**Supporting Information**

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**SI Text**

**Intracellular Ca\(^{2+}\) Monitoring: Population Level.** Neurons grown on poly-L-lysine-coated 96-well (9-mm; Costar) culture plates (1.5x \(10^5\) cells per well) were used for [Ca\(^{2+}\)]\(_i\) measurements as described (1, 2). Using neurons loaded with Fluo-3 AM (4 \(\mu\)M in Pluronic acid (0.04%), 96-well plates were excited at 488 nm and Ca\(^{2+}\)-bound Fluo-3 emission was recorded at 538 nm at 1.2-s intervals using a FlexStationII (Molecular Devices) bench-top scanning fluorometer.

**Ca\(^{2+}\) and Na\(^+\) Imaging: Single-Cell Level.** Neurons grown on 40-mm coverslips (0.8x \(10^5\) cells/mL) were loaded with either 4 \(\mu\)M Fluo-3/AM in Pluronic acid or 10 \(\mu\)M SBF-AM in 0.02% (wt/vol) Pluronic acid for Ca\(^{2+}\) imaging or 10 \(\mu\)M SBF-AM in 0.02% (wt/vol) Pluronic acid for Na\(^+\) imaging, in Locke’s buffer for 30 min at 37 °C and 5% CO\(_2\). Cells were then washed in Locke’s buffer to remove excess dye and incubated in Locke’s for another 15 min. Coverslips were mounted in a temperature controlled (37 °C) chamber (Bioptechs) on an inverted Olympus IX 71 microscope. Background-corrected fluorescent images (60x oil/NA 1.25; image sequence: 1 frame/s, 5- to 10-ms exposure time, 4 x 4 binning) were acquired with Slidebook (version 5.0) imaging software using the Fura-2 filter set (excitation, 340 and 380 nm; emission, 505 nm). Compounds were added as a bolus addition to a static bath (total volume, 1,000 \(\mu\)L). Pharmacological evaluation of the response to PbTx-2 (100 nM or 1 \(\mu\)M) was assessed in the presence of various antagonists as indicated.

**Calibration of [Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\).** An intracellular calibration of [Ca\(^{2+}\)]\(_i\) was performed postacquisition for each experiment. For measuring maximal ([R\(_{\text{max}}\)]) and minimal ([R\(_{\text{min}}\)]) Ca\(^{2+}\) ratios, cells were treated, respectively, with 4 \(\mu\)M A23187 (calcium) and 2.5 mM CaCl\(_2\) or 5 mM EGTA in Ca\(^{2+}\)-free Locke’s buffer. The [Ca\(^{2+}\)]\(_i\) was calculated from the following equation: [Ca\(^{2+}\)]\(_i\) = \(K_d \times (R_{\text{max}} - R) / (R_{\text{max}} - R_{\text{min}})\), where \(K_d\) is the dissociation constant, \(R_{\text{max}}\) for Fura-2 was 145 nM, and \(\beta\) is the ratio of the denominators of the minimum and maximum conditions.

Calibration media for in situ calibration of SBF-1 fluorescence contained 130 mM Na\(^+\) plus K\(^+\), 0.6 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 10 mM Heps, 30 mM Cl\(^-\), and 100 gluconate (pH 7.4); 5 \(\mu\)M gramicidin D, 10 \(\mu\)M mithenicol, and 100 \(\mu\)M ouabain were added to equilibrate extra- and intracellular [Na\(^+\)].

**Immunocytochemistry.** To assess the influence of PbTx-2 and NMDA on neurite outgrowth, neurons grown on poly-lysine-coated cover glass placed inside 24-well culture plates were used. Immunostaining of neurons was performed as described previously (1, 2) using fixation by 4% paraformaldehyde in PBS for 20 min. After permeabilization and blocking with PBS containing 0.15% Triton X-100 and 2% FBS for 30 min, neurons were incubated with protein gene product 9.5 (anti–PGP 9.5) primary antibody overnight at 4 °C and then with secondary antibody for 1 h at room temperature. Slides were mounted, and images were acquired using an Olympus IX 71 inverted microscope equipped with a 40x oil immersion objective. Total neurite length was quantified using Imaris software, and at least 30 randomly chosen neurons from different cultures were evaluated for each treatment group. The identity of treatment groups for all images was unblinded subsequent to image analysis.

**Double Immunofluorescence.** Neurons (1.0x \(10^6\) cells/mL) grown on poly-D-lysine-coated cover glass (22 x 22 mm) were used. PbTx-2 was added to the culture medium at 3 h after plating. The neurons on the cover glass were fixed, washed, permeabilized, and blocked. Neurons were incubated overnight at 4 °C with primary antibodies, synaptophysin (1:800; Invitrogen), and PSD-95 (1:400; Thermo Fisher Scientific) in 3% serum followed by secondary antibodies conjugated with Alexa fluor 488 (1:600) and Alexa fluor 568 (1:600).

**Image Acquisition and Colocalization Analysis.** Images were acquired using an Olympus spinning-disk confocal microscope attached to a Hamamatsu ORCA-ER digital camera with 60x oil immersion objective in 1344 x 1024 pixel (144 x 110 \(\mu\)m) frames and scanned at 0.2- \(\mu\)m intervals along the z axis with a depth of 5 \(\mu\m (25 planes). Double immunostaining with synaptophysin (green), a presynaptic marker, and PSD-95 (red), a postsynaptic marker, yielded reliable quantification of colocalized puncta (yellow) as an indication of the presence of a synapse. Automated colocalization analysis was performed using Slidebook version 5.0 software, which analyzes the entire two-channel confocal stack by measuring the intensity of each label, voxel by voxel.

**Quantification of Colocalized Puncta.** The confocal stacks were deconvolved, and the image background was corrected using the same threshold values for all images to be analyzed. A mask segmentation of fluorescent objects was created in both channel images, and the merged image was statistically analyzed using cross-mask and cross-channel functions. To exclude neuronal soma, a size-exclusion limit was defined. Pearson’s correlation coefficient (≥0.7) was used to determine colocalized puncta. Pearson’s correlation coefficient indicates the extent to which the intensity of the two labels increases together in the same voxels. It varies between +1 and −1, with positive values indicating a positive correlation, values near 0 indicating no correlation, and negative values indicating an inverse correlation. To determine synapse density, total dendritic length 5 \(\mu\m away from neuronal soma was measured in each frame and normalized to the number of synapses per 20 \(\mu\m neurite length.

**Dioleic Labeling and Quantification of Dendritic Branching and Filopodial Protrusion Density.** Neurons grown on poly-D-lysine-coated glass coverslips placed inside 24-well culture plates (0.1x \(10^5\) cells per well) were used. A Helios Gene Gun System (Bio-Rad) was used to deliver Dio-coated tungsten particles (1.1 \(\mu\m) (Bio-Rad) into paraformaldehyde-fixed cerebriocortical neurons as described previously (2). Z-stacked images were acquired using an Olympus spinning-disk confocal microscope, and each neuron was scanned at 0.2- \(\mu\)m intervals along the z axis with a depth of 5 \(\mu\m (25 planes).

**Measurement of Dendritic Branching and Filopodial Protrusion Density.** For quantitative analysis of the super module of Imaris software (Bitplane Scientific Software). For the analysis of dendritic arbor complexity, the dendritic tracings were quantified by an automated 3D Sholl analysis, where a set of concentric spheres (1 \(\mu\m apart) centered at the cell body was drawn, and the number of dendritic branch intersections at each sphere counted, using Imaris software to analyze arbor complexity (Bitplane Scientific Software).

The quantification of dendritic protrusions was initiated after the first branch point, and the values were normalized based on number and length of the dendrites quantified within each individual neuron. The rendered image after Gaussian smoothing was thresholded manually such that all visually discernable neuronal protrusions in 3D were identified. We used the filament module of Imaris software that quantifies spine density and head.
diameter. The minimum end segment diameter (spine head) was set at \( \geq 0.215 \mu m \) (i.e., the thinnest quantifiable spines were 0.215 \( \mu m \) in diameter). For automatic quantification of neuronal protrusions, the following algorithm was used: total number of protrusions from dendrites = \( \geq 0.215 \mu m \) head diameter and \( \leq 10 \mu m \) length; number of stubby filopodia = \( \geq 0.430 \mu m \) head diameter and \( \leq 5.375 \mu m \) length; number of long filopodia = total number of protrusions minus number of stubby filopodia.

**Immunoblotting.** Immunoblot analysis was performed in cells grown in 12-well plates (2.0 \( \times 10^8 \) cells per well) within 24 h postplating. Cells were washed two times with Locke’s incubation and then allowed to equilibrate in Locke’s buffer for 30 min. Cultures were then treated with the PbTx-2 in the absence and presence of antagonists at 37 °C for specified times. Cultures were then transferred to ice slurry. The cells were lysed using lysis buffer containing 50 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Nonidet P-40, 0.1% SDS, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 25 mM β-glycerophosphate and phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor mixture for 20 min at 4 °C. Cell lysates then underwent sonication and were centrifuged at 12,000 \( \times g \) for 15 min at 4 °C. The supernatant was retained for 20 min for 709. Cells were then sonicated and centrifuged at 100,000 \( \times g \) for 20 min and then incubated with streptavidin and protease inhibitors. The lysate was centrifuged at 12,000 \( \times g \) for 20 min at 4 °C and then incubated with streptavidin–Sepharose beads (Pierce) for 20 min with gentle agitation. Cells were washed three times with ice-cold PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂, and 5 mM EDTA (PBS⁺) and incubated with 1 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS⁺ with protease inhibitors for 20 min at 4 °C with gentle agitation. Cells were washed three times with ice-cold quenching buffer (50 mM glycine in PBS⁺) for 5 min each. The cells were then sonicated and centrifuged at 100,000 \( \times g \) for 20 min at 4 °C to obtain the crude membrane fraction. The crude membrane fraction was first lysed in 1% SDS for 30 min at 37 °C and then in 1% Triton X-100 at 4 °C, both in PBS with 5 mM EDTA and protease inhibitors. The lysate was centrifuged at 16,000 \( \times g \) for 20 min and then incubated with streptavidin–Sepharose beads (Pierce) for 2 h at 4 °C with gentle rotation. Beads were washed four times in washing buffer containing 1% Triton X-100 and then twice in PBS. Bound proteins were eluted in Laemmli sample buffer and immunoblotted with antibodies against GluN2B (1:1,000), TrKB (1:2,000), and α-tubulin (1:10,000) through activation of voltage-gated sodium channels. J Pharmacol Exp Ther 332(3): 698–709.


The addition of extracellular $[\text{Na}^+]$ containing calibration media (0–130 mM) evoked corresponding stepwise changes in fluorescence ratio. To convert the fluorescence ratio of emitted SBFI signals into a $[\text{Na}^+]$ value, the normalized data from our calibration experiments [expressed as changes in fluorescence ratio $\Delta R$ divided by the fluorescence ratio at 0 mM $[\text{Na}^+]$; $\Delta R/R_0$], were plotted in the form of the Michaelis–Menten equation, using $\frac{(\Delta R/R_0)_{\text{max}}}{\Delta R/R_0} = \frac{[\text{Na}^+]_{\text{max}}}{[\text{Na}^+] + K_d}$, where $\Delta R/R_0_{\text{max}}$ is the maximal change in fluorescence ratio, $K_d$ is the apparent dissociation constant of SBFI for $\text{Na}^+$, and $\beta$ is the ratio of the fluorescence of the free (unbound) dye to bound dye at the second excitation wavelength (380 nm). A linear fit ($r^2 = 0.99$) as a Hanes function ($[\text{Na}^+]/(\Delta R/R_0)$ vs. $[\text{Na}^+]$) yielded a $K_d$ of 36 mM and a $(\Delta R/R_0)_{\text{max}}$ value of 0.039. The Hill coefficient was close to 1 (1.007 ± 0.01), indicating that one $\text{Na}^+$ binds to one SBFI molecule.

$\text{Ca}^{2+}$ imaging of DIV-1 neurons loaded with Fura-2 AM showed concentration-dependent increment in $[\text{Ca}^{2+}]_i$ on exposure to PbTx-2. Data show the raw values of Fura-2 fluorescence in response to addition of each concentration of PbTx-2. PbTx-2 was added in incremental concentrations. Arrow indicates addition of PbTx-2.
Fig. S3. Pharmacological evaluation of 100 nM PbTx-2–induced Ca$^{2+}$ influx at the neuron population level. (A) Data are from a representative experiment performed in triplicate and repeated thrice. TTX (VGSC blocker), APV (NMDAR competitive antagonist), and nifedipine (L-type VGCC antagonist) were used to block distinct calcium influx pathways. (B) Histogram representing quantification of data shown in A. TTX (1 μM) and APV (100 μM) significantly blocked PbTx-2–induced Ca$^{2+}$ influx. These data indicate that 62% of Ca$^{2+}$ entry is through NMDAR channels and 38% of the increment in [Ca$^{2+}$]$_i$ is derived from non-NMDAR sources, of which only 7% represent Ca$^{2+}$ entry through VGCC.

Fig. S4. Ontogeny of spontaneous Ca$^{2+}$ oscillations in cerebrocortical neurons. (A) Quantification of spontaneous Ca$^{2+}$ oscillations at DIV-2, -4, -6, and -9 in Fluo-3–loaded neuronal cultures [mean ± SEM of peak response (amplitude)]. (B) Frequency of Ca$^{2+}$ oscillations [number of oscillations per 300 s; mean ± SEM; n ≥ 3 independent experiments]. These spontaneous Ca$^{2+}$ oscillations become prominent from DIV-6 onward both in amplitude and frequency of oscillations.
Fig. 55. PbTx-2–induced neuronal plasticity involves NMDAR-mediated CaMK signaling with downstream activation of CREB-dependent transcription of BDNF. (A) Time course of 100 nM PbTx-2–induced phosphorylation and activation of CaMKI (Thr177), CaMKIV (Thr196), Erk1/2, Akt (Ser473), CaMKII (Thr286), and CREB (Ser133) in DIV-1 cerebrocortical neurons. Representative immunoblots are shown. (B) Quantification of relative band densities of immunoblots. Each point represents mean ± SEM of three independent experiments. *P < 0.05 (Student’s t test comparing phosphorylation level of signaling proteins in PbTx-2 treated vs. control neurons). (C and D) Pharmacological evaluation of PbTx-2–induced CREB (Ser133) phosphorylation. The PbTx-2–enhanced phosphorylation of CREB was attenuated by TTX, D-APV, and STO-609 (δP < 0.05; *P < 0.05; Student’s t test). (E) Conventional RT-PCR showed a time- and concentration-dependent increase in BDNF mRNA expression following PbTx-2 exposure (n = 3). (F) Biotinylated surface proteins were isolated after 30 min of PbTx-2 exposure, resolved by SDS/PAGE, and probed with GluN2B, TrkB, and α-tubulin antibodies. A representative blot is shown. (G) Quantification of the biotinylation experiments. The immunoreactive signals for cell surface GluN2B and TrkB were normalized to the respective crude membrane fraction values and are presented as a histogram. (Mean ± SEM; ANOVA, P < 0.05; Dunnett’s post hoc test, *P < 0.05; n = 3.)