representative spectrograms of USVs recorded from P7 mouse pups electroporated in utero with scrambled shRNA (scr shRNA), SRPX2 shRNA (SRPX2 shRNA), SRPX2 shRNA and rescue construct (SRPX2 rescue), or SRPX2 dominant negative construct (SRPX2-DN).