SYNPLA: A synapse-specific method for identifying learning-induced synaptic plasticity loci

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Which neural circuits undergo synaptic changes when an animal learns? Although it is widely accepted that changes in synaptic strength underlie many forms of learning and memory, it remains challenging to connect changes in synaptic strength at specific neural pathways to specific behaviors and memories. Here we introduce SYNPLA (SYNaptic Proximity Ligation Assay), a synapse-specific, high-throughput and potentially brain-wide method capable of detecting circuit-specific learning-induced synaptic plasticity.

Changes in synaptic strength have been hypothesized to underlie learning and memory since before the time of Hebb. The best studied form of Hebbian synaptic plasticity is long-term potentiation (LTP), which underlies the formation of fear conditioning memories[1]. Work over the last two decades has uncovered the molecular mechanisms of LTP in great detail, revealing that the expression of plasticity is mediated by the rapid synaptic insertion of GluA1-subunit containing AMPA receptors[2]. These GluA1-containing receptors are then likely replaced by GluA1-lacking AMPA receptors within 15 to 72 hours[3–5], making GluA1 a marker of recently potentiated synapses. LTP appears to be very general at glutamatergic synapses in the central nervous system, including in cortex[3], amygdala[6], ventral tegmental area[7], nucleus accumbens[8], striatum[9] and lateral habenula[10]. Furthermore, plasticity mediated by trafficking of GluA1-subunit containing AMPA receptors appears to play an important part in several neuropsychiatric disorders, such as addiction[11], autism[12] and depression[10].

Relating behaviorally-induced plasticity to its synaptic substrate remains an important technical challenge. Electrophysiological or optical methods provide mechanistic insight, but are low-throughput and require specialized equipment[6]. Somatic expression of immediate early genes (IEG) such as *cfos* can be used to screen for brain areas activated during plasticity[13], but lack synaptic resolution. We therefore sought to develop a pathway-specific histological method for detecting behaviorally induced changes.

Here we present SYNPLA, a method that uses the proximity ligation assay (PLA) to detect synaptic insertion of GluA1-containing AMPA receptors in defined circuits. PLA is a highly sensitive and specific biochemical method that reliably detects the close (< 40 nm) juxtaposition of two proteins *in situ*[14] (Fig. 1**a**). To detect nearby molecules A and B, PLA uses a standard primary antibody raised against A, and another raised against B. These antibodies are detected by secondary antibodies conjugated to unique oligonucleotides (Ao, Bo). A second set of oligonucleotides, ABo1 and ABo2, that are complementary to parts of both Ao and Bo are added. Only when Ao and Bo are sufficiently close can ABo1 and ABo2 be ligated to form a circle. This circle is then amplified (>1,000-fold) via rolling-circle amplification to form a nanoball of DNA, which can be reliably probed with complementary fluorescent oligonucleotides and observed as a punctum with light microscopy. A key advantage of PLA over traditional immunostaining is the absolute requirement for a pair of proteins to produce a PLA signal—a logical AND—which confers high specificity and thus reduces the false positive rate. Moreover, the 1000x signal amplification renders each positive signal very bright and therefore easily distinguishable from background, allowing results to be imaged quickly, and at lower resolution than would otherwise be necessary for resolving synaptic contacts.

We first confirmed previous experiments[15] suggesting that PLA could be used to detect direct protein-protein interactions across the synaptic cleft in dissociated cultured neurons (Fig. 1**b-d**).

We exploited the developmental window (days 2-10) during which synapses are formed in this preparation[16]. In some neurons we expressed the presynaptic protein Neurexin1b with a myc tag (myc-NRXN) along with cytosolic GFP; in other neurons we expressed Neuroligin1, the normal postsynaptic binding partner of NRXN[17], with an HA tag (HA-NLGN) and cytosolic mCherry. Consistent with the normal time course of synapse formation[16], PLA reactions using antibodies to myc and HA produced an increasing number of PLA puncta, whereas cultures expressing only NLGN-HA led to minimal PLA products (Fig. 1**c-d**, Supplementary Fig. 1). These results indicate that PLA can specifically detect the interaction of NRXN and NLGN across the synaptic cleft.

We then reasoned that since the 20 nm synaptic cleft is less than the 40 nm PLA capture radius, SYNPLA could detect the apposition of presynaptic NRXN with GluA1 inserted into the postsynapse during LTP. In contrast, no PLA products should be produced between NRXN and the more distant extrasynaptic pool of uninserted GluA1 containing AMPA receptors (Fig. 1**e**). We expressed myc-NRXN in dissociated cultured neurons (Fig. 1**f-h**) or in region CA3 of cultured hippocampal brain slices[18] (Fig. 1**i-k**), and we performed PLA using antibodies to myc and the extracellular domain of GluA1 on the cultured neurons or region CA1. Although we observed few PLA puncta in control conditions, chemically induced LTP (cLTP)[19] greatly increased the number PLA puncta (Fig. 1**f-k**; 4-fold, $p<0.01$, paired t-test in dissociated cultures; 12-fold, $p<0.001$, paired t-test in organotypic cultures). The increase in the number of PLA puncta was blocked by addition of APV, a blocker of LTP induction[20], to neurons prior to cLTP (Fig. 1**g**,**h**; $p<0.01$, unpaired t-test). These results indicate that SYNPLA can detect (i) the apposition of presynaptic myc-NRXN and endogenous postsynaptic GluA1; and (ii) synaptic plasticity under cultured conditions.

To test if SYNPLA can detect synaptic plasticity following memory formation *in vivo*, we injected the auditory cortex and/or the medial geniculate nucleus of the thalamus of rats with a virus expressing myc-NRXN and cytosolic GFP. We then exposed the rats to a cued fear-paired conditioning protocol, wherein a 10 second tone (conditioned stimulus) is immediately followed by a brief foot shock (Fig. 2**a-c**). Such protocols have been shown to produce LTP-like plasticity at synapses onto the lateral amygdala (LA)[6]. Control animals received either no viral injection, no conditioning or unpaired conditioning, wherein the tone and the foot shock are not temporally paired. We perfused control or conditioned animals 30 minutes after conditioning, and then post-fixed and sectioned the brains at 50µm. We subjected tissue sections to SYNPLA and imaged the LA region containing GFP labeled presynaptic fibers (Fig. 2**d**). Uninjected, naïve or animals receiving unpaired conditioning displayed few PLA puncta; while animals receiving paired conditioning displayed on average three-fold increase in PLA puncta (Fig. 2**f-h**; $p<0.001$, paired t-test). The large fractional increase in GluA1-containing receptors at synapses during learning is consistent with a synaptic plasticity model wherein GluA1-containing receptors are added during plasticity and replaced within about 24 hours with GluA1-lacking receptors[3]. These results indicate that SYNPLA can detect synaptic plasticity induced by learning.

Here we have demonstrated that SYNPLA can reliably detect synaptic potentiation in circuits participating in newly formed memories. SYNPLA can be used to probe plasticity in anatomically defined pathways as in the present study, or in genetically defined pathways by limiting expression of myc-NRXN only to neurons expressing Cre recombinase. Simultaneous screening of multiple pathways in the same animal might also be possible by exploiting orthogonal PLA probes[21]. Unlike

IEG-based screening approaches, which assay cell-wide changes, SYNPLA is synapse- and pathway-specific and directly assesses synaptic plasticity. Moreover, it is fast and easy to perform, and so could be scaled up as a powerful brain-wide screen for behaviorally-induced plasticity.

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Author contributions

K.D., Y.P., J.S.P., S.A., H.Z. S.G., S.M, and J.M.K. performed the experiments and analyzed the data. K.D., A.M.Z, R.M. and J.M.K. wrote the manuscript and supervised the project.

Competing interests

The authors declare no competing financial interests.

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Figures

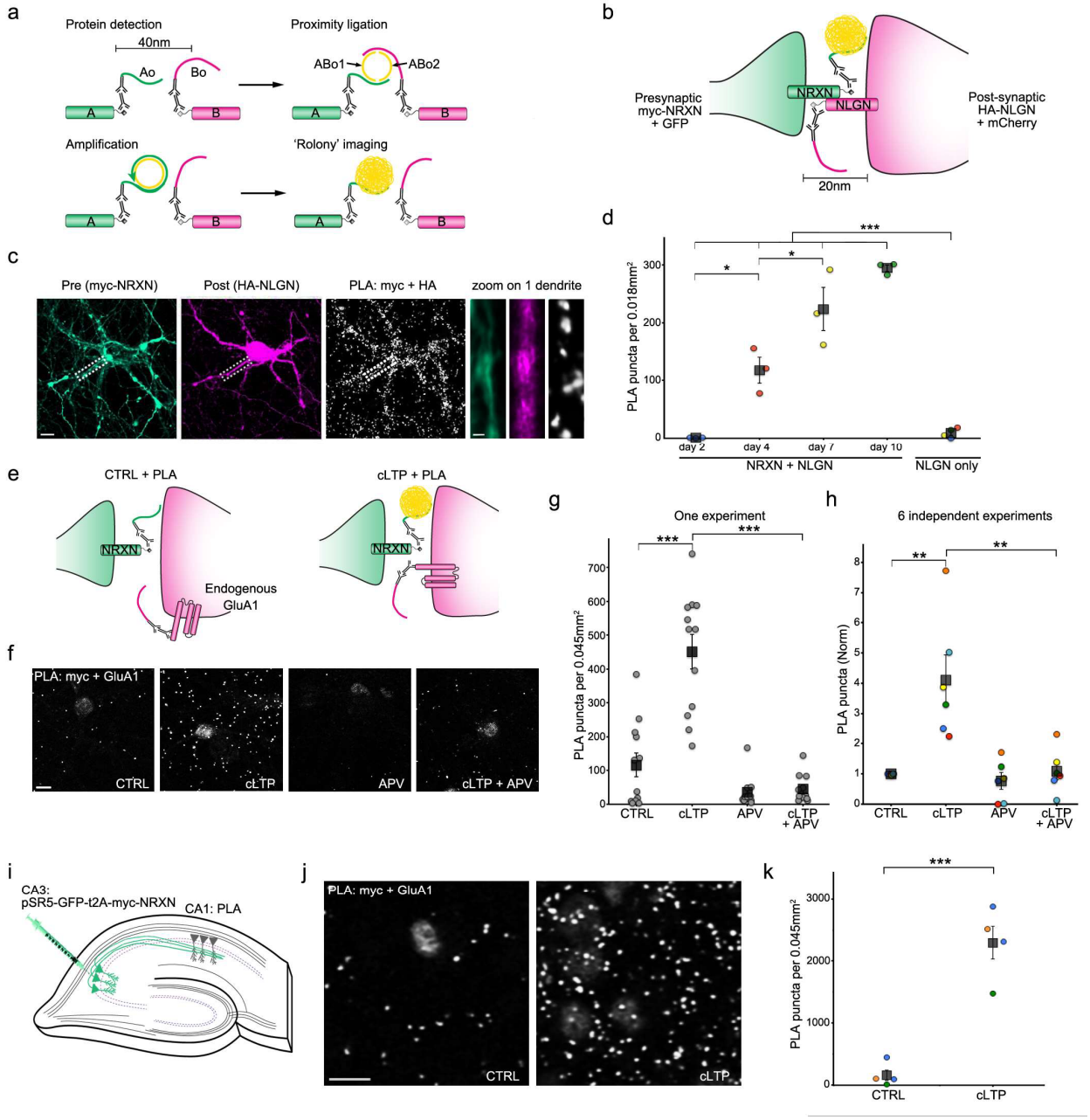

Figure 1: SYNPLA detects synapse formation and potentiation in cultured neurons and slices

a) Diagram of a general PLA reaction.
b) Diagram of PLA targeting recombinant presynaptic myc-NRXN and postsynaptic HA-NLGN.
c) PLA nanoballs (white dots) formed between a postsynaptic mouse hippocampal neuron in culture expressing HA-NLGN + mCherry (magenta; middle panel) and co-cultured presynaptic neurons expressing myc-NRXN + GFP (cyan; left panel). Scale bar: 10µm. A zoomed image of the indicated dendrite is shown on the right. Scale bar: 1µm.

d) Quantification of PLA puncta between cultured neurons expressing HA-NLGN and myc-NRXN or control cells expressing only HA-NLGN for the indicated days *in vitro*. Average number of PLA puncta in each sample (round) and average (square) ± SEM are shown (each color indicates data acquired on the same day). *** $p<0.001$, 2-way ANOVA (using days *in vitro* and expressed epitopes (myc and HA or HA only) as factors); * $p<0.05$, 1-way ANOVA followed by Tukey-Kramer post-hoc test.

e) Diagram of SYNPLA targeting presynaptic myc-NRXN and endogenous GluA1. cLTP induces trafficking of GluA1 to the post-synaptic density, decreasing the distance between the targeted proteins and permitting PLA.

f) Representative images of SYNPLA reactions performed on myc-NRXN expressing cultured rat hippocampal neurons at 14 days *in vitro* for the indicated conditions; PLA shown in gray. Scale bar: 10μm.

g) Quantification of a single SYNPLA experiment (as in **f**). The number of SYNPLA puncta detected in each field of view (round) and the average (square) ± SEM across all fields of view are shown; *** $p<0.001$, 1-way ANOVA followed by Tukey-Kramer post-hoc test.

h) Quantification of six independent experiments (indicated by different colors); the average number of SYNPLA puncta across 10-12 fields of view for each experiment (circles, normalized to CTRL) and the average PLA signal across experiments (square) ± SEM are shown; ** $p<0.01$, 1-way ANOVA followed by Tukey-Kramer post-hoc test.

i) Diagram of SYNPLA in rat organotypic hippocampal slices. Sindbis virus expressing myc-NRXN is injected in the presynaptic CA3 region SYNPLA between myc-NRXN and endogenous GluA1 is measured in the postsynaptic CA1 region.

j) Representative images of SYNPLA under the indicated conditions in region CA1 of organotypic slice cultures. Scale bar: 10μm.

k) Quantification of SYNPLA puncta in organotypic slices, from three independent experiments (indicated with different colors, 4 slices per condition (one circle per slice)); squares indicate average ± SEM across experiments; *** $p<0.001$, paired t-test.

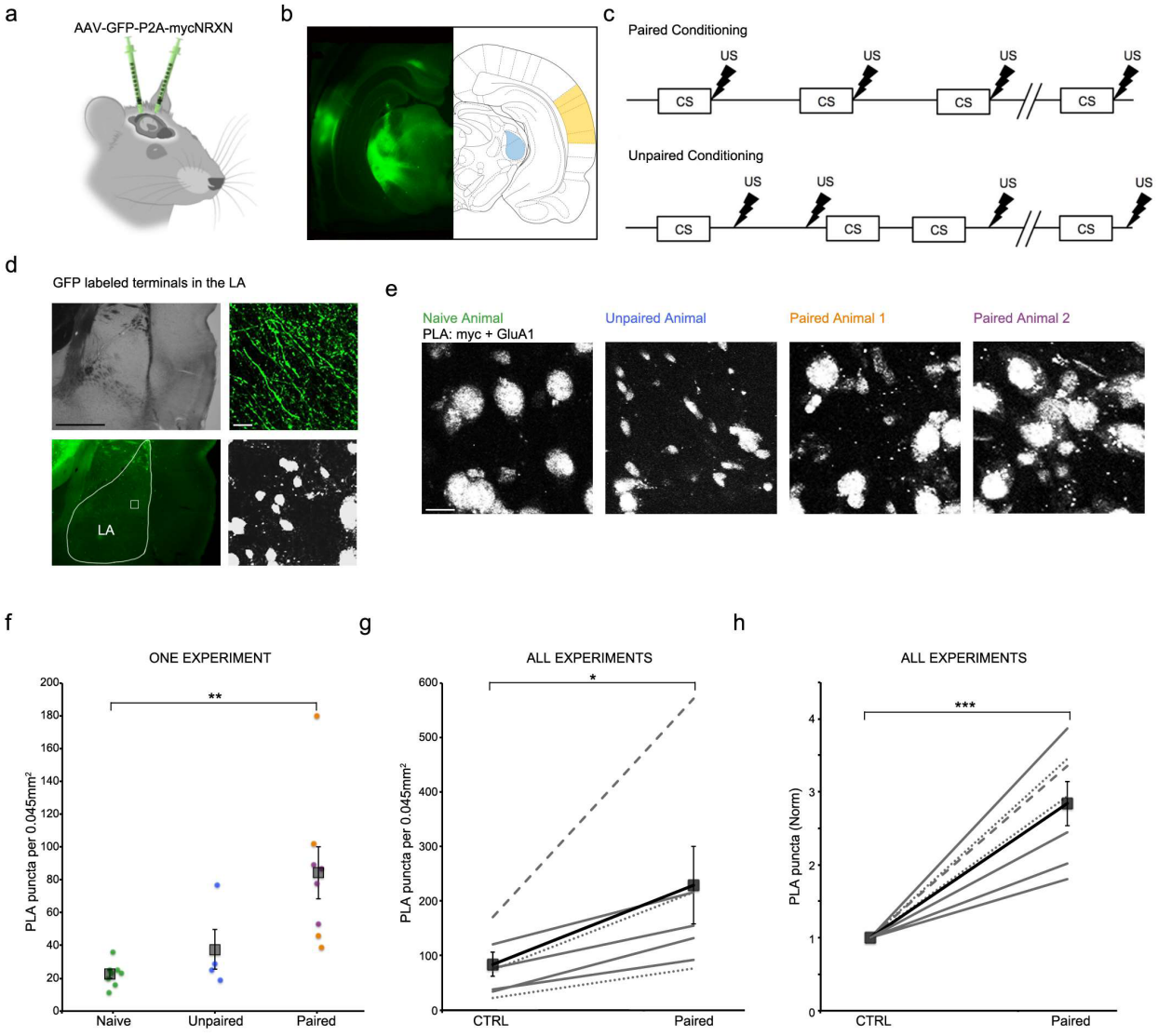

Figure 2: SYNPLA detects potentiated synapses between medial geniculate nucleus or auditory cortex and the lateral amygdala (LA) following fear conditioning.

a) Injection of AAV9-GFP:P2A:myc-NRXN into auditory cortex and/or medial geniculate nucleus.
b) GFP expression in the cell bodies at the injection sites (Auditory cortex: yellow, medial geniculate nucleus, blue).
c) Diagram of paired (top) and unpaired (bottom) fear conditioning paradigm; tone (CS; 10 seconds) and shock (thunderbolt) delivered where indicated.
d) Representative images of presynaptic GFP-labeled fibers terminating onto the LA under low (left; top: transmitted light; below: GFP signal, LA traced in white) and high (right; top: GFP signal; PLA shown below) magnification in an animal that received paired conditioning. Scale bar: left, 1mm; right, 10μm.
e) Representative images of a single SYNPLA experiment in the LA from animals subjected to the indicated conditions; colors correspond to symbols in **f**. Scale bar: 10μm.

f) Quantification of the experiment shown in **e**. Number of SYNPLA puncta detected in one field of view (circles) and average across all fields of view (squares) ± SEM; * $p<0.05$, ** $p<0.01$; unpaired t-test.
g) Quantification of all *ex vivo* experiments; SYNPLA puncta per animal (average across all fields of view; gray) 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. See Supplementary Fig. 3 for information regarding specific injection sites used in these experiments.
h) Same data as in **g**, normalized to control, ** $p<0.01$; paired t-test.