Data supplement for MacDonald et al., Selective Loss of Smaller Spines in Schizophrenia. Am J Psychiatry (doi: 10.1176/appi.ajp.2017.16070814)

Human Subjects:

Tissue from two cohorts (**Supplemental Table 1**) comprised of subjects diagnosed with schizophrenia or schizoaffective disorder (together referred to as Sz) and controls matched on the basis of sex, and as closely as possible for age, post-mortem interval (PMI), and handedness.[1-4]

Human Tissue Processing:

At the University of Pittsburgh postmortem the right and left hemispheres of a postmortem brain are processed differently. Tissue blocks from the left hemisphere are fixed in paraformaldehyde, while blocks from the right hemisphere are fast-frozen in isopentane. Thus, tissue from the left hemisphere was utilized for Immunohistochemistry while tissue from the right hemisphere was utilized for targeted mass spectrometry.

Tissue Preparation for Immunohistochemistry: Brains from individuals in cohorts 1 and 2 were bisected and the left hemisphere was cut into 1-2 cm thick coronal blocks, which were then immersed in 4% paraformaldehyde in phosphate buffer for 48 hours, equilibrated in a series of graded sucrose solutions, and stored at -30˚C in an antifreeze solution.

The left superior temporal gyrus (STG) of each subject was dissected from fixed coronal blocks; reassembled in their in vivo orientation, and cut into 3 mm thick slabs as previously described[5]. Every other slab was selected, sectioned exhaustively, and adjacent sections stained for parvalbumin, acetylcholinesterase, and Nissl substance for determination of the boundaries of the primary auditory cortex[5]. For each human subject, the borders of layers 2/3 and 3/4 were identified on the mapping sections to determine the total layer 3 area for each subject. A contour outline of the deepest one third of layer 3 was drawn in Stereo Investigator (Figure 1, MicroBrightField Inc., Colchester, Vermont). These contours were then aligned to the tissue sections used in the current study using pial surface fiduciaries. For cohort 1, the primary auditory cortex was dissected from the unused slabs, and further subdivided into 3 mm wide blocks which were sectioned at 50 µm in an orientation perpendicular to the pial surface, and stored in antifreeze solution at -30˚C until selected for use in this study as described previously[6,7]. For cohort 2, 60 µm sections adjacent to the mapping sections were sampled systematic uniform random for assay.

Tissue Preparation for Targeted Mass Spectrometry: Grey matter was harvested from the auditory cortex as previously described[8,9]: Tissue slabs containing the superior temporal gyrus with Heschl's Gyrus (HG) located medial to the planum temporal were identified, and the superior temporal gyrus removed as single block. Grey matter was collected from HG by taking 40 µm sections, and frozen at -80 °C[8].

Subject Characteristics						
	Cohort 1		Cohort 2		Total	
	Control	Sz	Control	Sz	Control	Sz
n	12	12	8	8	20	20
Mean Age, Years (SD)	45.2 (12.9)	47.3 (13.4)	46.4 (14.0)	46.5 (12.4)	45.8 (13.0)	46.9 (13.4)
Range	19–65	27–71	24–62	25–62	19–65	25–71
Sex (F/M)	3/9	3/9	4/4	4/4	7/13	7/13
Handedness (R/L/A/U)	11/1/0/0	6/2/1/3	8/0/0/0	5/3/0/0	19/1/0/0	11/5/1/3
PMI (SD)	18.1 (6.5)	17.9 (8.8)	13.7 (6.5)	15.6 (6.8)	16.4 (6.7)	17.0 (7.9)
Storage Time, Months (SD)	155.0 (27.2)	145.5 (29.8)	97.1 (22.4)	92.8 (14.0)	131.8 (38.2)	124.4 (35.9)
Illness Duration, Years (SD)		22.1 (14.7)		22 (13.3)		22.1 (13.8)
Range		3–50		4–41		3–50
Age at Onset, Years (SD)		25.2 (7.7)		24.5 (9.6)		24.9 (8.3)
Suicide, n (%)		2 (16.7%)		2 (25.0%)		4 (20.0%)
Schizoaffective, n (%)		4 (33.3%)		2 (25.0%)		6 (30.0%)
Alcohol/Substance Abuse ATOD		5 (41.7%)		0 (0%)		5 (25.0%)
Anticonvulsant ATOD, n (%)		5 (62.5%)		1 (12.5%)		6 (30.0%)
Antidepressant ATOD, n (%)		3 (37.5%)		5 (62.5%)		8 (40.0%)
Antipsychotic ATOD, n (%)		11 (91.7%)		6 (75.0%)		17 (85.0%)
Benzodiazepine ATOD, n (%)		1 (8.3%)		3 (37.5%)		4 (20.0%)
History of Cannabis Use, n (%)		5 (41.7%)		2 (25.0%)		7 (35.0%)
Tobacco ATOD, n (%)	4 (33.3%)	8 (66.7%)	3 (37.5%)	6 (75.0%)	7 (35.0%)	14 (70.0%)

Supplemental Table 1. Each Sz subject in cohorts 1 and 2 was previously matched to a normal control subject based on sex and as closely as possible for age and postmortem interval. There were no diagnostic group differences in age [$t_{38} = -.333$, $p = .741$] or postmortem interval [$t_{38} = -.272$, $p = .787$]. The distribution of handedness between diagnostic groups reached trend level ($\chi^2 = 8.800$, $p = .066$). Mean tissue storage time did not differ between diagnostic groups (cohort

1 [t_{22} = .817, p = .423], cohort 2 [t_{14} = .461, p = .652]).

A, ambidextrous; ATOD, at time of death; F, female; L, left-handed; M, male; PMI, postmortem interval; R, right-handed; SD, standard deviation; Sz, schizophrenia; U, unknown.

	Effect of Confounds on the Percent Difference within a pair (C-S)/C								
Spine Size	covariate	Parameter	Estimate	StdErr	tValue	df	Probt	(C-S)/C in M or Y	(C-S)/C in F or N
1	sex	M-F in sex	0.323	0.340	0.951	18	0.354	0.234	-0.089
	suicide	Y-N in suicide	0.117	0.414	0.282	18	0.781	0.214	0.097
	schizoaffective	Y-N in schizoaffecti	-0.155	0.360	-0.429	18	0.673	0.012	0.167
	antidepressants	Y-N in antidepressan	0.079	0.338	0.234	18	0.818	0.168	0.089
	antipsychotic	Y-N in antipsychotic	-0.655	0.439	-1.494	18	0.153	0.022	0.677
	Anticonvulsants	Y-N in Anticonvulsan	0.053	0.362	0.147	18	0.885	0.158	0.105
	Benzodiazepines	Y-N in Benzodiazepin	0.275	0.410	0.670	18	0.511	0.340	0.066
	Other_medications	Y-N in Other_medicat	0.101	0.361	0.279	18	0.783	0.151	0.050
	Alcohol_dependence_abuse_ATOD	Y-N in Alcohol_depen	-0.255	0.411	-0.621	18	0.542	-0.084	0.172
	Cannabis_history	Y-N in Cannabis_hist	0.410	0.334	1.226	18	0.236	0.387	-0.023
	tobacco_atod	Y-N in tobacco_atod	-0.349	0.291	-1.199	18	0.247	0.134	0.482
	Other_substance_abuse_ATOD	Y-N in Other_substan	0.230	0.551	0.418	18	0.681	0.328	0.098
	ageofonset	Slope of ageofonset	0.000	0.021	-0.005	18	0.996		
	durationofdisease	Slope of durationofd	-0.016	0.012	-1.316	18	0.205		
2	sex	M-F in sex	0.241	0.267	0.903	18	0.378	0.252	0.011
	suicide	Y-N in suicide	0.045	0.325	0.137	18	0.892	0.204	0.159
	schizoaffective	Y-N in schizoaffecti	-0.090	0.283	-0.318	18	0.754	0.105	0.195
	antidepressants	Y-N in antidepressan	0.018	0.265	0.068	18	0.946	0.179	0.161
	antipsychotic	Y-N in antipsychotic	-0.527	0.342	-1.539	18	0.141	0.089	0.616
	Anticonvulsants	Y-N in Anticonvulsan	0.022	0.284	0.079	18	0.938	0.184	0.161
	Benzodiazepines	Y-N in Benzodiazepin	0.133	0.323	0.410	18	0.687	0.274	0.141
	Other_medications	Y-N in Other_medicat	0.076	0.283	0.268	18	0.792	0.191	0.115
	Alcohol_dependence_abuse_ATOD	Y-N in Alcohol_depen	-0.147	0.323	-0.454	18	0.655	0.051	0.197
	Cannabis_history	Y-N in Cannabis_hist	0.332	0.261	1.274	18	0.219	0.384	0.052
	tobacco_atod	Y-N in tobacco_atod	-0.315	0.227	-1.387	18	0.183	0.166	0.481
	Other_substance_abuse_ATOD	Y-N in Other_substan	0.218	0.430	0.508	18	0.618	0.365	0.146
	ageofonset	Slope of ageofonset	0.000	0.016	0.004	18	0.997		
	durationofdisease	Slope of durationofd	-0.013	0.009	-1.402	18	0.178		

Supplemental Table 2. For the categorical confound effect analysis, the percent change of spine density within each pair was calculated and the percent changes were compared between the group with response Y to the confound variable and that with response N. A one-way ANOVA model was applied and the percent changes in the Y and N groups were estimated. There are two estimates in the last two columns in the table: the mean percent change within

pairs with response Y to the confound variable and mean percent change within pairs with response N to the confound variable.

Immunohistochemistry

Tissue from the 20 pairs was divided into two cohorts: Cohort 1 (n = 12 pairs) and Cohort 2 (n = 8 pairs) (Supplemental Table 2). These two cohorts were assayed independently. Tissue with in each cohort was processed together over a series of immunohistochemical runs. In order to visualize dendritic spines, we used two markers in combination: a polyclonal antibody directed against spinophilin (Millipore AB5669, Billerica, MA), a protein that is highly enriched in spine heads., and the f-actin binding mushroom toxin phalloidin (Invitrogen A12380, Carlsbad, CA), which is also highly enriched in dendritic spines.

Image Collection

Matched pairs from each cohort were imaged during the same session by an experimenter blinded to diagnostic or antipsychotic exposure group. All images were taken using a confocal microscope equipped with a 60X oil supercorrected objective (equipment details found in the supplement). Tissue thickness was measured at each sampling site and did not differ by diagnostic group ($F_{1,18.5}$=0.05, p=0.83) or cohort ($F_{1,18.6}$=2.35, p=0.14). Image stacks were taken beginning 12.5 µm below the tissue surface closest to the coverglass, stepping up 0.25 µm with each image until the tissue surface was reached. This produced an image stack comprised of 50 individual planes, each 512 x 512 pixels in size. Exposure times for 488nm and 568 nm excitation wavelengths were set to optimize the spread of the intensity histogram for each cohort, then were kept consistent for all subjects within cohorts.

Image Processing

Images were processed using Slidebook software version 5.027 with keystrokes automated by Automation Anywhere software (Automation Anywhere, Inc. San Jose, CA). Camera background was subtracted from channels 488 and 568 prior to processing. Underlying gray level values were extracted from the mask objects.

Image stacks were deconvolved using the AutoQuant adaptive blind deconvolution algorithm (MediaCybernetics, Rockville, MD). After deconvolution, edges were sharpened by taking the difference between images convolved at two standard deviations of the Gaussian distribution (σ_1=0.7; σ_2=2.0) as previously described, then subjected to iterative intensity/morphological segmentation. Spinophilin-IR and phalloidin puncta with intensity measures above the RC defined minimum threshold derived value in Slidebook were selected and contiguous pixels were defined as a ‘mask object.’ Spinophilin-IR and phalloidin mask objects with volumes between 0.1 and 0.8 μm^{-3} and 0.04 and 1.5 μm^3, respectively, were selected at each iteration. Due to lower spinophilin-IR intensity in cohort 2, it was necessary to begin with a minimum threshold value at 1/3 the RC defined value. At each of 100 iterations, the threshold intensity was increased and the mask objects combined with those of the prior iteration.

Calculation of Spine Density, Number, and Area

While both spinophilin-IR and phalloidin binding are strongly localized to spines, each has some off target label Therefore, identification of putative dendritic spines required co-localization of spinophilin-IR and phalloidin label **(Fig. 2)**, operationalized as phalloidin mask objects that overlapped (≥ 1 voxel) with a spinophilin-IR mask object Spine density (N_v) in cohort 1 was calculated as previously described with minor modification:

$$N_v := \frac{\bar{t}_{wQ^-}}{h} \cdot \frac{\sum(Q_i^- \cdot w_i)}{BA \cdot a \cdot \sum(P_i \cdot w_i)}$$

Where *a* is the area of the counting frames, Q_i^- is the count of dendritic spines within the *i*th block, P_i is the count of the associated points hitting the region of interest in the *i*th block, *h*= disector height (see supplemental methods for additional details), *BA* is the cryostat block advance (50 µm for cohort 1 and 60 µm for cohort 2, $\bar{t}_{wQ^-}$ is the block-and-number-weighted mean section thickness calculated using this formula:
(2)

$$\bar{t}_{wQ^-} := \frac{\sum(t_j \cdot q_j^- \cdot w_i)}{\sum(q_j^- \cdot w_i)}$$

where t_j is the local section thickness measured centrally in the *j*th sampling frame and q_j^- is the corresponding count of dendritic spines in the *j*th frame. w_i is the block weight—i.e. either 1 or 1/3. Because for cohort 2, sections adjacent to the mapping sections were sampled, calculation of N_v was as above but omitting the block weighting.

Targeted Mass Spectrometry

Sample Preparation

Tissue homogenates were prepared from fresh frozen human A1 grey matter described above. Total protein was extracted using SDS extraction buffer (0.125 M Tris – HCl (pH 7), 2% SDS, and 10% glycerol) at 70°C. Using bicinchoninic acid assay (Micro BCA™ Protein Assay, Pierce) protein concentration was measured. A pooled technical replicate sample composed of homogenate aliquots from all subjects was also prepared. 20 ug of total protein from the gray matter homogenate or pooled sample was mixed with Lysine $^{13}C_6$ Stable Isotope Labeled Neuronal Proteome Standard[10] ($^{13}C_6$ STD; 20 ug) for on gel trypsin digestion. Samples were organized in a block distribution. Each block was run on a single 10 well 4-12% BisTris gel with two SeeBlue® Plus2 Pre-stained Protein Standards (Invitrogen, Carlsbad, CA). On-gel trypsin digestion was performed as previously described[10] with samples being run 4 cm into the gel and divided into two fractions (above and below 65kd).

LC-SRM/MS

The selection process for the proteins included in this SRM assay has been extensively described[10]. Briefly: proteins were chosen from recently published discovery proteomics analyses of the voltage-dependent calcium channel interactome[11]. Peptides from these proteins were chosen and then filtered by the following criteria: 1) the presence of a lysine, the amino acid labeled in the $^{13}C_6$STD, 2) non-redundant to a selected protein or protein group

(determined by BLAST search) and 3) 100% homology across mouse and human sequences (determined by BLAST search).

LC-SRM/MS analyses of target peptides was conducted as previously described[10,12]. In brief, SRMs were monitored using a TSQ Quantiva triple stage quadrupole mass spectrometer (Thermo Scientific) with an UltiMate 3000 Nano LC Systems (Thermo scientific). 2 µl (~1 µg protein) of sample was loaded/desalted on a PepMap100 Nano-Trap column (Thermo scientific) at 8 µl/min for 2 min and separated on a Reprosil-pur 3 µm PicoChip column (New Objective) at 400 nl/min over a 20 min gradient from 2-35% mobile phase B (Acetonitrile containing 0.1% formic acid). SRM transitions were timed using 1.5 min retention windows. Transitions were monitored, allowing for a cycle time of 1 sec, resulting in a dynamic dwell time, never falling below 2 msec. The MS instrument parameters were as follows: capillary temperature 275 °C, spray voltage 1100 V, and a collision gas of 1.4 mTorr (argon). The resolving power of the instrument was set to 0.7 Da (Full Width Half Maximum) for the first and third quadrupole. Using Skyline, integrated peak areas for both "light" human peptides and the "heavy" $^{13}C_6$STD peptides were calculated for each of the peptide sequences. The light:heavy integrated area ratio was calculated to obtain peptide measures using multiple transitions per peptide. We have previously established the linearity of peptide quantification over a broad range of expression magnitude using this approach[10]. The expression of tryptic peptides from over 150 neuronal proteins, including PSD and calcium signaling proteins, were measured. Some of these findings have been previously reported[12]. Of these only one peptide from voltage gated calcium channels, ALFDFLK from CACNB1-4, was assayed.

Primary Neuronal Culture

Primary cortical neuron cultures were prepared from embryonic day 17 (E17) Sprague Dawley rats (Envigo and Charles River Laboratories). The pregnant rat was anesthetized by exposure to isoflurane (2-5%) for 5 minutes, all embryos were removed, decapitated, and their brains were extracted and placed in HBSS (1X Hank's Balanced Salt Solution (Ca2+ and Mg2+ free; Gibco; Catalog #: 14185)) on ice. Under a dissecting scope, the cortical tissue was dissected, collected via pipette and placed in a 15 mL conical tube on ice. After removing HBSS, the tissue was resuspended in 5 mL of pre-warmed (37°C) papain (Papain (Suspension; Worthington Biochemical; Catalog #: LS003126) diluted to a concentration of 20 units/mL in 1X Hank's Balanced Salt Solution with a few grains of L-Cysteine (Sigma-Aldrich; Catalog #: C7352) and 400 µM Sodium Hydroxide (Solution 1N; Fisher Scientific; Catalog #: SS266-1)), and incubated at 37°C in a water bath for 4 minutes. The papain solution was removed, and the tissue was quickly washed in 5 mL of pre-warmed trypsin inhibitor (Trypsin Inhibitor (Ovomucoid Source; Worthington Biochemical; Catalog #: LS003086) dissolved to a concentration of 1% (w/v) in 1X Hank's Balanced Salt Solution with 400 µM Sodium Hydroxide). The tissue was washed an additional three times with the trypsin inhibitor solution at 37°C for 2 minutes before being placed in 5 mL of temperature-equilibrated (37°C) plating medium (Minimum Essential Medium with Earle's salts, Gibco, Catalog #: 41090-036), 100 mM pyruvic acid (Sigma-Aldrich; Catalog #: P-2256), 20% glucose (Sigma-Aldrich; Catalog #: G6152), 10% Horse serum (Gibco;

Catalog #: 26050-070), 2 mM GlutaMAX-I (Gibco, Catalog #: 35050-061)) and triturated with a 10 mL serological pipette. After trituration an additional 5 mL of plating media was added and cell numbers were estimated using exclusion of trypan blue (Sigma-Aldrich; Catalog #: T8154). Neurons were plated at 450,000 cells/well in 12-well plates containing poly-D-lysine (PDL)-laminin coated glass coverslips (neuVitro; Catalog #: GG-18-laminin) in 1 mL of plating media per well. After 4 hours, the plating media was aspirated and replaced with B27 maintenance media (Neurobasal Medium (Minus Phenol Red; Gibco; Catalog #: 12348-017) supplemented with 2% B27 Supplement (Gibco; Catalog #: 17504-44), penicillin/streptomycin (100 U/mL and 100 mg/mL, respectively; Gibco; Catalog #: 15145-014), 2 mM GlutaMAX-I). Every 3-4 days, half of the media in each well was exchanged for fresh maintenance media. Neurons were incubated at 37 °C, with 5% CO_2.

Transfection

On day in vitro (DIV) 12, the media in each well was removed and saved. Each well was washed twice with 1 mL of Neurobasal medium. After a final 1 mL of Neurobasal medium was added to each well, the neurons were returned to the incubator. Pre-warmed (37°C) Opti-MEM Medium (Reduced Serum; Gibco; Catalog #: 31985-062) was added to a 50-mL centrifuge tube (50 µL for each well to be transfected) followed by Lipofectamine LTX Reagent (With PLUS Reagent; Invitrogen; Catalog #: 15338-100; 5 µL for each well to be transfected). OptiMEM Medium (50 µL for each well to be transfected) was added to a separate 50-mL centrifuge tube. The sequence of the CACNB4 plasmid (OriGene; Catalog #: RR204310) was confirmed (Figure S?) and it and a GFP plasmid (gift of Ryan Logan, University of Pittsburgh, PA) were added at a ratio of 2:1 (1 µg total DNA per well to be transfected), followed by PLUS Reagent (1 µL per well to be transfected). The plasmid-PLUS solution was combined with the LTX solution and allowed to incubate at room temperature for 5 minutes. 100 µL of the transfection solution was added to each well, and the neurons were returned to the incubator for 4 hours before the media containing the transfection solution was aspirated. Each well was washed twice with pre-warmed Neurobasal Medium and the original conditioned media (half of which had been replaced with fresh media) was pre-warmed to 37°C and returned to the transfected wells (1 mL per well).

Fixation and Immunohistochemistry

On DIV 15, after a quick wash in cold Hank's Balanced Salt Solution (HBSS), coverslips were immersion-fixed in cold 4% paraformaldehyde for 20 minutes, then washed three times for 5 minutes each in HBSS and stored in HBSS at 4°C until staining.

Fixed cells were permeabilized with 0.2% Triton for 10 minutes and rinsed three times for 3 minutes each in HBSS. Coverslips were incubated for 20 minutes in HBSS containing 2% normal goat serum, followed by a 45 minute incubation at room temperature in HBSS containing 1% normal goat serum (Jackson ImmunoResearch Laboratories, Inc.) and a 1:1000 dilution of mouse anti-c-Myc antibody (monoclonal 9E10; Santa Cruz Biotechnology, Inc.). After 3 washes with HBSS, coverslips were incubated for 45 minutes at room temperature in HBSS containing

1% normal goat serum and a 1:1000 dilution of goat anti-mouse secondary antibody conjugated to Alexa Fluor 568 (Invitrogen). After a final three rinses in HBSS, the coverslips were mounted onto glass slides with Vectashield HardSet Mounting Medium (Vector Laboratories, Burlingame, CA).

To verify CACNB4 overexpression, separate coverslips were blocked in 2% normal donkey serum and then incubated in a primary antibody solution containing 1% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc.), a 1:100 dilution of mouse anti-CACNB4 (monoclonal S10-7; antibodies-online Inc.), and a 1:100 dilution of goat anti-c-Myc (polyclonal; Novus Biologicals). The secondary antibody solution contained 1% normal donkey serum, 1:100 donkey anti-mouse Alexa Fluor 568 (Invitrogen) and 1:100 donkey anti-goat Alex Fluor 647 (Invitrogen).

Microscopy and Image Processing

Data acquisition was performed on an Olympus (Center Valley, PA) BX51 WI upright microscope equipped with an Olympus spinning disk confocal (SDCM) using an Olympus PlanAPO N 10X 0.40 NA air objective and a 1.42 numerical aperture 60X oil supercorrected objective. The SDCM was equipped with an ORCA-R2 camera (Hamamatsu, Bridgewater, NJ), MBF CX9000 front-mounted digital camera (MicroBrightField, Inc., Natick, MA), BioPrecision2 XYZ motorized stage with linear XYZ encoders (Ludl Electronic Products Ltd., Hawthorne, NY), excitation and emission filterwheels (Ludl Electronic Products Ltd.), and equipped with a Sedat Quad 89000 filter set (Chroma Technology Corp., Bellows Falls, VT). The microscope was controlled by Slidebook (Intelligent Imaging Innovations) software and illuminated using a Lumen 220 metal halide lamp (Prior Scientific, Rockland, MA).

First, 10x fixed images were taken of healthy GFP-positive neurons with pyramidal neuron morphology. Neurons were categorized as either CACNB4-overexpressing or GFP-only controls based on c-Myc intensity. Across three experiments, a total of 56 GFP-only neurons and 47 Myc+/GFP+ neurons were imaged. Two dendrites per neuron were chosen for 60x imaging. Selected dendrites were secondary or tertiary branches emanating from the apical dendrite and were greater than 50 µm from the soma. Additionally, each segment had a length of ~50 µm before the next branch point. Fixed 60x image stacks, which traversed the entire depth of the dendritic segment, were taken with a step size of 0.1 µm. Each two-dimensional image in the stack consisted of an image plane that was 512 X 512 pixels (~3,058 μm^2). At each plane, data were collected in the 488 channel. Exposure time was optimized for each dendrite based on the center plane of the stack. A sum projection image was created for each stack, and the histogram and gamma for the 488 channel were manually adjusted to aid in spine counting. For every image, an approximately 50 µm-long segment was marked for spine counting and the exact length recorded. The marked projection image was exported from SlideBook as a TIFF file.

To quantify Myc intensity, a mask was generated in each 10x image of the neuron's soma using the 488 channel. A background mask, which was the inverse of the soma mask, was also created. Intensity statistics were extracted for both the soma and background masks. The median 568 intensity of the background mask was subtracted from the median 568 intensity

of the soma mask. The resulting values were adjusted for differences in exposure times by dividing by the 568 channel exposure time.

GFP-positive neurons on coverslips stained for both c-Myc and CACNB4 were imaged at 10x. Exposure times for the 488 channel were optimized whereas the 568 and 647 channels were shot at fixed exposures of 447 ms and 3000 ms, respectively.

Spine Counting

The TIFF files of all dendrites were imported into StereoInvestigator, (MicroBrightField, Inc.) for counting. Protrusions from the dendritic shaft were manually assigned to one of the following categories, blind to experimental condition: short mushroom spine, long mushroom spine, short stubby spine, long stubby spine, or filopodia. Criteria for assignment were previously described (Richardson et al. 2009). Briefly, mushrooms spines had distinct heads while stubby spines did not. Long spines had a length greater than maximal width and the rest were short. Filopodia were longer than 2 μm, thinner than 0.3 μm, and lacked a distinct head. For a given dendrite, the number of protrusions in each category was recorded and the densities per μm of dendrite length for all spines, mushroom and stubby subtypes, and filopodia were calculated.

Spine Masking

A subset of the 60x dendritic images used for spine counting was randomly selected, blind to condition, for spine area analysis. In total, 35 GFP-only dendrites and 38 Myc+/GFP+ dendrites were analyzed. The raw stack files were processed using a no neighbors deblurring algorithm, and maximum projection images were created. GFP signal is sometimes higher within the dendritic shaft, which can interfere with automated masking algorithms. To improve dendritic spine segmentation, spines were first masked by hand so that only the fluorescence signal associated with dendritic spines could be imported to MatLab. Within MatLab, a custom intensity/morphological segmentation algorithm was used to make mask objects of dendritic spines as previously described[7] with one exception, a dynamic size gate was used. Following segmentation, the new mask (“mask1”) was imported back into SlideBook. Although this approach was ideal for masking larger objects, it failed to mask some smaller objects (you might want to say that smaller spines are inherently less bright). To mask the smaller spines, we first took the square root of each pixel in the MatLab matrix containing the spine fluorescence data. This was done to increase the brightness of the smaller spines with respect to the larger brighter spines. Then the Otsu thresholding algorithm[13] was used to mask the image (“mask2”). Although in mask2, small spines were ideally masked, larger spines were over masked. Therefore, to create the final mask (“fmask”), mask operations between mask1 and mask2 were performed such that only masked objects in mask2 pertaining to spines not masked in mask1 were added to mask1. Next, multiple objects belonging to a single spine were connected by hand. Object area (μm^2) statistics were extracted for the final mask in each image. The final masks were visually inspected, and if any object masked more than a single spine, the number of spines in that object was recorded.

Reference for Spine Counting:

Richardson RJ, Blundon JA, Bayazitov IT, Zakharenko SS (2009) Connectivity Patterns Revealed By Mapping of Active Inputs on Dendrites of Thalamorecipient Neurons in the Auditory Cortex. J Neurosci 29(20):6406-6417.

Statistical Analysis

Human spine density by volume:

The data was analyzed through a linear mixed model with the pair effect taken into account. Spine density was assumed to be normally distributed. In the model, pair, cohort, diagnosis, spine size category, cohort*spine size category and diagnosis*spine size category were the fixed effects. Subject was treated as a normal random effect to account for the repeated measures within each subject. Insignificant interaction terms were not included in the final model. The Kenward-Roger method was used to adjust for the denominator degrees of freedom. The analysis was implemented in SAS 9.4 with the PROC MIXED procedure.

Human Spine Density Categorical Confound Effects:

For the categorical confound effect analysis, the percent change of spine density within each pair was calculated ((C-S)/C) x100% and the percent changes were compared between the group with that responded “yes”(Y) to the confound variable and that responded “no”(N). A two-sample t-test was applied and the percent changes in the Y and N groups were estimated. There are two estimates in the last two columns in the table: the mean percent change within pairs with response Y to the confound variable and mean percent change within pairs with response N to the confound variable.

For example, in spines with size 1, for confound variable sex (marked in red in the following table), the percent change (calculated as (C-S)/C) x100% in spine density within the males (M) is estimated to be 0.234 and the percent change in spine density within the females (F) is estimated to be -0.089. That is to say, in the M pairs, Sz patients were about 23.4% lower in spine density compared to C. Similarly, in the F pairs, Sz patients were about 8.9% greater in spine density compared to C. The difference in the two percent changes (percent change in M – percent change in F) was 0.323 and was insignificant (p=0.354).

Human Spine Density Continuous Confound Effects:

For the continuous confound effect analysis, the percent change of spine density within each pair was calculated ((C-S)/C) x100% and a simple linear regression analysis was done upon each of the two confound variables separately (age of onset and duration of disease). The slope of the percent change of spine density on each confound variable was estimated and the p-value to test whether the slope was significantly different from 0 was provided in the table.

Protein Level Correlations with Spine Density by Size:

Person's Correlation was used to determine the relationship between peptide expressions and spine density by volume.

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