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Supplementary Information for

SYNPLA, a method to identify synapses displaying plasticity after learning

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Supplemental Materials and Methods

Experimental model

Primary neuronal cultures. For the time course of interacting proteins shown in Fig. **1CD**, we obtained single cell suspension of hippocampal neurons from E18 CD1 (Charles River) mouse embryos as previously described (1). We separately nucleofected these neurons with CAGs:gfp-P2A-myc-Nrxn1B (jk95; available on Addgene with accession 120176) or CAGs:mcherry-P2A-HA-Nlgn1 (jk96; available on Addgene with accession 120177) using a Lonza Nucleofector and Mouse Neuron transfection kit (Lonza) according to the manufacturer's instructions. We then either mixed cells nucleofected with CAGs:gfp-P2A-myc-Nrxn1B or CAGs:mcherry-P2A-HA-Nlgn1 and co-plated them, or plated cells nucleofected with CAGs:mcherry-P2A-HA-Nlgn1 only as a negative control. We fixed samples from the same cell suspension (thus controlling for variations in cell density) in 4% PFA at 2, 4, 7 and 10 days *in vitro* (DIV) for 15 min at room temperature and performed PLA without delay.

For experiments shown in Fig. **1EF** and **2B-D**, we prepared primary rat hippocampal neurons from postnatal day 0-1 rat pups as previously described (2). At 12-15 DIV, we infected the neurons with a Sindbis virus expressing mCherry-t2A-myc-Nrxn1B. Eighteen to 24 hours later, we subjected neurons to cLTP for 10 min (in HBSS based solution, 100 nM rolipram, 50 μM forskolin, 100 μM picrotoxin, 2 mM Ca^{2+}, 0 mM Mg^{2+}) (26). Control neurons were kept in HBSS for 10 min. When indicated, 100 μM APV was added. Immediately after cLTP, we fixed the neurons in 4 % PFA for 10 min at room temperature and performed PLA without delay.

Organotypic hippocampal slices. For experiments shown in Fig. **2E-G**, we prepared organotypic hippocampal slices from postnatal day 7-8 rat pups as previously described (3). Slice cultures were infected in the CA3 region with the mCherry-t2A-myc-Nrxn1B Sindbis at 14–18 DIV. Eighteen to 24 hours later, we subjected the slices to cLTP or mock stimulation similar as with the primary rat hippocampal cultures. Briefly, we incubated the slices in ACSF+drugs (118 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 20 mM glucose, 0 mM $MgCl_2$, 2 mM $CaCl_2$, 100 nM rolipram, 50 μM forskolin, 100 μM picrotoxin) or regular ACSF (118 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM glucose) for 16 min, and immediately fixed the slices in 4 % PFA for 4 hours at 4ºC. To make sure that this cLTP protocol produced functional synaptic potentiation, we performed evoked field recordings in 4 independent slices (see Figure **S5**). For electrophysiology, fEPSPs were recorded until 20 minutes of a stable baseline was acquired. Following the stable baseline, ACSF was washed out and replaced with cLTP ACSF for 16 minutes, during which time no electrical stimulation was applied. The cLTP solution was subsequently washed out and replaced with ACSF as described in the previous section, and electrical stimulation was resumed at a frequency of 0.1 Hz.

Animals for ex vivo SYNPLA. We injected 6- to 8-week-old WT Sprague-Dawley rats in the medial geniculate nucleus (AP: -6.0 mm, ML: - 3.4 mm, DV: 5.4 mm) and/or the auditory cortex (AP: -5.0 mm, ML: -7.2 mm, DV: 4.5 mm) with 500 nl of AAV9-GFP-P2A-myc-Nrxn1B (construct jk95; UNC Vector Core). See Fig. **S6** for details on the different injection sites for all animals tested. Virus was injected over a 4-minute period and the injection pipette was kept in place for an additional 10 minutes to ensure focal infection. After a minimum of 4 weeks, injected animals were subjected to paired or unpaired tone-conditioning paradigm (Fig. **3C**). Briefly, in the paired condition, a 10 second tone (1.2 kHz, 70 dB) was followed immediately by a 1 second footshock (0.8 mA delivered via Coulbourn

Precision Animal Shocker) with an inter-trial interval of 60 seconds with a 20 second jitter. In the unpaired condition, the animal was on the same shock schedule but the tones were played at pseudorandom intervals such that they did not predict the shock. This behavioral paradigm was programmed via a custom MATLAB script and connected to the Shocker via a National Instruments DAQ. Thirty minutes after conditioning, we anaesthetized the animals with isoflurane and transcardially perfused them with 4 % PFA. We postfixed the brains for 36 hours at 4C, and then transferred them to PBS before cutting 50 μm coronal sections on a vibratome. We performed PLA on the tissue section where the lateral amygdala (LA) was most visible (as shown in Fig. **3D**). In total, we performed 6 independent *ex vivo* experiments. Five of these experiments consisted of one control animal (uninjected, naïve or unpaired conditioning) and one animal that received paired conditioning. In one experiment (shown in Fig. **3EF**), however, we processed 4 animals (2 controls and 2 paired) at the same time. For summary graphs shown in Fig. **3G-H**, each control animal of this experiment was randomly paired with one animal from the same experiment that received paired conditioning.

Animal procedures were approved by the Cold Spring Harbor Laboratory or UCSD Animal Care and Use Committee and were carried out in accordance with National Institutes of Health standards.

Colocalization analysis. For experiments shown in Fig. **1EF**, we expressed myc-NRXN/cytosolic mCherry in 14 DIV hippocampal neurons then induced cLTP (as described in the Primary neuronal cultures section) in order to deliver endogenous GluA1 to synapses. Neurons were fixed and PLA between myc-NRXN and endogenous GluA1 was performed. At the same time, we also immunolabeled for PSD-95 (mouse monoclonal antibody MA1-045, ThermoFisher), a well-established synaptic marker. To determine if PLA signal colocalizes with PSD-95, we conducted the following analytical process, performed with a MATLAB script. For each image, we identified the location of PLA puncta in a given region of interest. Then we progressively increased the masking threshold for the presynaptic fiber (mCherry) and postsynaptic (PSD-95 immunolabel) channels, setting image values below this threshold to zero. For each identified PLA punctum, and for each masking level, we quantified the number of non-zero pixels within 0.14 μm (1 pixel) of the PLA punctum in the presynaptic AND postsynaptic image. We then repeated this analysis 30 times, but using randomly placed puncta instead of PLA signals. See Fig. **1EF** and Fig. **S3** for more details.

Statistics. For experiments shown in Fig. **1D**, we tested for statistical significance of increased PLA puncta when both epitopes were present using a 2-way ANOVA (using DIV, expressed epitopes (myc and HA or HA only) as factors). We used a 1-way ANOVA and Tukey-Kramer post-hoc testing to assess significance of PLA puncta increase over DIV. For other experiments where we compared multiple conditions (Fig. **2CD** and Fig. **3F, K-M**) we used a 1-way ANOVA and Tukey-Kramer post-hoc testing to assess significance. Unpaired or paired t-test were used elsewhere as indicated.

Supplemental References

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2. Dore K, Aow J, & Malinow R (2015) Agonist binding to the NMDA receptor drives movement of its cytoplasmic domain without ion flow. *Proceedings of the National Academy of Sciences of the United States of America* 112(47):14705-14710.

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Supplemental Figures

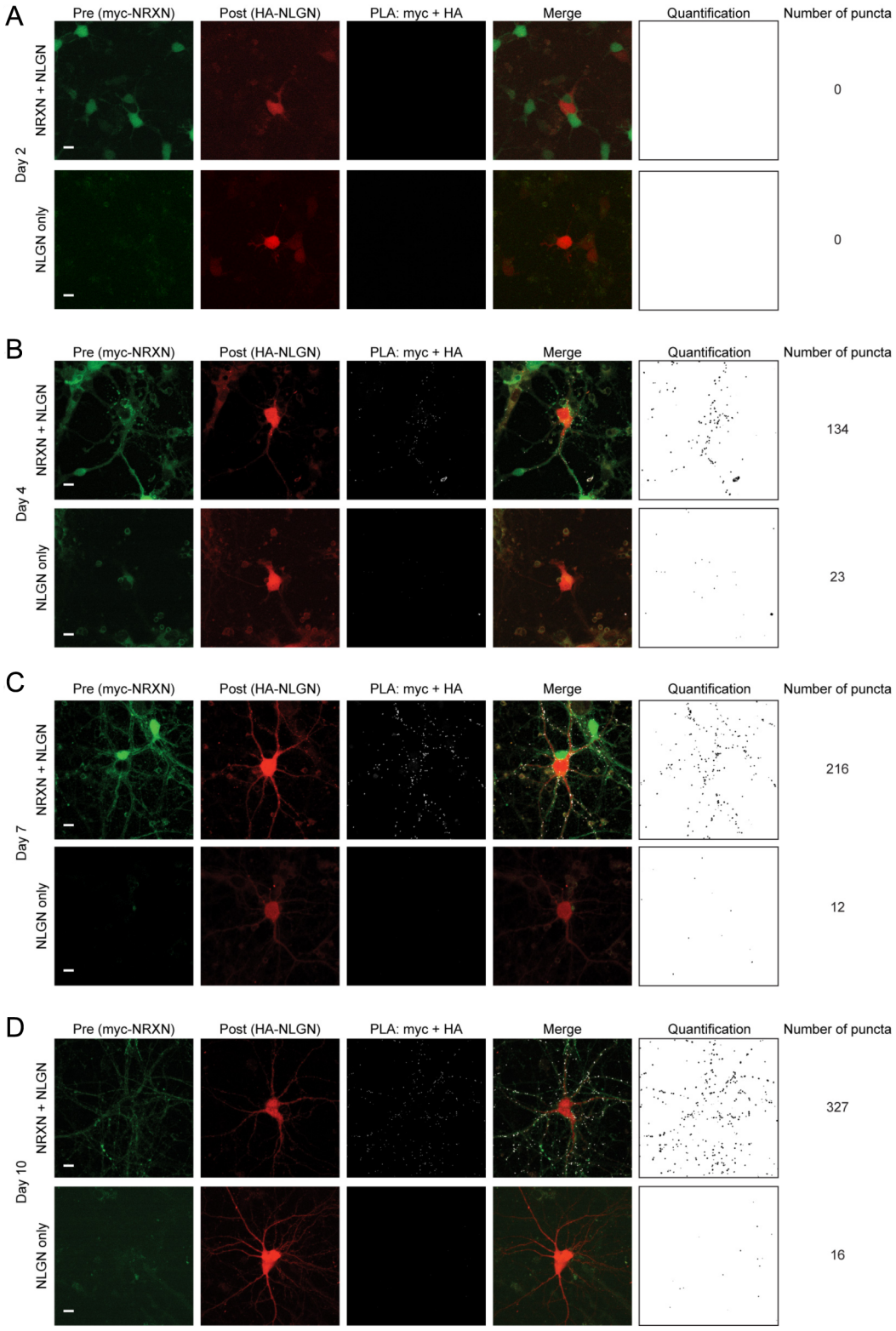

Figure S1 (related to Figure 1B-D): SYNPLA between myc-NRXN and HA-NLGN across time in culture

Representative images of PLA signals (3rd column) generated by probing for HA and myc epitopes in co-cultures of HA-NLGN+mCherry expressing neurons neurons (2nd column) and myc-NRXN+GFP expressing neurons; 1st column), or in control cultures of only HA-NLGN+mCherry expressing neurons, after different days in culture. **A**) 2 days *in vitro*, **B**) 4 days *in vitro*, **C**) 7 days *in vitro*, **D**) 10 days *in vitro*. Automatically detected PLA puncta are shown in the last column, together with the number of puncta detected in each image. Scale bar is 10μm.

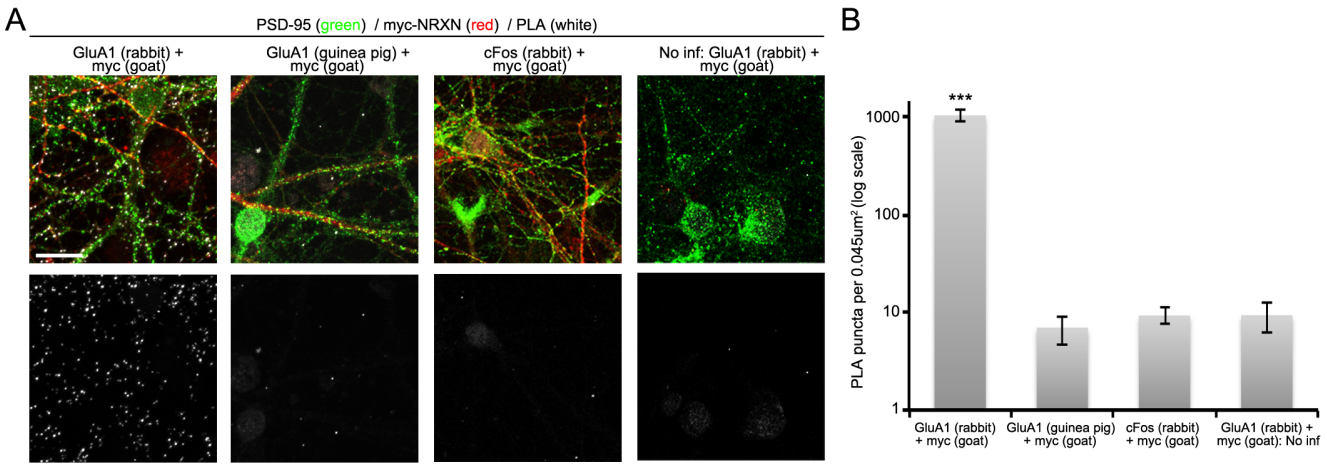

Figure S2 (related to Figures 1 and 2) SYNPLA displays high specificity and very low background

A) Example images of SYNPLA in 15 DIV cultured neurons, probed with different primary antibodies; target, and animal used to raise antibody indicated; PLA probes used are always anti-rabbit and anti-goat. Top: Composite images showing PSD-95 staining in green, cytosol of myc-NRXN-expressing neuron in red and PLA in white. Images shown below displays PLA signal only. Scale bar is 20μm.

B) Quantification of SYNPLA puncta in the indicated conditions, a log scale is used for better data visualization. Note that all negative controls yield ~100-fold less PLA signal. *** $P < 0.001$; one-way ANOVA with Tukey-Kramer post hoc test.

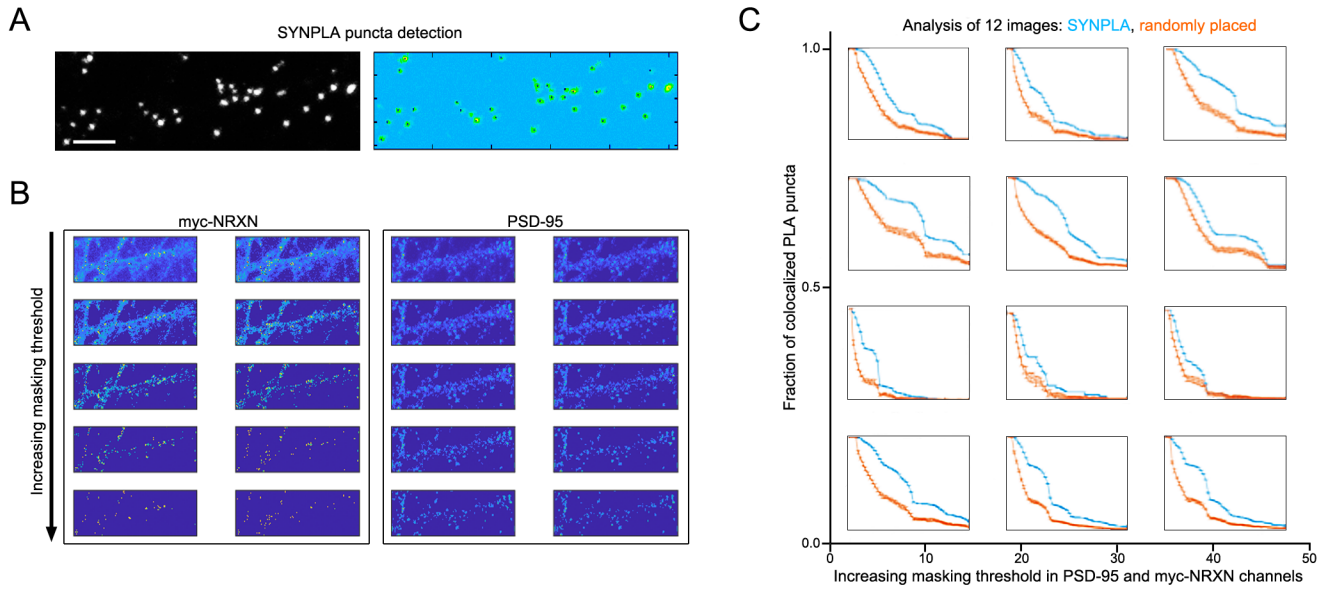

Figure S3 (related to Figure 1EF): Approach to detect colocalization of SYNPLA signal with pre- and post-synaptic signals

A) Automatic detection of SYNPLA puncta. Scale bar is 5µm.

B) Increasing masking threshold on presynaptic signal (myc-NRXN) and postsynaptic signal (PSD-95).

C) Result of colocalization analysis on 12 different images, SYNPLA shown in blue and randomly placed puncta (30 trials) shown in orange.

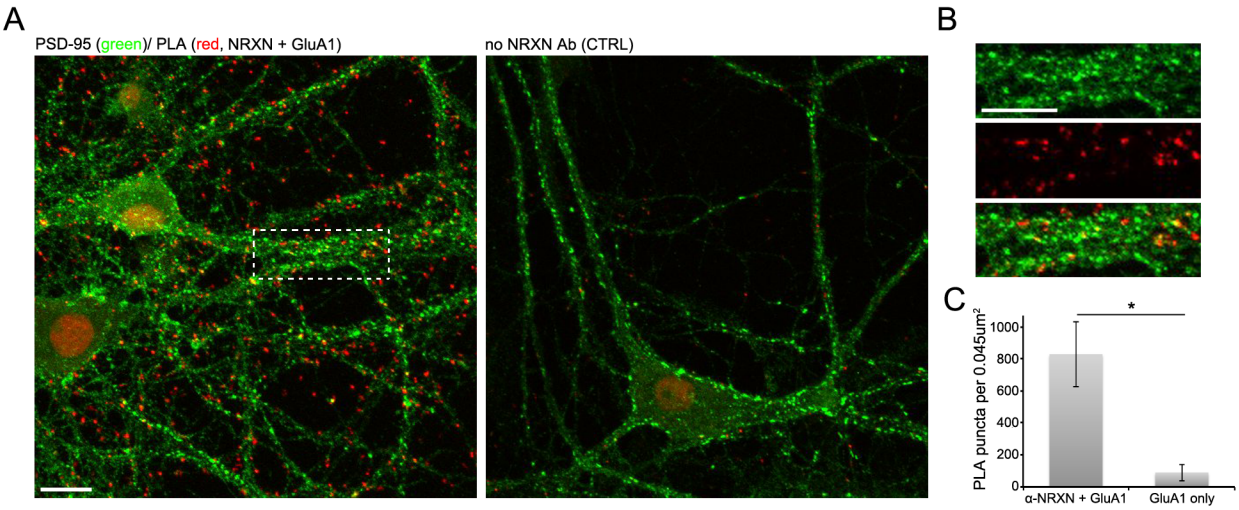

Figure S4: SYNPLA between endogenous Neurexin and GluA1

SYNPLA in cultured hippocampal neurons (after cLTP) between endogenous NRXN and GluA1.

A) Example images of endogenous SYNPLA (left) or negative control where the NRXN antibody was omitted (right). Synaptic labeling is demonstrated by a PSD-95 co-staining in green. Scale bar is 10µm.

B) Inset of SYNPLA shown in **A)** with separated channels. Scale bar is 10μm.

C) Quantification of SYNPLA puncta in the indicated conditions, * $P < 0.05$; unpaired T-test.

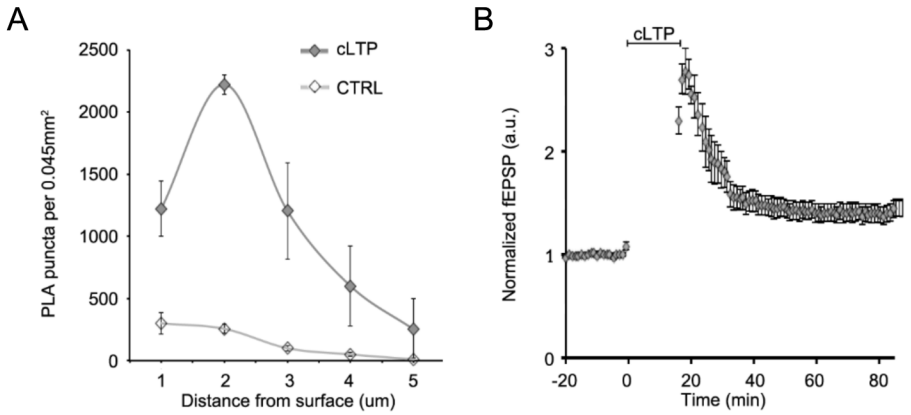

Figure S5 (related to Figure 2E-G): SYNPLA between myc-NRXN and endogenous GluA1 in organotypic slices

A) Number of PLA puncta quantified in each 1μm z-section in one representative experiment in hippocampal organotypic slices, 2 slices (2 z-stacks per slice) in each condition.

B) Evoked field potential recordings were performed in organotypic slices subjected to the cLTP protocol, which led to robust potentiation, n=4 slices.

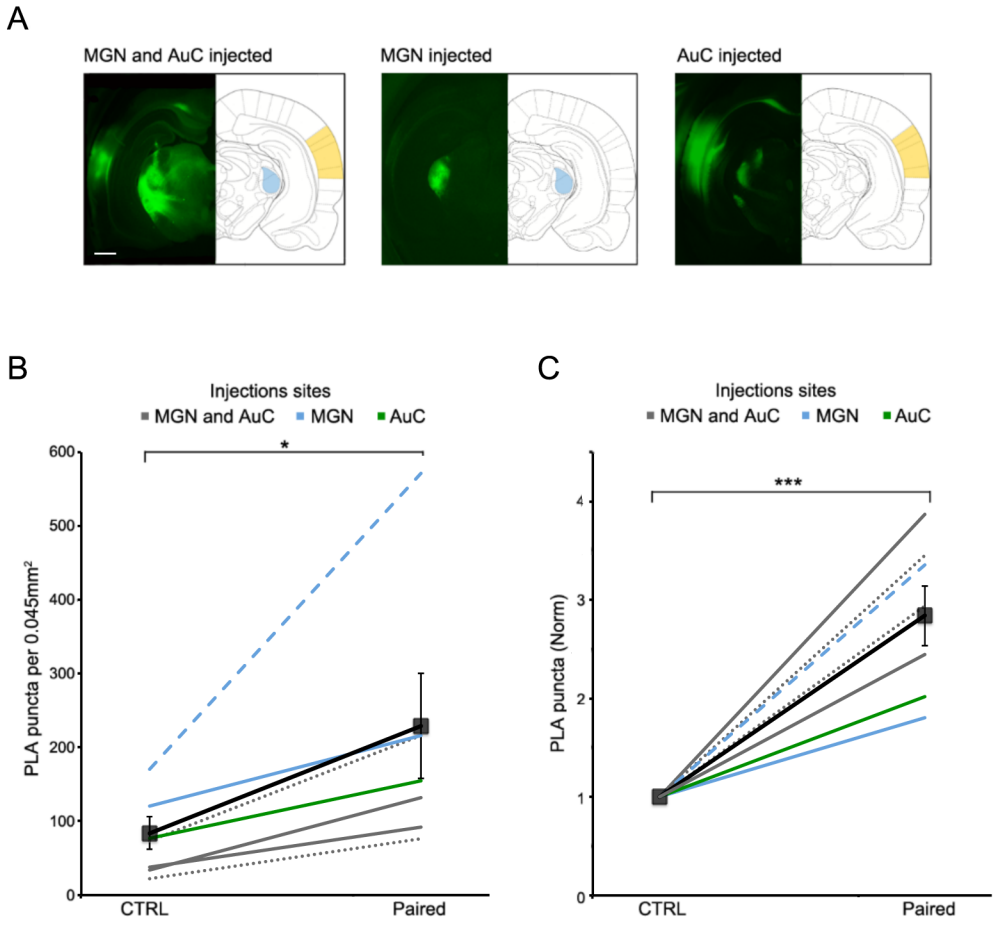

Figure S6 (related to Figure 3): Injection sites used for *ex vivo* SYNPLA

A) Representative images of the injection sites used in this study, scale bar is 1mm.

B) Quantification of all *ex vivo* experiments; SYNPLA puncta per animal (average across all fields of view) under the indicated conditions (CTRL: uninjected (dashed line), naïve (dotted lines) or unpaired (solid lines)) and the average across animals (square) ± SEM are shown; * $p<0.05$; paired t-test. Animals that were injected in the medial geniculate nucleus (MGN) and auditory cortex (AuC) are shown in gray; animals injected in the MGN only are shown in blue and animals injected in the AuC only are shown in green.

C) Same data as in **B)**, normalized to control, *** $p<0.01$; paired t-test.

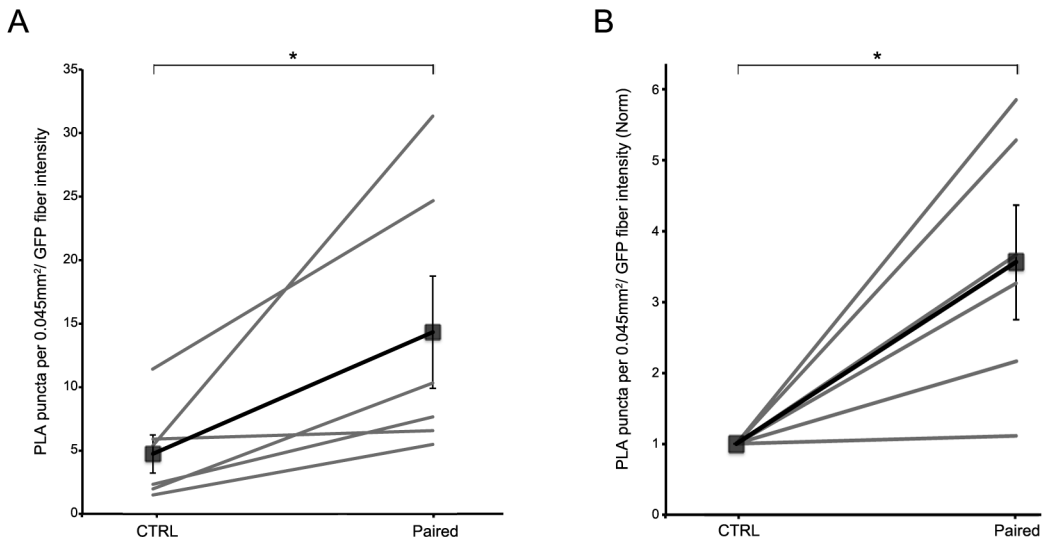

Figure S7 (related to Figure 3): *Ex vivo* data normalized to myc-NRXN green fiber intensity

A) Quantification of 6 of the 7 *ex vivo* experiments (one experiment was removed because the control animal was uninjected); number of SYNPLA puncta per animal (average across all fields of view) normalized to the pre-synaptic GFP fiber intensity in the same field of view. Each experiment is shown as a gray line and the average across animals is shown in black (square) ± SEM are shown; * $p<0.05$; paired t-test.

B) Same data as in **A)**, normalized to control, * $p<0.05$; paired t-test.

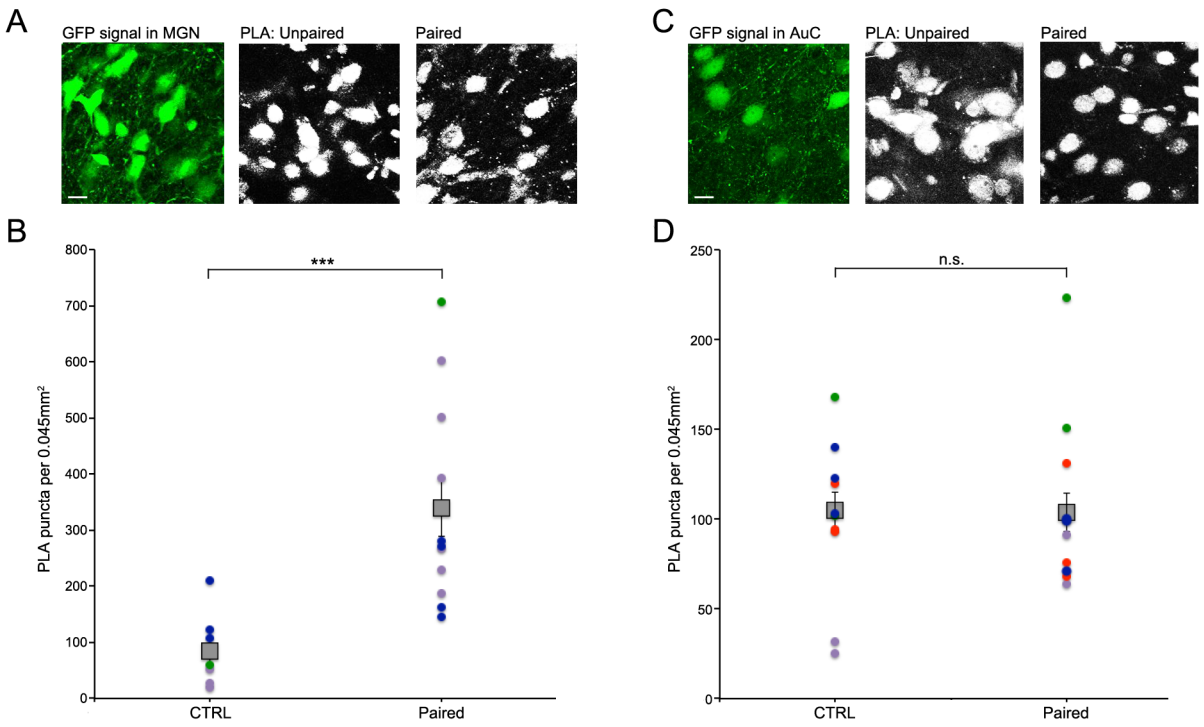

Figure S8 (related to Figure 3): *Ex vivo* SYNPLA in injection sites

A) Representative images of *(left)* GFP signal in the MGN, *(middle)* corresponding PLA signal in an animal that received Unpaired conditioning or (right) Paired conditioning. Scale bar is 10μm.

B) Number of SYNPLA puncta detected in the MGN in each field of view. Each color is one independent experiment. In total, results obtained from 7 animals are shown in this graph, all were injected in the MGN, 3 received unpaired conditioning, 4 paired conditioning. *** $p<0.001$.

C) Representative images of *(left)* GFP signal in the AuC, *(middle)* corresponding PLA signal in an animal that received Unpaired conditioning or (right) Paired conditioning. Scale bar is 10μm.

D) Number of SYNPLA puncta detected in the AuC in each field of view. Each color is one independent experiment. In total, results obtained from 8 animals are shown in this graph, all were injected in the AuC, 4 received unpaired conditioning, 4 paired conditioning.