Expanded View Figures

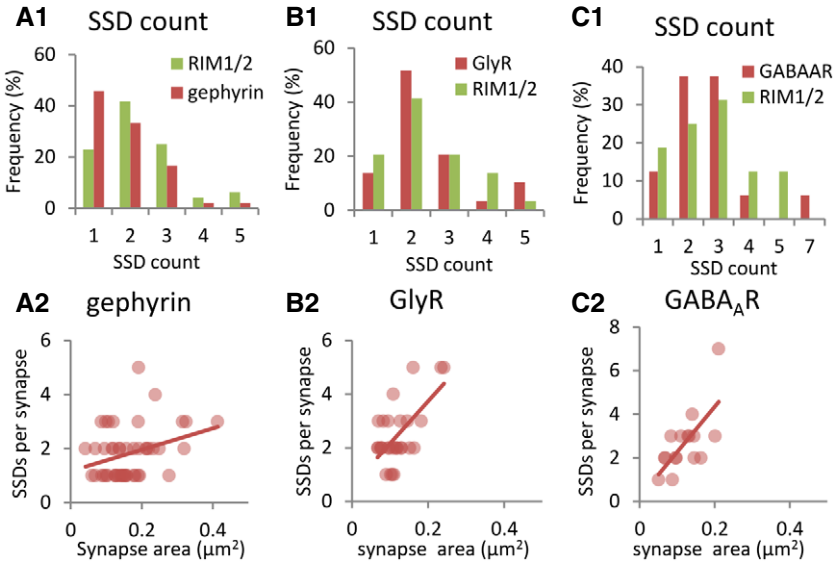

Figure EV1. Sub-synaptic domains of inhibitory synaptic proteins.

A–C Number of SSDs per synapse. (A1–C1) Histograms of SSD numbers of RIM1/2 and gephyrin, GlyRs, and $GABA_A$Rs, respectively. (A2–C2) Correlation between SSD numbers and synaptic cluster sizes of different post-synaptic proteins. Spearman *R* test, $P > 0.5$ in (A2) and (B2), $P < 0.5$ in (C2). Quantification was done on synapses with side view profiles as shown in Fig 2. Number of synapses: $n = 48$ (A1, A2), $n = 30$ (B1, B2), $n = 16$ (C1, C2).

Source data are available online for this figure.

Figure EV2. Two-color dSTORM imaging of $GABA_A$Rs and GlyRs.

A–D Workflow of the dual-color dSTORM experiments. (A1–D1) Epifluorescence images of two fields of views of spinal cord neurons labeled for $GABA_A$Rs (with Alexa 647) and GlyRs (with Cy3B). The brightness and contrast between A1 and C1, and between B1 and D1 are adjusted to the same dynamic range. Scale bar: 10 μm. (A2–D2) Pointillist images showing the dSTORM detections within the regions in the yellow boxes in A1–D1. Scale: 2 μm. (A3–D3) Rendered images from the detections shown in A2–D2. Super-resolution $GABA_A$R clusters are shown in red hot false colors, GlyR clusters in cyan hot. Arrows indicate the synaptic clusters shown in the magnified images A4–D4. Scale: 1 μm. (A4–D4) Detections (blue dots) overlaid with synaptic masks (in white) in the pickpointsSR program in Matlab. Synaptic masks were produced by binarizing the corresponding super-resolution clusters indicated by arrows in A3–D3. Scale: 500 nm. (A5–D5) Correlation between the total fluorescence intensity of synaptic clusters measured in epifluorescence images and the number of detections per synapse in dSTORM. Spearman *R* test, $P < 0.0001$ in A5, B5, C5, $P < 0.05$ in D5. Each data point represents one synapse ($n = 33$ in A5 and B5, $n = 35$ in C5 and D5).

Source data are available online for this figure.

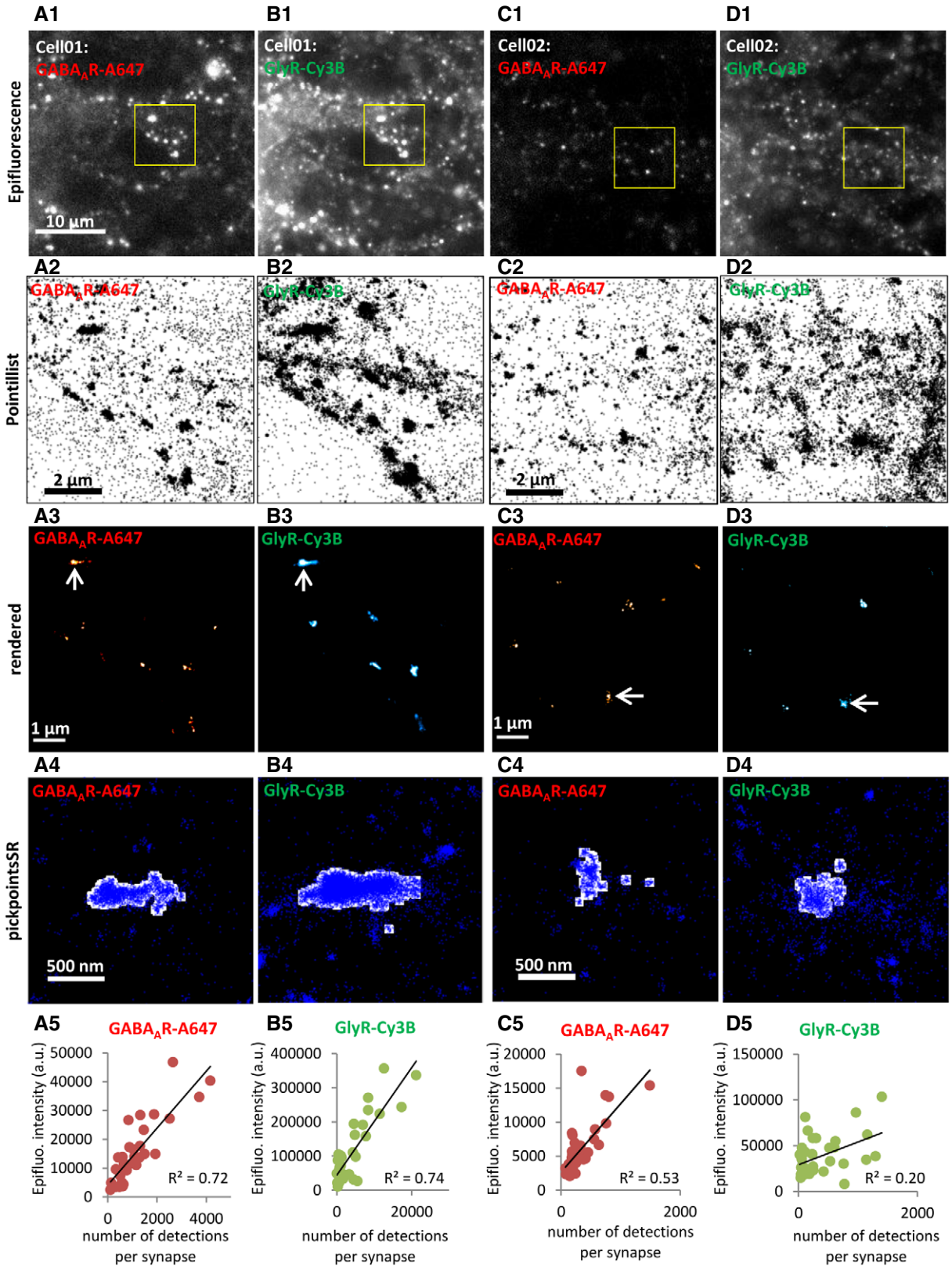

Figure EV2.

Figure EV3. Calcium imaging and immunocytochemistry (ICC) of cultured spinal cord neurons in response to changes in network activity.

A Calcium signals before treatment (pre) and after a 5-min application of TTX (top), 4-AP (middle), or strychnine/gabazine (bottom, false color). The brightness and contrast were adjusted to the same range across all the images. Note that cells active at baseline were chosen in order to better illustrate the effects of the treatments. Scale bar: 20 μm.

B Measurement of calcium signals at the beginning of the recording (pre) and after 5 and 20 min of bath application of TTX (top, blue traces), 4-AP (middle, red traces), or strychnine/gabazine (bottom, purple traces) to induce changes in neuronal network activity.

C, D Quantification of the amplitudes and frequency of the calcium signals. The amplitude was calculated as the average intensity of calcium transients per cell during the 3 min recordings. Before treatment (pre), baseline activity had a frequency of 0.15 ± 0.06 Hz/0.12 ± 0.07 Hz/0.14 ± 0.06 Hz, and an amplitude of 12.72 ± 9.21/12.34 ± 8.04/11.33 ± 5.25 (in cells treated with TTX, 4-AP, and strychnine/gabazine, respectively). TTX blocked all neuronal activity, as judged by the lack of calcium signals after application. Five minutes after 4-AP treatment, the frequency of calcium transients was greatly increased (0.33 ± 0.21 Hz, Friedman test with Dunn's *post hoc* test, $P < 0.0001$) and the amplitude was unchanged (14.56 ± 9.43, $P = 0.31$). On the other hand, 5 min strychnine/gabazine treatment increased both the frequency (0.20 ± 0.06, $P < 0.0001$) and the amplitude (52.68 ± 39.23, $P < 0.0001$). Number of cells: $n = 117$ for 4-AP (red bars), $n = 70$ for TTX treatment (blue bars), $n = 114$ for strychnine/gabazine (purple bars), from three independent experiments, data are represented as box plots showing the median, 25 and 75% quartiles, as well as the minimum and the maximum of the population.

E–J Differential regulation of GlyRs, $GABA_A$Rs, and gephyrin through altered network activity. (E–G) Quantification of synaptic levels of GlyRs, $GABA_A$Rs, and $GABA_A$R/GlyR ratios using ICC. Some of these data are the same as in Fig 4 (TTX and 4-AP treatment). (H) Treatment of cultured spinal cord neurons with 4-AP strongly reduced gephyrin immuno-labeling (KW test, $P < 0.0001$). Gephyrin was detected with mAb7a antibody that recognizes the phosphorylated S270 epitope of gephyrin. (I, J) Intensity ratios of gephyrin/GlyR and $GABA_A$R/gephyrin, showing their relative changes at the same synapses (KW test, $P < 0.0001$ in I and J). The control (CTRL) condition was without any pharmacological treatment. Number of synapses: $n = 9{,}416$ in TTX (blue traces), $n = 6{,}949$ in 4-AP (red), $n = 8{,}856$ in CTRL (black), $n = 8{,}150$ in strychnine/gabazine conditions (purple) from three independent experiments. KW test/Dunn's test, *P* values indicate the comparison to the TTX condition. ns: not significant, ****$P < 0.0001$.

Source data are available online for this figure.

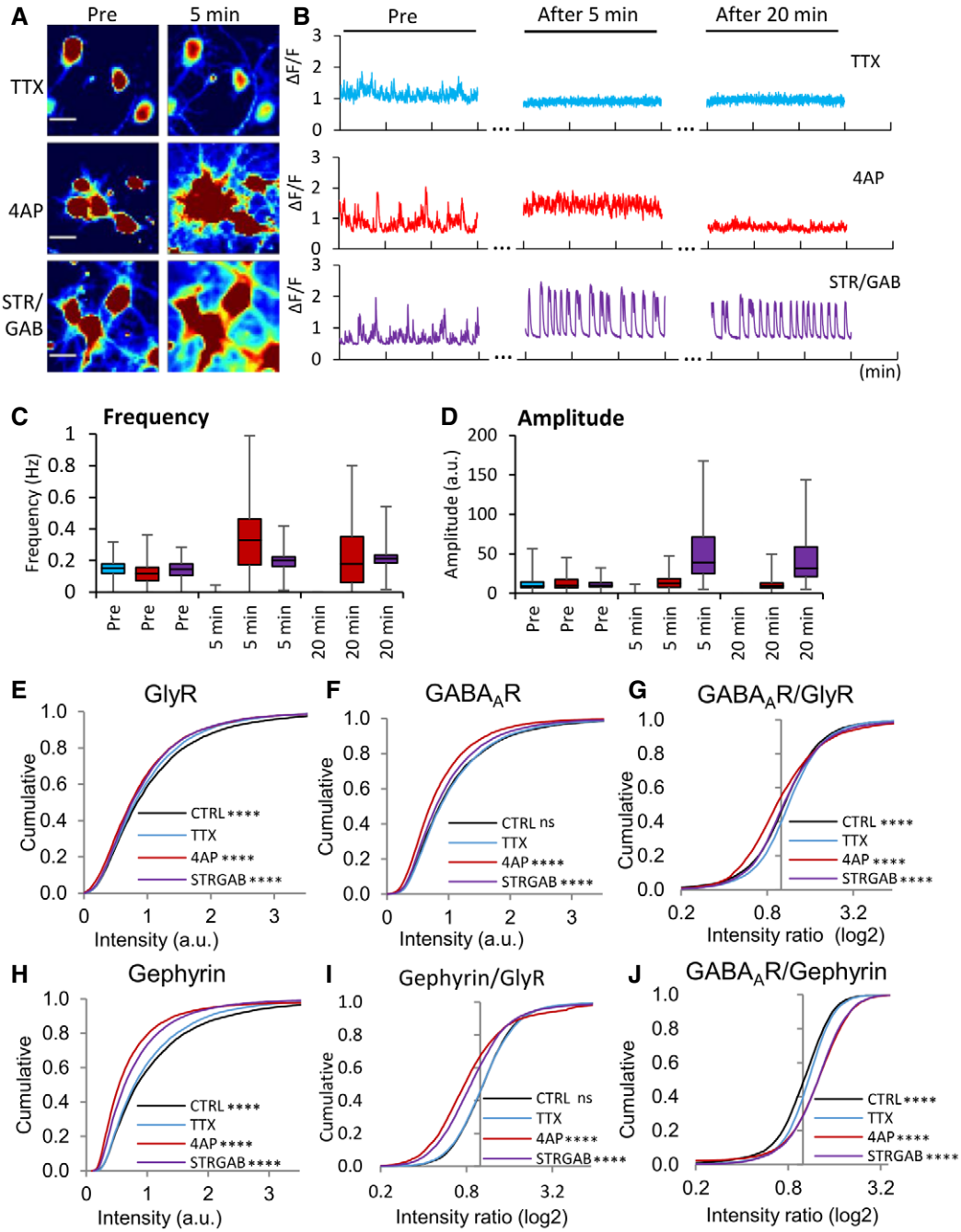
A
Pre
5 min
TTX
4AP
STR/
GAB
B
Pre
After 5 min
After 20 min
TTX
4AP
STR/GAB
ΔF/F
(min)
C
Frequency
Frequency (Hz)
D
Amplitude
Amplitude (a.u.)
Pre
5 min
20 min
E
GlyR
F
GABA$_A$R
G
GABA$_A$R/GlyR
H
Gephyrin
I
Gephyrin/GlyR
J
GABA$_A$R/Gephyrin
Cumulative
Intensity (a.u.)
Intensity ratio (log2)
CTRL ****
CTRL ns
TTX
4AP ****
STRGAB ****

Figure EV3.

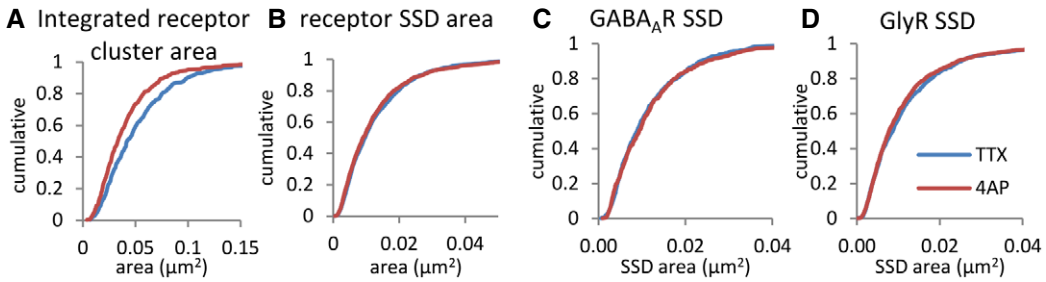

Figure EV4. Size measurement of synaptic clusters and SSDs in dSTORM.

A Synaptic receptor areas were calculated from the combined clusters of GABA$_A$Rs and GlyRs in dSTORM images.

B Receptor SSDs were segmented from the combined receptor clusters.

C, D GABA$_A$R SSDs were segmented from rendered dSTORM images of GABA$_A$R detections, and GlyR SSDs from dSTORM images of GlyR detections. 4-AP treatment led to a decrease in the total synapse area (A, KS test, $P < 0.0001$), but not the size of receptor SSDs (B, KS test, $P = 0.21$), GABA$_A$R SSDs (C, KS test, $P = 0.50$) or GlyR SSDs (D, KS test, $P = 0.23$).

Source data are available online for this figure.

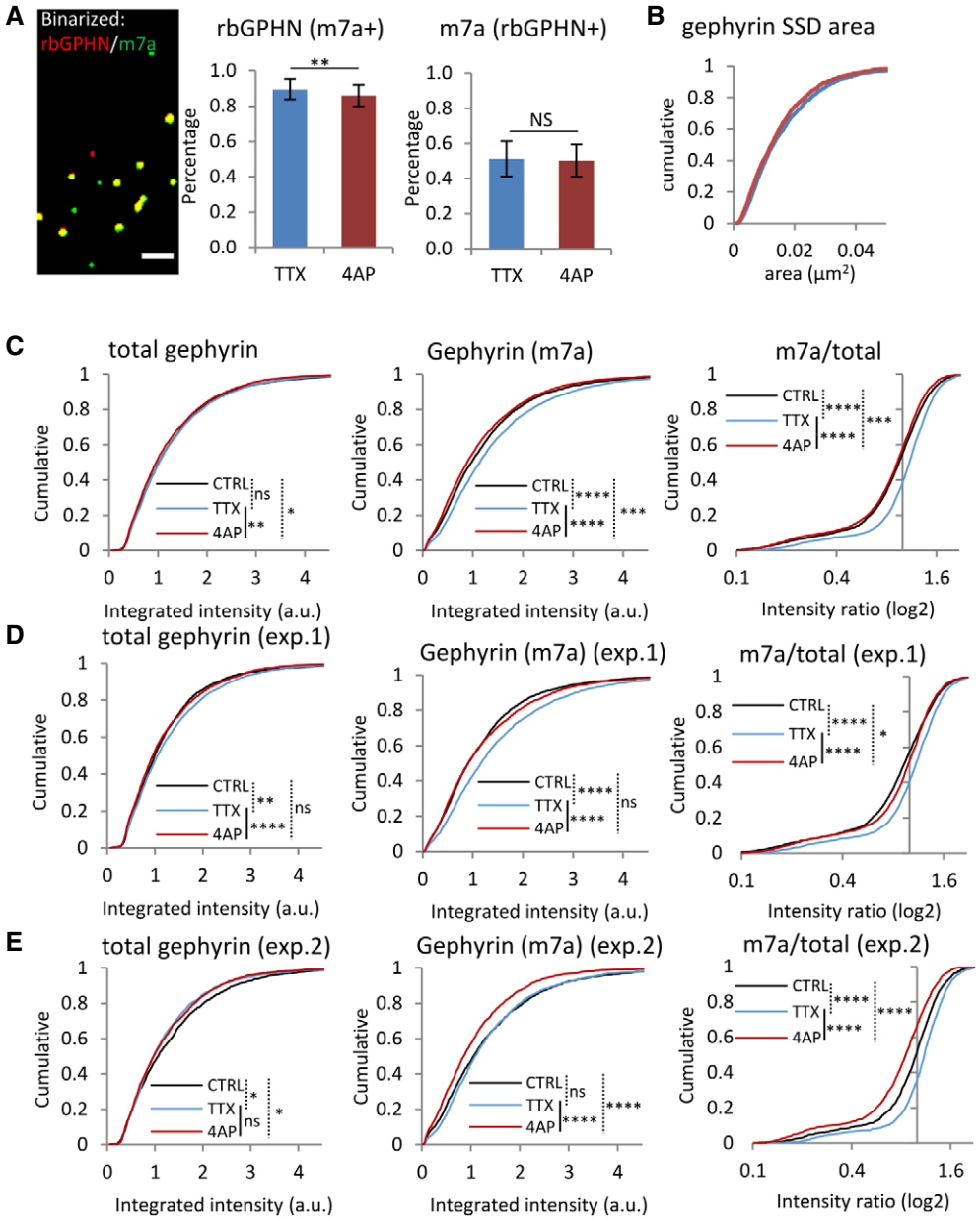

Figure EV5. Co-localization of pS270 gephyrin and total gephyrin at synapses.

A Co-localization of total gephyrin (rbGPHN) and phosphorylated pS270 gephyrin clusters (mAb7a) in conventional epifluorescence images. This is a binarized version of the image shown in Fig 7A. Scale bar: 2 µm. After 4-AP treatment, the percentage of rbGPHN clusters positive for mAb7a (binarized) showed only a minor reduction (middle panel, MW test, $^{**}P < 0.01$), possibly as a result of decreased mAb7a immunoreactivity. The percentage of mAb7a clusters positive for rbGPHN was not changed (right panel, MW test $P = 0.74$). mean ± SD; TTX: $n = 4{,}040$ synapses, 4-AP: $n = 3{,}818$, from two independent experiments.

B Gephyrin SSDs segmented from mAb7a clusters in dSTORM images did not show differences in size after 4-AP treatment (KS test, $P = 0.05$, $n = 810$ synapses in TTX and $n = 727$ in 4-AP conditions from three experiments).

C–E Variability of network activity in the control condition (CTRL, no treatment) in primary spinal cord neuron cultures. (C) The pooled results of two independent experiments (shown in D and E) show that the immunoreactivity of pS270 gephyrin (mAb7a antibody) is differentially modulated by pharmacological treatments that increase (4-AP, red traces) or decrease (TTX, blue) network activity, relative to the control condition (CTRL, black). Some of these data (TTX and 4-AP) are the same as the ones shown in Fig 7B and C. Number of synapses: $n = 4{,}040$ in TTX (blue traces), $n = 3{,}818$ in 4-AP (red), $n = 3{,}946$ in the CTRL condition (black) from two independent experiments. (D, E) Separate analysis of the two experiments shows that while the difference between the TTX and 4-AP conditions is consistent, the control can vary substantially between experiments. Number of synapses: $n = 2{,}362$ in CTRL, $n = 2{,}577$ in TTX and $n = 2{,}283$ in 4-AP conditions in (D), and $n = 1{,}584$ in CTRL, $n = 1{,}463$ in TTX and $n = 1{,}535$ in 4-AP in (E). KW test/Dunn's test, ns: not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

Source data are available online for this figure.