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Authors
Reissner, Kathryn J
Pu, Lu
Schaffhausen, Joanna H
et al.

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A Novel Postsynaptic Mechanism for Heterosynaptic Sharing of Short-Term Plasticity

Kathryn J. Reissner[1], **Lu Pu**[1], **Joanna H. Schaffhausen**[2], **Heather D. Boyle**[1], **Ian F. Smith**[1], **Ian Parker**[1], and **Thomas J. Carew**[1,3,*]

[1]Department of Neurobiology and Behavior, University of California, Irvine, Irvine, CA 92697

[2]ABC News Medical Unit, Needham, MA, 02494

[3]Center for the Neurobiology of Learning and Memory

Abstract

Postsynaptic release of Ca^{2+} from intracellular stores is an important means of cellular signaling which mediates numerous forms of synaptic plasticity. Previous studies have identified a postsynaptic intracellular Ca^{2+} requirement for a form of short-term plasticity, post-tetanic potentiation (PTP) at sensory-motor neuron (SN-MN) synapses in *Aplysia*. Here we show that postsynaptic IP_3-mediated Ca^{2+} release in response to a presynaptic tetanus in a SN that induces PTP can confer transient plasticity onto a neighboring SN synapse receiving subthreshold activation. This heterosynaptic sharing of plasticity represents a dynamic, short-term synaptic enhancement of synaptic inputs onto a common postsynaptic target. Heterosynaptic sharing is blocked by postsynaptic disruption of Ca^{2+} and IP_3-mediated signaling, and conversely, it is mimicked by postsynaptic injection of non-hydrolysable IP_3, and by photolysis of caged IP_3 in the MN. The molecular mechanism for heterosynaptic sharing involves mGluR and Homer-dependent interactions, indicating that Homer can facilitate the integration of Ca^{2+}-dependent plasticity at neighboring postsynaptic sites and provides a postsynaptic mechanism for spread of plasticity induced by presynaptic activation. Our results support a model in which postsynaptic summation of IP_3 signals from suprathreshold and subthreshold inputs results in molecular coincidence detection that gives rise to a novel form of heterosynaptic plasticity.

Keywords

Homer; calcium; mGluR; postsynaptic; *Aplysia*; posttetanic potentiation

Introduction

Synaptic plasticity exists in a wide range of temporal domains. Activity-dependent short-term plasticity, lasting on the order of seconds to minutes, includes paired pulse facilitation, paired-pulse depression, and post-tetanic potentiation (PTP). While long-lasting forms of plasticity, such as LTP and LTD, are generally considered to provide a means of information storage in neurons, these short-term forms of plasticity are critical for dynamic, moment-to-moment adjustments of synaptic strength during online processing of neural information. At most synapses, PTP is typically thought to be mediated solely by presynaptic mechanisms (Zucker and Regehr, 2002). However, a major component of PTP at sensory-motor (SN-MN) synapses in *Aplysia* requires postsynaptic Ca^{2+} (Bao et al., 1997; Schaffhausen et al.,

*Address correspondence to Thomas J. Carew, tcarew@uci.edu..

2001; Jin and Hawkins, 2003). The fact that postsynaptic Ca^{2+} contributes significantly to PTP at SN-MN synapses in *Aplysia* led us to propose that the induction of PTP at one synapse might transiently facilitate induction of plasticity at neighboring synapses onto the same MN, via shared postsynaptic Ca^{2+}-dependent mechanisms (Schaffhausen et al., 2001). Here we show that heterosynaptic sharing of PTP can indeed occur when an input sufficient for PTP induction (20 Hz activation of a SN) is paired with a subthreshold input to a neighboring SN (activated at 7 Hz). The mechanism for heterosynaptic sharing is dependent upon postsynaptic Ca^{2+} and Homer-mediated interactions. Heterosynaptic sharing is blocked by postsynaptic injection of BAPTA, as well as inhibitors of intracellular Ca^{2+} release including heparin, thapsigargin, and caffeine. Moreover, heterosynaptic sharing is facilitated by postsynaptic injection of non-hydrolyzable IP_3 and by photo-activated caged IP_3. Finally, heterosynaptic sharing is blocked by disruption of Homer-mediated protein interactions following postsynaptic injection of a synthetic peptide containing the Homer binding sequence of the *Aplysia* IP_3R.

These findings provide evidence for a novel form of short-term heterosynaptic plasticity that arises from postsynaptic interactions between presynaptic inputs, and indicates that activity-dependent spread of plasticity is not limited to the long-term time domains. Moreover, this unique form of plasticity provides a mechanism for dynamic, temporally restricted amplification of synaptic inputs onto a common postsynaptic target.

Materials and Methods

General Methods

Wild-caught *Aplysia californica* (120–250 g) were obtained commercially (Marinus, Long Beach, CA). Animals were anesthetized by injection of isotonic $MgCl_2$, and isolated ganglia were pinned and desheathed in dishes containing 1:1 $MgCl_2$/artificial seawater (ASW) (containing in mM: 460 NaCl, 55 $MgCl_2$, 11 $CaCl_2$ 10 KCl, and 10 Tris, pH 7.6).

Protein quantification was performed using the BCA Protein Assay (Pierce). Western blots were performed by electrophoresis using the Invitrogen NuPAGE gel system. Blotting conditions were varied depending on primary antibody used. Blocking was performed in 3% Blotto in TBST and blots were probed overnight in primary antibody in blocking solution, followed by 1 h at room temperature in HRP-conjugated secondary antibody at 1:2500. Blots were developed using the ECL Western Blotting Analysis System by Amersham.

Cloning

For use in degenerate RT-PCR, whole RNA was prepared from pleural and pedal ganglia using Trizol Reagent (Invitrogen). Initial forward and reverse degenerate Homer primer sequences were CAYGTNTTYCANATHGAYCC and CCAYTGNCCRAAMTTYTG, respectively. Subsequent RT-PCR was performed using a separate degenerate forward primer (ATGGGNGARCARTTYATHTT) and a gene-specific reverse primer from within the initially identified sequence. PCR was then performed on *Aplysia* sensory neuron or CNS libraries (generously provided by Kelsey Martin and Wayne Sossin, respectively) with different combinations of forward gene-specific primers and T3 or T7 vector primers. Typically, PCR products were cloned into the Invitrogen pCR2.1 sequencing vector and submitted for DNA sequencing (Laguna Scientific). This approach yielded a total of 353 amino acids. While a termination codon was not identified within the library screen, alignment of ApHomer with *Drosophila* and vertebrate Homers indicated that the sequence obtained likely represented >95% of the coding region of ApHomer. Translation start site was confirmed using 5' RACE with the GeneRacer kit from Invitrogen, using pleural/pedal RNA as starting material.

Cloning of the IP_3 receptor in *Aplysia* was performed by degenerate RT-PCR from pleural/pedal ganglia and PCR of an *Aplysia* CNS library using gene-specific and vector primers, as described for ApHomer. Initial degenerate forward and reverse primers were CCICCNAARAARTTYMG and AAWTTYTCYTGYTCNGCRTG. DNA sequence obtained from the initial fragment of *Aplysia* cDNA was used to make gene-specific primers which were then coupled to vector primers for use in amplification from the *Aplysia* CNS library. For expression in bacteria, the first 258 amino acids of $ApIP_3R$ were cloned into the pENTR vector from Invitrogen. Following recombination into the pDEST15 GST expression vector, recombinant protein was expressed in BL21(DE3)Star cells. Bacterial pellets were resuspended in lysis buffer (PBS+0.5% Triton X-100, 10% glycerol, 10 mM imidizol), and sonicated. Soluble protein was recovered by centrifugation and couple to glutathione agarose (Pierce). Constructs for the expression of recombinant ApHomer were prepared using the EcoRI and NotI sites in the pGEX4T-3 vector (Amersham), containing either amino acids 1–335 or 163–335. Following expression, bacterial cell pellets were resuspended and lysed by sonication in a smiliar fashion. The accuracy of all constructs was confirmed by DNA sequencing (Laguna Scientific, Cogenics).

Tissue Distribution and ApHomer Expression

Regulation of Homer expression was investigated using four separate methods: injury, *in vivo* 5-HT, KCl depolarization, and tail-shock. To look at the effect of injury, the animal was anesthetized and the pleural and pedal ganglia were immediately excised into Trizol for RNA preparation. One hour later, the other half was removed and processed identically. For *in vivo* 5-HT treatment, animals were placed in 6 liters of ASW or 6 liters ASW containing 250 μM 5-HT for 1.5 hour at 18°C. After 1.5 h, animals were either taken for tissue or moved to ASW. Tail-shock was performed as previously described (Sharma et al., 2003). Five shocks were given to one side of the tail with a 10 min inter-shock interval. The unshocked side served as control to that animal. Pleural and pedal ganglia were dissected into Trizol. In all cases, reverse transcription was performed using 3.0 μg total RNA, and 1.0 μL cDNA was used in the PCR reaction. Cycle number was optimized for each primer set, to ensure amplification was kept within the linear range for comparison between control and experimental groups. In all cases, histone H4 was used as a negative control, and the immediate early gene C/EBP was used as a postive control. C/EBP has previously been demonstrated to be rapidly transcribed following injury and *in vivo* 5-HT exposure (Alberini et al., 1994). RT-PCR was also used to assess tissue distribution, similarly as described for investigation of experience-dependent expression of ApHomer.

Antibody Preparation & Detection of Protein Interactions

Frozen rat brains were obtained from Pel Freez Biologicals. MGluR antibody was from BD Biosciences, and IP_3R antibody was from Affinity BioReagents. Recombinant ApHomer proteins were coupled to 0.1 mL packed glutathione-agarose overnight at 4 °C. As described by Tu et al. (Tu et al., 1998), rat cerebellar extracts were prepared by sonication in PBST plus protease inhibitors, followed by centrifugation at 15,000 × g for 10 min. Glutathione beads were washed 3 times with 0.3 mL PBST plus protease inhibitors, and 40 mg of cerebellar extract was allowed to bind for 4 h at 4 °C. Beads were again washed once with PBS followed by two additional washes with PBST, and bound material was removed by boiling in 100 μL SDS-PAGE sample buffer. Equal amounts of eluted material was separated by gel electrophoresis for Western blotting with antibodies against mGluR1a and type 1 IP_3R.

In the case of recombinant GST-$ApIP_3R$, soluble proteins were similarly coupled to glutathione-agarose overnight at 4 °C. Ganglia from 4–6 animals were homogenized with a glass-teflon motor driven homogenizer into 1.0–1.2 mL PBST plus 1:100 Sigma mammalian

protease inhibitor cocktail, 3 mM Na orthovanadate, 30 mM NaF, and centrifuged for 10 min at 10,000 × g. A small amount of supernatant was saved for use as input material, and then divided equally for addition to agarose-coupled recombinant proteins. To inhibit the Homer-IP_3R interaction, a synthetic peptide corresponding to *Aplysia* IP_3R amino acids 41–52 (GDLNNPPKKFRD; mutant peptide GDLNNPLKKRRD) was synthesized by Invitrogen. Peptide was added to *Aplysia* CNS extract prior to addition of the extract to glutathione-coupled recombinant protein.

Following binding on a rotator for 4 h at 4 °C, unbound material was removed by three washes of PBST plus inhibitors. Bound material was eluted by addition of SDS-PAGE sample buffer and boiling for 5 min, followed by analysis by Western blot for ApHomer. A custom affinity-purified ApHomer antibody was prepared by Covance Research Products, Inc. against the ApHomer peptide, -LQDSQRQLQDGRSSRDQEHRE (amino acids 324–344). The synthetic peptide was conjugated to KLH and used as immunogen for antibody generation in rabbits.

Electrophysiology

For electrophysiology, pleural and pedal ganglia were desheathed in 1:1 isotonic $MgCl_2$:ASW to expose the tail sensory and motor neuron clusters. $MgCl_2$ was removed by perfusion of the preparation with ASW for at least 15 min. Intracellular recording was performed using glass microelectrodes containing 3 M KCl with electrode resistance of 8–12 MΩ. Motor neurons were hyperpolarized to approximately −70 mV. The resting potential and input resistance of the SN and MN were monitored throughout each experiment. Baseline EPSP amplitudes were determined by injection of current to elicit a single test spike in the SN in each of the two presynaptic SNs (~200 ms apart). One minute later, a tetanizing stimulus (generated by individual current pulses [10 nA] at 20 or 7 Hz for 2 sec) was applied to the SNs. A posttest EPSP was measured 10 sec after the offset of the stimulus train. 200 mM BAPTA (tetrapotassium salt, Molecular Probes, Eugene, OR), 25 μM heparin (Calbiochem, San Diego, CA), and 125 μM IP_3 were dissolved in 3 M KCl, 10 mM HEPES, and 3 mM Fast Green dye (Fisher Scientific). Drugs were injected into the MN with 2–4 pulses (20 ms, 20–40 psi) from a picospritzer (General Valve, Fairfield, NJ) and at least 20 minutes were allowed for drugs to take effect before pretest values were obtained. For caffeine experiments, caffeine (5 μM, Sigma, St. Louis, MO) was dissolved in ASW and perfused over the ganglion through the experiment. Thapsigargin (5 μM, Sigma, St. Louis, MO) was prepared in 0.1% DMSO and continually perfused as in the case of caffeine.

For mGluR1 blocking experiments, LY367385 (300 μM) and *S*-MCPG (500 μM) (Tocris) were prepared in 110 mM NaOH and diluted 1:250 in ASW immediately prior to each experiment. In vehicle control experiments, 200 μL 110 mM NaOH was added to 50 mL ASW (1:250) and perfused in a similar manner as drug treated preparations. Following removal of $MgCl_2$ by perfusion, drug or vehicle was bath applied for at least 30 min, at a flow rate of 0.5–1 mL/min, before the commencement of recording.

Synthetic ApIP_3R peptide (active and mutant) was prepared at a concentration of 1.0 mM in 0.5 M KOAc, 10 mM TrisCl pH 7.5, 15 mM Fast green (~1.0%). Peptides were injected into the MN by 28–32 pulses, 10–40 msec each, at 20 psi. The number and duration of pulses was determined depending upon resistance of injection electrode and size of the cell. Following injection, cells were continually perfused in ASW for at least 45 min before recording from MN. Monosynaptic EPSPs were measured and PTP performed as described above. Short-term facilitation (STF) induced by a single pulse of 5-HT was also assessed following 3 pretests taken at 15 min intervals. Ten min after the last pretest, 50 μM 5-HT was perfused for 5 min, and a single post-test was taken immediately.

Input resistance and excitability of the injected MN were monitored in all experiments, and any preparations which indicated possible cellular damage were discarded prior to onset of the stimulus train (approx 10% of cases).

Cell culture

Cell culture was performed based on a modified protocol as described (Gruenbaum and Carew, 1999; Lyles et al., 2006). Briefly, pleural and abdominal ganglia from 80–100 g *Aplysia* were first incubated in 10 mg/ml of Protease Type IX (Sigma) at 34.5 °C for 2 hr 15 min and 2 hrs 10 min, respectively. The ganglia were then desheathed. SNs were isolated from the pleural ganglia, and LFS MNs were isolated from the abdominal ganglia. Identified SNs or MNs were then plated in culture dishes. The sensory neuron processes were carefully manipulated to contact the motor neurons. Culture dishes were pre-coated with poly-*L*-lysine (0.5 mg/ml). Culture medium consisted of 50% *Aplysia* hemolymph and 50% L-15 medium supplemented with salts (NaCl, 260 mM; $CaCl_2$, 10 mM; KCl, 4.6 mM; $MgSO_4$, 25 mM; $MgCl_2$, 28 mM; $NaHCO_3$, 2 mM; HEPES, 15 mM) and D-glucose (34.6 mM). Additionally, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 U/ml) were also included in the culture medium. Cultured cells were left on the microscope stage overnight and then transferred to an 18 °C incubator. After 4 days, Ca^{2+} imaging was performed on MNs and synaptic strengths between the SN-MN co-cultures were measured. Electrophysiological recordings were performed similarly to that in the intact CNS.

Photolysis of Caged IP_3 and Calcium Imaging in MN neurites

Cultured MNs were injected with 1 mM caged-IP_3 (Invitrogen) and 10 mM cell impermeable Fluo-4 Ca^{2+} indicator dye (Invitrogen) using a picospritzer, similar to the microinjection procedure described in the intact ganglia. In order to aid visualization of calcium release in MN neurites, cells were pretreated with a high K^+ (100 mM), high calcium (55 mM), and low magnesium (11 mM) ASW for 5 min (Malkinson and Spira, 2010). This conditioning depolarization loads intracellular calcium stores (Garaschuk et al., 1997; Hong and Ross, 2007). Imaging was accomplished using a home-built microscope system based around an Olympus IX 50 microscope equipped with an Olympus 60× oil objective (N.A. 1.45), employing excitation by a solid-state 488 nm laser (Spectra Physics sapphire) and imaging emitted fluorescence through at λ>510nm by a Cascade 650 electron-multiplied c.c.d. camera (Roper Scientific). Fluorescence signals are expressed as ratios (F/F_0 or $\Delta F/F_0$) of the fluorescence (F) at each pixel relative to the mean resting fluorescence (F_0) prior to stimulation. IP_3 was photoreleased from a caged precursor by delivering flashes of UV light (350 – 400 nm) focused uniformly throughout the field of view. Image data were streamed to computer memory and then stored on disc for offline analysis using the MetaMorph software package (Molecular Dynamics).

Photolysis of Caged IP_3 and electrophysiology in SN-MN co-culture

The electrophysiological studies using SN-MN co-cultures were performed on a different experimental setup than that used to visualize Ca^{2+} transients in neurites (as described above). Following microinjection with caged IP_3 and fluo-4, cultured cells were again subject to a conditioning depolarization by treatment with a high K^+ (100 mM) ASW for 5 min in order to load intracellular Ca^{2+} stores and then subject to a 2s UV flash. This conditioning depolarization pretreatment, followed by washout, had no effect on subsequent baseline synaptic transmission. Flashes of UV light were derived from a Zeiss Xite 120 metal halide light source via a UV filter set in a Zeiss Axiovert 200 inverted microscope (Leissring et al., 1999) and were delivered to the MN soma and proximal neurites.

EPSPs of sensory-motor synapses before and after (i) 20 Hz stimuli, (ii) 7 Hz stimuli, (iii) 7 Hz stimuli paired with the UV flash, (iv) UV flash alone, and (v) no stimulation were then compared.

Data analysis and statistics

Postsynaptic EPSP amplitudes were measured as the peak voltage of the EPSP. In the case of 5HT-induced facilitation, baseline EPSP was determined based on the average of three pretests in ASW, and only EPSPs with pretests within 20% of the mean were used for further analysis. In the case of PTP, a single pretest was taken as described (Schaffhausen et al., 2001). All EPSP amplitudes are expressed as a percent of the baseline mean. Two-tailed t-tests were used for data analysis. All data are shown as ± standard error of the mean (SEM).

Results

PTP can be shared at SN-MN synapses

To directly test the hypothesis that convergent inputs might result in postsynaptic sharing of PTP, we first asked whether presynaptic activation of a SN at a level that is ineffective in producing PTP might gain the ability to induce PTP when paired with effective activation of a neighboring presynaptic SN. Intact ganglia (abdominal or pleural-pedal, in separate experiments) were excised and neurons impaled for intracellular recording. By eliciting a single presynaptic action potential and recording the evoked postsynaptic EPSP, two presynaptic SNs were identified which made synapses onto a common MN. At least 15 min after identification of convergent synapses, each SN was activated with a train (2 s in duration) of either 7 Hz (which produces no PTP), or 20 Hz (which induces PTP) (Fig. 1, inset). EPSPs were then measured 10 s after the offset of the trains. While 7 Hz alone produced no PTP, when paired with a 20 Hz train to the other SN, the 7 Hz train induced a robust enhancement of synaptic strength (Fig. 1*A*, *B*). Additional experiments with no activation of one SN and 20 Hz activation of the other revealed that non-activated synapses do not passively inherit this enhancement (Fig. 1*B*), indicating that the 7 Hz train likely generates a subthreshold postsynaptic signal which summates with the signal initiated by the 20 Hz train. Thus, the temporal registry of trains from both cells is required for the “sharing” of plasticity between synapses.

We next examined the time course of heterosynaptic sharing and found that it decayed faster than that induced by 20 Hz (Fig. 1*C*). By one min after simultaneous activation, the EPSP associated with the 7 Hz train was no longer significantly different from baseline, while the corresponding 20 Hz EPSP was still significantly elevated at 10 min. Next, to determine the temporal constraints on induction of heterosynaptic sharing, we varied the time between 7 and 20 Hz activation of the two SNs. Sharing was observed when the onset of trains were separated by 3 sec or less (Fig. 1*D*), and was independent of the order of activation, indicating that the two SNs need not be coactive, but that their activation must occur within a restricted time window. The symmetrical and relatively broad (3 sec) permissive time window for heterosynaptic sharing contrasts with previously described forms of associative plasticity, such as spike timing dependent plasticity (STDP) and input timing dependent plasticity (ITDP) which have much more restricted temporal windows (in the range of ms), and even for associative LTD in the cerebellum, in which pairing of inputs can occur on the order of hundreds of ms (Sjostrom and Nelson, 2002;Wang et al., 2003;Dan and Poo, 2004;Dudman et al., 2007;Sarkisov and Wang, 2008). This dissociation of temporal requirements suggests that heterosynaptic sharing represents a unique form of synaptic interaction whereby activity at one set of inputs can change the gain for local information flow at other synaptic sites.

Heterosynaptic sharing requires postsynaptic Ca^{2+}

To examine the postsynaptic Ca^{2+}-dependency of heterosynaptic sharing, we injected the Ca^{2+} chelator BAPTA into the MN, and 20 Hz and 7 Hz trains of activation were delivered to two SNs. In contrast to the control condition, synaptic enhancement in the 7 Hz SN was entirely blocked (Fig. 2*A*). We and others have previously shown that NMDA receptor activation is not required for induction of PTP at the SN-MN synapse (Schaffhausen et al., 2001;Jin and Hawkins, 2003). Moreover, Jin and Hawkins (2003) have previously shown that PTP in SN-MN culture is dependent on intracellular Ca^{2+} release, but not on voltage-gated Ca^{2+} influx or extracellular Ca^{2+} concentration. Thus we next examined the contribution of Ca^{2+} from intracellular stores. Perfusion of caffeine (which causes Ca^{2+} store depletion via the ryanodine receptor) and thapsigargin (a SERCA pump antagonist), as well as postsynaptic injection of heparin (an IP_3R antagonist), also resulted in a total block of heterosynaptic sharing (Fig. 2*A*). Interestingly, while injection of heparin (25 μM) completely blocked heterosynaptic sharing, this concentration had no effect on PTP. However, when we injected a higher concentration of heparin (100 μM) we observed a significant attenuation of PTP (Fig. 2*A*), which is consistent with previous observations by Jin and Hawkins (2003) who also observed a significant attenuation of PTP in cultured SN-MNs by injecting this concentration of heparin into the MNs. The remaining PTP in the heparin (100 μM) experiments (Fig. 2*A*) is likely due to the presynaptic contribution to PTP at these synapses (Schaffhausen et al., 2001).

While perfusion of thapsigargin and caffeine in the bath could affect both pre- and postsynaptic Ca^{2+}, the block observed by these reagents, taken together with the block observed following injection of heparin into the MN, are consistent with a possible role of IP_3-mediated postsynaptic plasticity. To directly test this idea we injected non-degradable IP_3 into the MN. In this case a 7 Hz SN tetanus alone induced significant PTP (Fig. 2*B*). Collectively, these results suggest that effective (20 Hz) SN activation generates IP_3-dependent release of Ca^{2+} from internal stores in the MN, which facilitates the induction of sharing at the neighboring (7 Hz) synapse.

Photolysis of caged-IP_3 induces Ca^{2+} transients in the MN neurites

As demonstrated above, non-degradable IP_3-7Hz pairing induces PTP (Fig. 2*B*). One potential mechanism that may account for this observation involves IP_3-mediated Ca^{2+} release. To test this hypothesis, MNs were injected with Fluo-4 and caged IP_3. As shown in Figure 3*A*,*B* repetitive, localized Ca^{2+} transients in neurites were evoked by photorelease of IP_3 following a UV flash. These observations are representative of results in four cells, obtained at 16 locations up to 500 μm away from the cell body. These localized Ca^{2+} transients likely arise from clusters of IP_3Rs and are reminiscent of puff activity as reported in mammalian cells (Smith et al., 2009).

Heterosynaptic sharing is mimicked by pairing 7Hz activation and photolysis of caged-IP_3

In order to further elucidate the mechanisms of heterosynaptic sharing, we used the photolysis of caged IP_3 to mimic the *in situ* generation of an IP_3 signal induced by 20 Hz stimuli. We predicted that if IP_3-mediated intracellular Ca^{2+} release is a critical component of the mechanism for sharing, then the transient Ca^{2+} release induced by the photo-activation of caged IP_3 in the MN, when paired with a train of sub-threshold 7 Hz tetanus in the SN, should mimic facilitation induced by heterosynaptic sharing. In SN-MN co-cultures, MNs were injected with caged-IP_3 and the calcium indicator Fluo-4. A UV flash, which liberates free IP_3 and induces calcium transients in the MN neurites (Fig. 3) was delivered to the MNs. We then examined the synaptic strengths at the SN-MN synapses before and after: (i) a 20 Hz train, (ii) a 7 Hz train, (iii) a 7 Hz train paired with the photolysis of caged IP_3, (iv) photolysis alone, and (v) no stimulation (No Stim) (Fig. 4). We observed that the SN-

MN synapses were significantly potentiated by 20 Hz stimuli alone (Fig. 4*A*,*B*). The significant potentiation exhibited following 20 Hz stimulation is all the more striking when compared to the No Stim control (Fig. 4*B*), which exhibited synaptic decrement typically observed in SN-MN cultured synapses (Eliot et al., 1994;Bao et al., 1997;Jin and Hawkins, 2003). Thus the 20 Hz-induced potentiation is superimposed on a progressively decrementing baseline (a similar result was previously observed by Jin and Hawkins, 2003). SN-MNs exposed to either 7 Hz alone or photolysis alone also exhibited synaptic decrement that was comparable to the No Stimulation condition (Fig. 4*B*). In contrast, the pairing of 7Hz stimuli and photolysis of caged IP_3 induced significant synaptic potentiation (Fig. 4*A*,*B*). The facilitation induced by pairing 7 Hz-with IP_3 photolysis was significantly greater than that induced by the 7 Hz stimuli alone, and was not significantly different from the facilitation induced by the 20 Hz stimuli alone (Fig. 4*B*). These data support the hypothesis that the postsynaptic intracellular Ca^{2+} transients induced by IP_3 receptor activation are critically involved in heterosynaptic sharing.

Molecular mechanisms of heterosynaptic sharing require postsynaptic Homer-mediated protein interactions

We next explored the postsynaptic molecular mechanisms underlying sharing. Previous work by Jin and Hawkins (2003) indicated that mGluRs are required for PTP in cell culture. Thus, we examined the effects on monosynaptic PTP in pleural/pedal ganglia of two type I mGluR inhibitors, LY367385 and *S*-MCPG. While control synapses demonstrated robust PTP, both LY367385 and *S*-MCPG caused a significant attenuation of PTP, with no effect on baseline synaptic transmission (Fig. 5*A*,*B*). The remaining PTP observed in the presence of inhibitors is likely due to the presynaptic component of PTP at these synapses (Schaffhausen et al., 2001).

Given the mGluR/Ca^{2+} dependency and IP_3 involvement in sharing, an attractive molecular candidate for mediating PTP and heterosynaptic sharing was Homer, a well described protein in the vertebrate postsynaptic density. Homer proteins mediate a series of interactions with other proteins including mGluRs as well as IP_3 receptors (Tu et al., 1998; Worley et al., 2007). Moreover, Homer proteins have been demonstrated to both couple mGluR signaling with intracellular Ca^{2+} release in vertebrate neurons (Tu et al., 1998), and to localize IP_3Rs within dendritic subregions, allowing for synaptically localized IP_3-mediated Ca^{2+} release (Sala et al., 2001; Sala et al., 2005). However, there was no available evidence to indicate that a Homer ortholog is expressed in *Aplysia*, or whether it might play a role in the spread of plasticity.

Thus, to directly explore the possible involvement of Homer in sharing, we first cloned *Aplysia* Homer (ApHomer) using a combination of degenerate RT-PCR and library screening (Fig. 6*A, B*). ApHomer shares the domain organization of other Homer proteins, including an N-terminal EVH1 domain and a C-terminal coiled-coil region. In vertebrates, Homer has been predominantly studied in the nervous system, but is also expressed in tissues outside the CNS, as observed for ApHomer (Fig 6*C*). In comparison, the gene for *Aplysia* MAGUK protein ApSAP is expressed predominantly within the CNS (Reissner et al., 2008). Because Homer was first identified as an activity-dependent IEG (Brakeman et al., 1997; Kato et al., 1997), we used semi-quantitative RT-PCR to assess message levels in response to injury, *in vivo* 5-HT, KCl depolarization, or tail-shock (which induces behavioral sensitization). None of these treatments induced a significant change in the cDNA levels of histone H4 or Homer, but did induce a substantial increase in a positive control, C/EBP (Fig. 6*D*). These findings thus indicate the lack of an activity-dependent splice variant of Homer, and suggest that there is no change in the amount of the constitutively expressed splice form of Homer following experience. These results are consistent with findings in *Drosophila*, where there appears to be only one gene and one

transcript for Homer (Diagana et al., 2002). However, we did identify an alternative splice variant at the extreme 5' end of the mRNA, which would translate into proteins differing among the first six amino acids (Supplemental Figure 1).

A prerequisite for our hypothesis that ApHomer has a mechanistic role in heterosynaptic sharing is that Homer-mediated interactions observed in vertebrates, particularly with mGluRs and IP_3Rs, should be conserved in *Aplysia*. We found that recombinant ApHomer could successfully co-precipitate rat mGluR1α in a GST pull-down experiment (Fig. 7). However, a low signal to noise result made it difficult to interpret pull-down results for IP_3R (Fig. 7, bottom right). To directly examine a possible interaction between ApHomer and *Aplysia* IP_3R, we determined the coding sequence for the initial 154 amino acids of ApIP_3R. A cDNA for *Aplysia* IP_3R has also been reported, matching the sequences we obtained (Cummins et al., 2007). The consensus sequence for Homer binding is PXXFr (Tu et al., 1998), and in rat IP_3R is found at amino acid positions 49–54. This sequence is 100% conserved between *Aplysia* and other vertebrates and invertebrates (Fig. 8*A*).

The first 256 amino acids of ApIP_3R were expressed as a GST fusion protein for a pull-down assay as described above. A custom antibody was generated against ApHomer amino acids 324–344 which resulted in immunoreactivity against multiple bands in *Aplysia* CNS extract (Fig. 8*B*, lane 1). However, Western blot following the GST pull-down assay indicated that, while the anti-ApHomer antibody recognizes a complex series of bands in CNS extract used as input, only a single band of approximately 47 kD is observed in samples eluted from beads coupled to GST-ApIP_3R (Fig. 8*B*, lanes 3–6). This is in agreement with the ~45–50 kD size of mammalian and *Drosophila* Homers and the expected size of ApHomer. Moreover, inclusion of a 12 amino acid peptide matching the Homer-recognition sequence within ApIP_3R competed away this interaction in a concentration-dependent manner, while a mutant peptide had no effect (Fig. 8*B*).

The synthetic peptide used to inhibit the ApIP_3R-ApHomer interaction *in vitro* now provided a powerful experimental tool to directly explore Homer-mediated IP_3 activation in sharing. We pressure injected MNs with either the ApIP_3R peptide or mutant peptide, in which two critical amino acids in the Homer-binding sequence were substituted. One hour post-injection, two SNs were activated with either 20 or 7 Hz to explore PTP and sharing (Fig. 8*C*). Following injection of the mutant peptide, 20 Hz activation induced significant PTP, and simultaneous 7 Hz activation induced significant heterosynaptic sharing. In contrast, following injection of the ApIP_3R peptide into the MN, the 20 Hz PTP was reduced, indicating a partial block by the active peptide, which is consistent with our previous observation that 20 Hz PTP has both pre- and postsynaptic components (Fig. 5; see also (Schaffhausen et al., 2001). Importantly, heterosynaptic sharing induced by simultaneous activation with 7 Hz was completely blocked. No effect was observed on baseline synaptic transmission. Finally, the attenuation of PTP and block of sharing by the peptide was restricted to short-term, activity-dependent plasticity at the SN-MN synapse. Short-term facilitation induced by a single pulse of 5-HT was unaffected by the peptide (Supplemental Figure 2).

Discussion

Our results elucidate a series of molecular steps that provide a mechanism through which short-term plasticity may be shared between two neighboring synapses. Collectively, our findings support the hypothesis that low level (e.g. 7 Hz) activation of a SN alone leads to postsynaptic release of IP_3 which is subthreshold for the induction of PTP, but can summate with IP_3 liberated by high level activation (e.g. 20 Hz) of another presynaptic input (Fig. 9). The idea that IP_3 summation can result in molecular integration has been previously

suggested by Parker and colleagues, who found that caged IP_3 responses can summate at distances up to 20 μm (Parker, 1989;Parker and Miledi, 1989;Parker and Ivorra, 1992). SN-MN synapses in *Aplysia* commingle with inter-synaptic distances in many cases well within this 20 μm range (Marinesco and Carew, 2002), supporting the idea that subthreshold IP_3 released in the postsynaptic neuron by low level activation may summate with additional IP_3 from high level input to generate a form of molecular coincidence detection that induces a sufficient Ca^{2+} signal to give rise to heterosynaptic sharing.

In the long-term time domain (e.g. LTP and LTD), Ca^{2+}-dependent heterosynaptic expansion of synaptic plasticity has been observed in other systems (Nishiyama et al., 2000; Royer and Pare, 2003). In addition, communication between proximal hippocampal CA1 synapses has been revealed by facilitation of LTP at a minimally activated synapse following LTP induction at a nearby synapse (Harvey and Svoboda, 2007). However, the heterosynaptic amplification of hippocampal LTP is not dependent on intracellular Ca^{2+}, whereas the heterosynaptic sharing we observe requires a postsynaptic Ca^{2+} response coupled to Homer- and IP_3R-dependent protein-protein interactions.

The current study raises two important questions regarding Ca^{2+} signaling. First, how might postsynaptic Ca^{2+} mediate the induction of PTP at strongly activated synapses, and second, how might postsynaptic Ca^{2+} contribute to heterosynaptic sharing at weakly activated synapses? Considering the first question, one candidate mechanism for the induction of PTP at strongly activated synaptic sites is CaMKII, since it is well established that Ca^{2+}-dependent kinases can be critical mediators of synaptic plasticity. For example, CaMKII regulates the conductance of AMPA receptors during LTP by phosphorylation of GluR1 at Ser^{831}, and also may indirectly mediate insertion of AMPA receptors into the membrane by phosphorylation of interacting molecules which govern membrane insertion (for reviews, see (Bredt and Nicoll, 2003; Malenka, 2003; Boehm and Malinow, 2005). Consistent with this possibility, Jin and Hawkins (2003) have found that postsynaptic CaMKII is required for PTP in cultured SN-MN synapses in *Aplysia*, supporting the hypothesis that Ca^{2+}-activated CaMKII may contribute to a postsynaptic mechanism for the induction of PTP we observe at strongly activated central synapses. Considering the second question, how might Ca^{2+} contribute to sharing? It is possible that spread of IP_3 from strongly activated synapses to neighboring weakly activated ones could induce Ca^{2+}release at the weakly activated synapses and thus engage the same CaMKII-mediated signaling events at those neighboring sites as are responsible for the induction of PTP at the “donor” synapse. However, we cannot exclude the possibility that the postsynaptic mechanisms contributing to the induction of PTP at strongly activated synapses could differ from those required for heterosynaptic sharing. What we can say however, is that both classes of plasticity require postsynaptic Ca^{2+}. Other Ca^{2+} targets beyond CaMKII may contribute both to the induction of PTP and to heterosynaptic sharing as well. For example, it has recently been shown that Ca^{2+}-dependent Ras activation occurs rapidly within individual spines (Harvey et al., 2008), and is thus a candidate for PTP induction at the strongly activated site. Moreover, activated Ras can spread to neighboring spines (Harvey et al., 2008), making it also an attractive candidate for mediating signaling contributing PTP sharing. Thus, a number of interactive Ca^{2+}-dependent postsynaptic signaling events may contribute both to the initial induction of PTP as well the sharing of PTP that we observe.

Our data also indicate that ApHomer-mediated protein interactions are critical for heterosynaptic sharing, since the synthetic peptide which interrupts Homer-IP_3R interactions completely blocks sharing. Interestingly, injection of the ApIP_3R peptide into the MN also tends to reduce the level of PTP (although not to a level that reaches statistical significance), suggesting that, as in the case of heparin, blockade of the signaling pathway between Homer and intracellular Ca^{2+} release is more readily impaired in the case of heterosynaptic sharing

as compared to PTP. Another difference between heterosynaptic sharing and PTP is their time course. Sharing is significantly attenuated after 60 seconds, whereas PTP lasts longer than 10 minutes. Thus, while not conclusive, the differential sensitivity of heterosynaptic sharing and PTP to both heparin concentration and the $ApIP_3R$ peptide, taken together with their differential time courses, suggest that these two forms of synaptic plasticity are not mechanistically identical.

What are the mechanisms by which disruption of Homer-mediated interactions might inhibit induction of PTP and heterosynaptic sharing? An obvious candidate is postsynaptic Ca^{2+} release at the activated synapse, localized by a protein scaffold linking type I mGluRs, Homer, and IP_3Rs. However, since the conserved Homer binding sequence is present in additional Homer ligands, these interactions are also likely to be disrupted by the synthetic peptide as well. For example, a number of Homer ligands containing the PXXFr sequence have been identified including the Ryanodine receptor, Shank, Dynamin III, PI3 kinase enhancer (PIKE-L), and TRPC (Tu et al., 1998; Feng et al., 2002; Rong et al., 2003; Yuan et al., 2003; Lu et al., 2007). These interactions serve to regulate release of Ca^{2+} from intracellular stores, activation of PI3 kinase, maturation of dendritic spines, as well as the localization of synaptic AMPA receptors and modulation of synaptic strength (Sala et al., 2001; Rong et al., 2003; Sala et al., 2005; Lu et al., 2007). While our results support a model in which mGluR-Homer-IP_3R interactions mediate a local Ca^{2+} signal near activated synapses, which may travel to nearby synapses as part of the mechanism of sharing (Fig. 9), we cannot rule out other Homer-dependent mechanistic components to heterosynaptic sharing.

Finally, an intriguing question arises from the present work: What is the functional significance of heterosynaptic sharing? The SN-MN synapses examined in this study mediate the monosynaptic component of the tail-elicited tail withdrawal reflex in *Aplysia*, in which a tactile stimulus to the tail gives rise to the withdrawal of the tail from the site of stimulation. Since there is an approximate topographic arrangement of SN-mediated tactile input from the tail skin onto the tail MNs (Walters et al., 2004), the sharing of plasticity we observe could allow the animal to be transiently more responsive to a weak stimulus applied to a neighboring area on the tail where a strong stimulus recently occurred. This enhancement of sensitivity within the net receptive field of the reflex would be constrained to a restricted time window commensurate with the duration of the net plasticity induced at neighboring SN presynaptic inputs to the tail MNs. In this fashion, the functional tactile receptive field of the reflex would be dynamically and rapidly expanded following a focal strong stimulus to the tail, with a time course sufficient for the animal to make an integrated, adaptive behavioral response.

In principle, one could expand this general notion to any site in a neural network where there is convergent presynaptic input onto a common postsynaptic target. At these convergent sites, heterosynaptic sharing of short-term plasticity could provide a potential mechanism for the time-limited rapid and dynamic expansion of the functional “receptive field” of a postsynaptic neuron. From this perspective, inputs whose activation are below threshold for the induction of plasticity can, by virtue of temporal coincidence with other suprathreshold inputs, subsequently acquire plasticity and thus more effectively drive the postsynaptic target. In this fashion the net enhanced synaptic input onto the postsynaptic neuron is dynamically expanded, within a constrained time course dictated by the duration of the composite plasticity at those presynaptic sites. This general mechanism could allow for a novel form of integration in a neural network by providing a means of rapid and transient sharing of plasticity arising from temporally correlated inputs to a common postsynaptic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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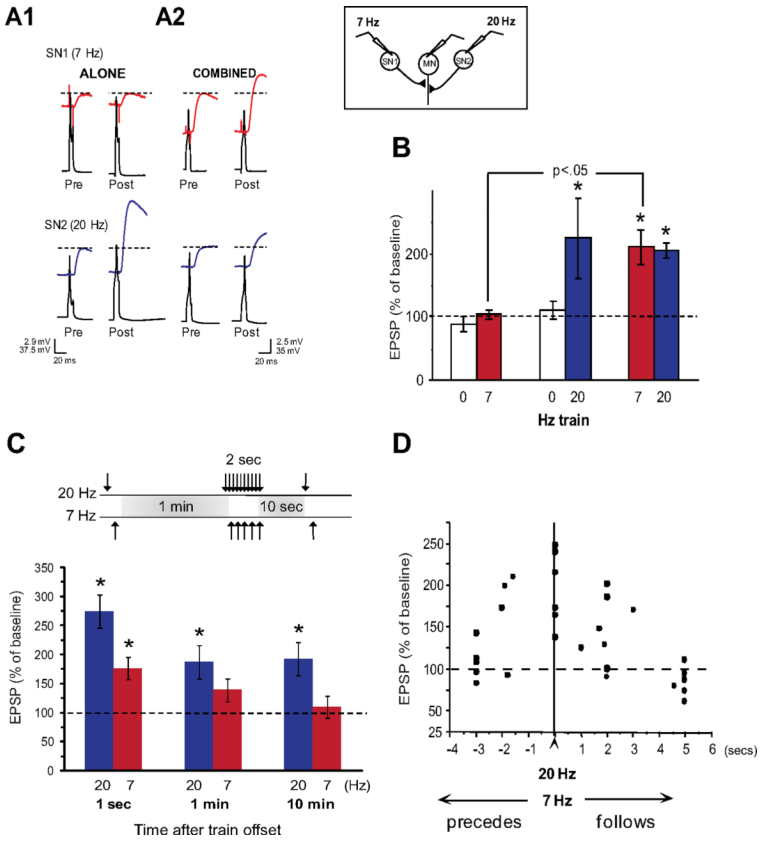

Figure 1. Induction of PTP can be shared with a neighboring synapse receiving sub-threshold activation

A, Inset: experimental design: two presynaptic SNs are identified which synapse onto the same postsynaptic MN. 20 Hz and 7 Hz trains are delivered intracellularly to the two SNs, while postsynaptic EPSPs are recorded in the MN. Representative traces are shown from synapses receiving 7 Hz alone (*A1*, top left), 20 Hz alone (*A1*, bottom left), or 7 Hz paired with 20 Hz (*A2*, top and bottom respectively). . ***B,*** Histogram of results indicating that a SN receiving a 7 Hz train acquires the ability to induce heterosynaptic sharing, but only when paired with a 20 Hz train to a neighboring SN. Previously we have shown that small synapses (<6.2 mV) exhibit an enhanced form of PTP, which is more robust than the magnitude of PTP observed across synapses of all sizes (~160% versus 285%) (Schaffhausen et al., 2001). Here we observed a similar result, in which 20 Hz PTP varied from ~200% when tested among small and large synapses alike compared with ~300% when experiments were restricted to small synapses (Fig. *1B* v. Fig. *1C*, 4A, 7C). Interestingly, the magnitude of the shared, 7 Hz PTP is consistently ~200%, regardless of the degree of potentiation at the "donor" SN. * indicates p<0.05, N=4–7 per group. ***C,Top,*** schematic diagram of experimental protocol. Pre-tests of both SNs are taken ~100 msec apart, 1 min before the onset of the overlapping 7 Hz and 20 Hz trains (each 2 sec in duration). Post-test measurements are typically taken 10 sec after the offset of the trains (e.g. as in 2*B*). For time course experiments a single post-test was taken for each synapse at either 1 sec, 1 min, or 10 min after the overlapping trains. As shown in part *C* (bottom), while 20 Hz PTP remains significantly above baseline at 10 min, the shared 7 Hz PTP is no longer significantly above baseline by 1 min (N=4–7 per group). ***D,*** Sharing of PTP requires that the 7 Hz and 20 Hz trains must occur within a narrow time window.

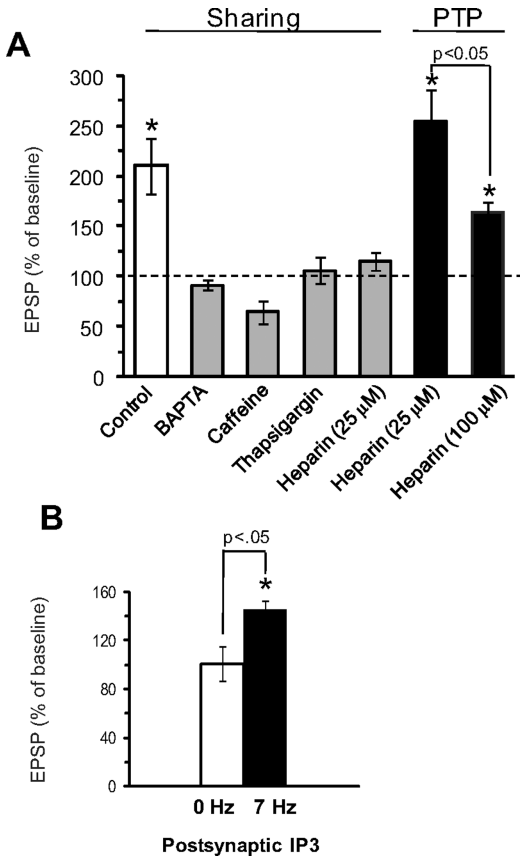

Figure 2. Heterosynaptic sharing requires postsynaptic intracellular calcium

A, Degree of sharing (control, open bar, N=7) is significantly blocked in the presence of Ca^{2+} inhibitors BAPTA (N=8), caffeine (N=5), heparin (N=7), and thapsigargin (N=5). While 25 μM heparin is sufficient to block sharing, that concentration is without effect on PTP (black bar, N=6). In contrast, 100 μM heparin results in significant, attenuation of PTP (N=4). ***B,*** Injection of non-degradable IP_3 into an MN has no effect on baseline synaptic transmission from an unstimulated SN (0 Hz, open bar), but when paired with a 7 Hz train delivered to the SN, results in significant PTP (N=7 per group).

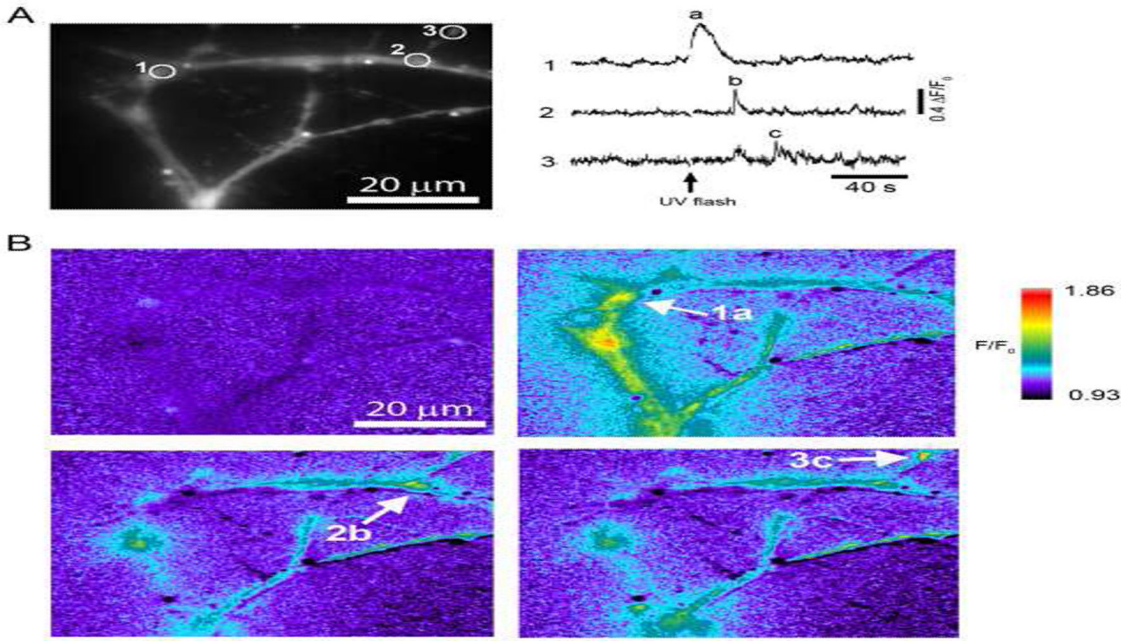

Figure 3. Photolysis of caged IP_3 induces Ca^{2+} release in MN neurites

A. Monochrome image of resting fluo-4 fluorescence in neurites from a motor neuron. ***A,*** Circled white areas indicate regions of interest from which intensity profiles over time were generated (shown at right). Traces depict local Ca^{2+} transients from each region evoked by photo-release of IP_3 using a UV flash (indicated by the arrow). ***B,*** Pseudocolored Ca^{2+} images (F/F_0) prior to (top left) and at different times after (a, b and c) photorelease of IP_3. Images are taken from the same experiment as in (***A***), and were captured at times indicated by the corresponding letters by the traces. Arrows indicate the neuritic regions where Ca^{2+} transients were captured. Each image is an average of 8 consecutive frames acquired at an exposure time of 200 ms.

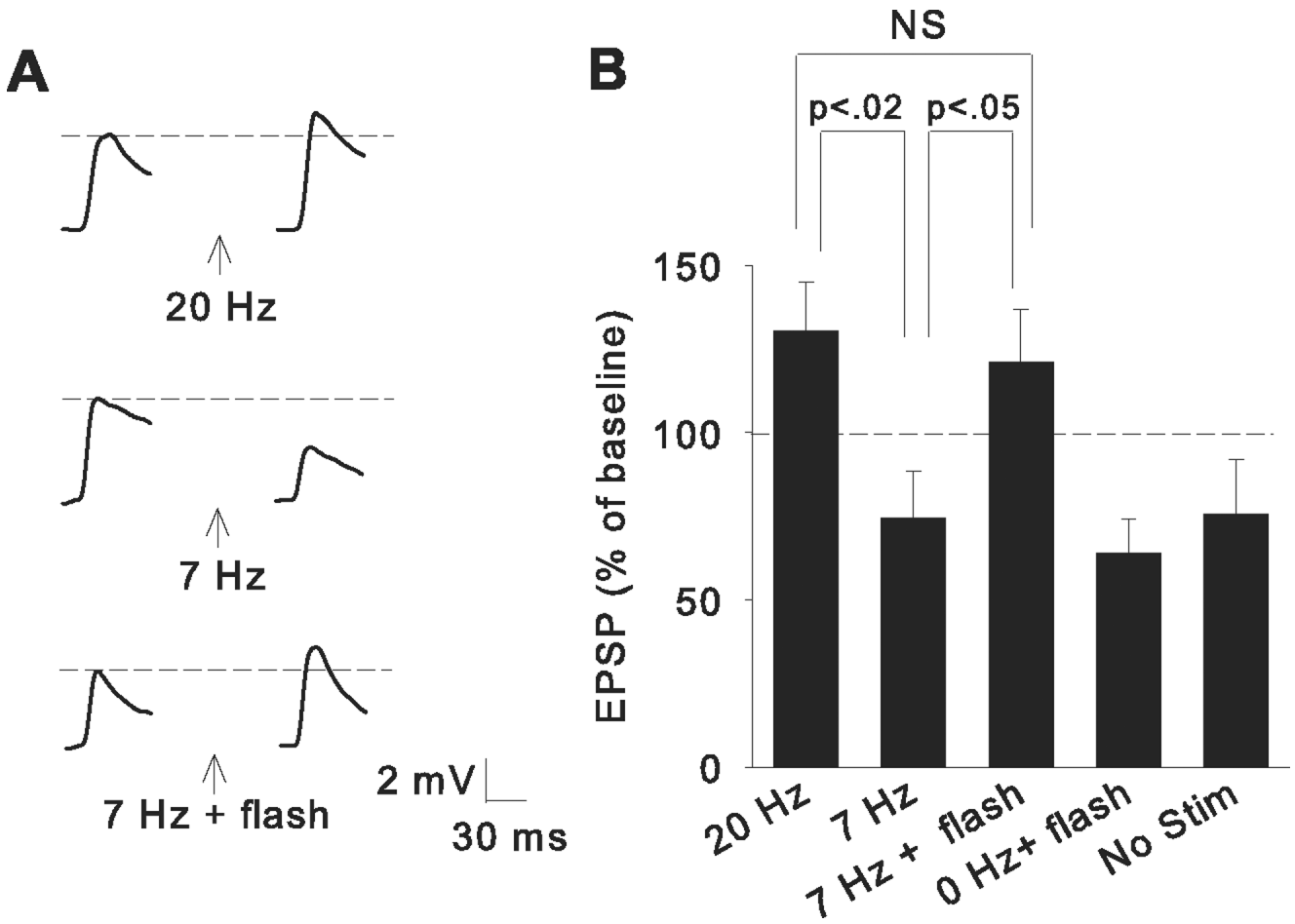

Figure 4. Sharing-like potentiation is induced by pairing 7Hz activation with photolysis of caged IP_3

SN-MN pairs are co-cultured. 20 Hz or 7 Hz trains are delivered extracellularly to the SN, while EPSPs are recorded in the MN. The MN is microinjected with caged-IP_3 and receives a UV flash (Fig. 3). ***A.*** Representative EPSPs are shown from SN-MN synapses before and after a 20 Hz train (top), a 7 Hz train alone (middle), or a 7 Hz train paired with a photolysis flash (bottom). ***B.*** Summary histograms indicating that 7Hz activation paired with the photolysis of caged IP_3 in the postsynaptic MN acquires the ability to induce potentiation comparable to a 20 Hz train. In contrast, 7Hz alone or photolysis alone exhibit synaptic decrement comparable to unstimulated SN-MN pairs. N=4–8 in each group.

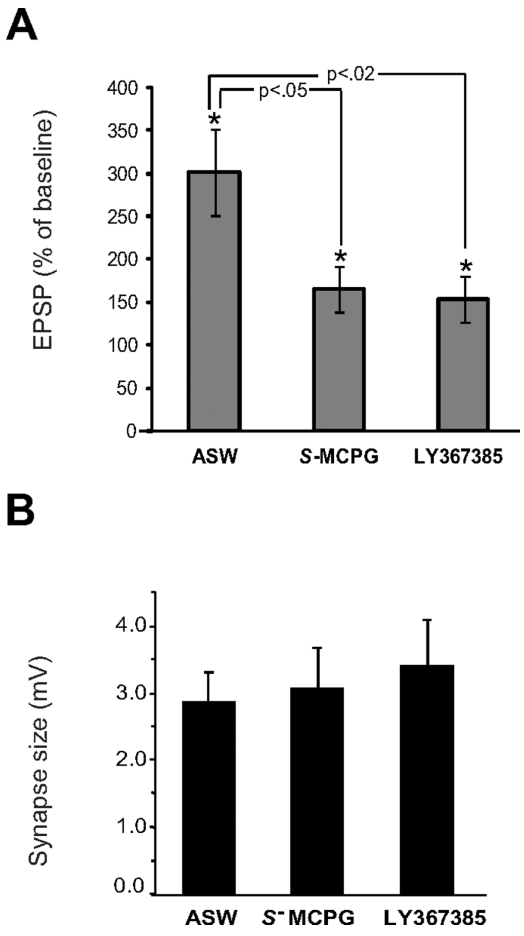

Figure 5. PTP in *Aplysia* requires type I mGluRs

A, PTP is significantly attenuated in the presence of mGluR inhibitors *S*-MCPG or LY367385 (ASW N=10, *S*-MCPG/LY367385, N=7 per group). ***B,*** the blockers had no effect on synapse size (N=7–10 per group).

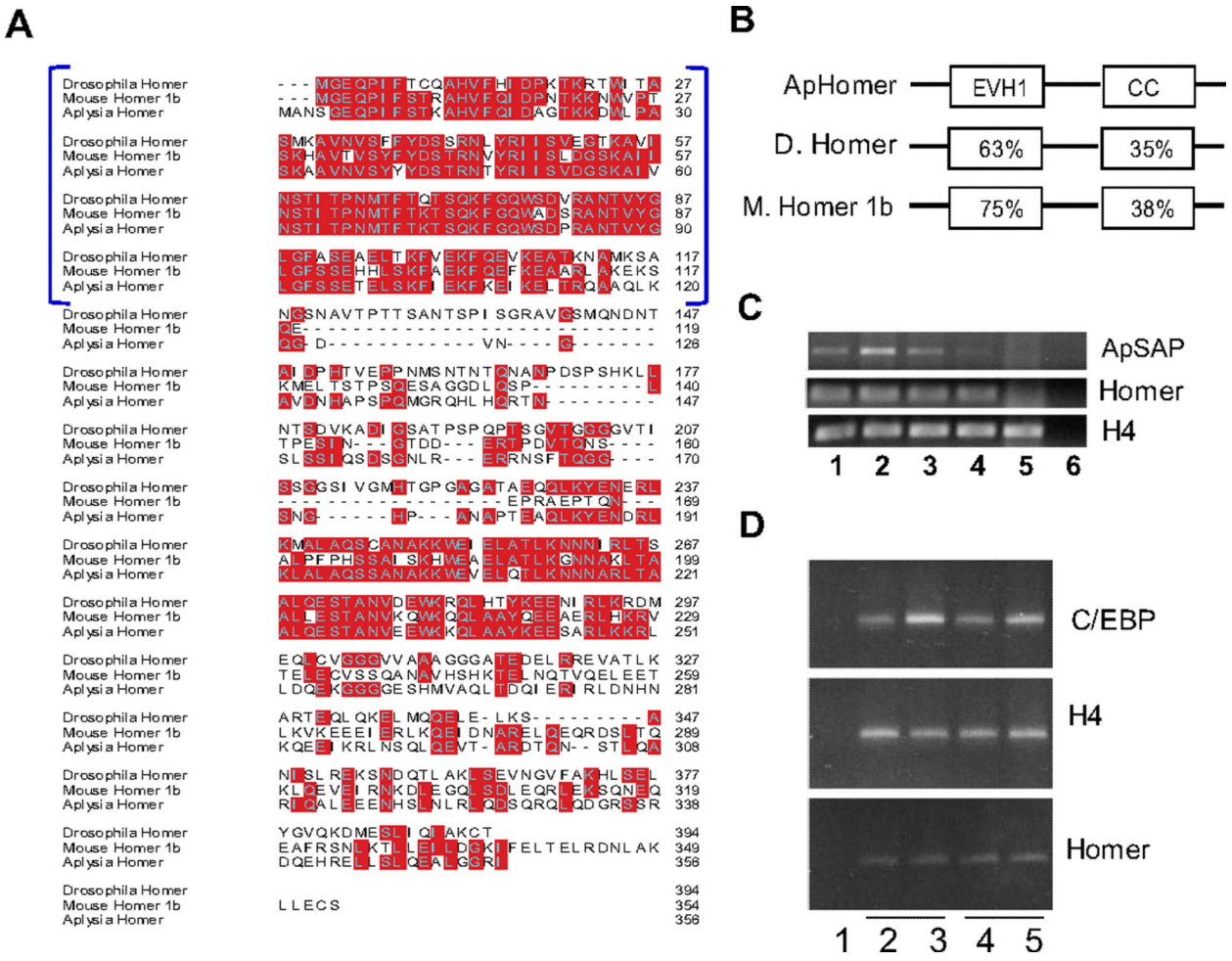

Figure 6. Identification of a Homer ortholog expressed in *Aplysia*

A, Amino acid sequence of ApHomer is shown in alignment with Drosophila Homer and mouse Homer 1b. Identical amino acids are shaded in red. The EVH1 region is bracketed in blue. The cDNA sequence for ApHomer is available under Genbank accession number EU999995. ***B,*** Schematic comparison of *Aplysia* Homer with mouse and *Drosophila* Homers. Alignment within the EVH1 domain shows a particularly high degree of sequence identity among Homer family members. Percents shown indicate the degree of sequence identity within that region between *Aplysia* and *Drosophila* Homer, or *Aplysia* and mouse Homer 1b. ***C***, Semi-quantitative RT-PCR indicates expression of Homer message in a variety of tissues. CDNA was prepared from buccal mass (lane 1), CNS (lane 2), heart (lane 3), muscle (lane 4), and sperm sac (lane 5). Lane 6 was amplified from water as template. ApSAP was amplified as a CNS-enriched control, and histone H4 was amplified as a ubiquitously expressed control. ***D***, Semi-quantitative RT-PCR of RNA from pleural and pedal ganglia. Lane 1: water control template, lane 2: cDNA prepared from RNA taken from an ASW-treated animal, compare with lane 3: cDNA prepared from an *in vivo* 5-HT treated animal; lane 4: cDNA prepared from RNA taken immediately from an anesthetized animal, lane 5: cDNA prepared from the other half of the the same animal, one hour later. No change is observed in amplification of histone H4 or Homer, while C/EBP levels are robustly increased. Similar results were observed following tail shock and KCl (100 mM) depolarization (not shown).

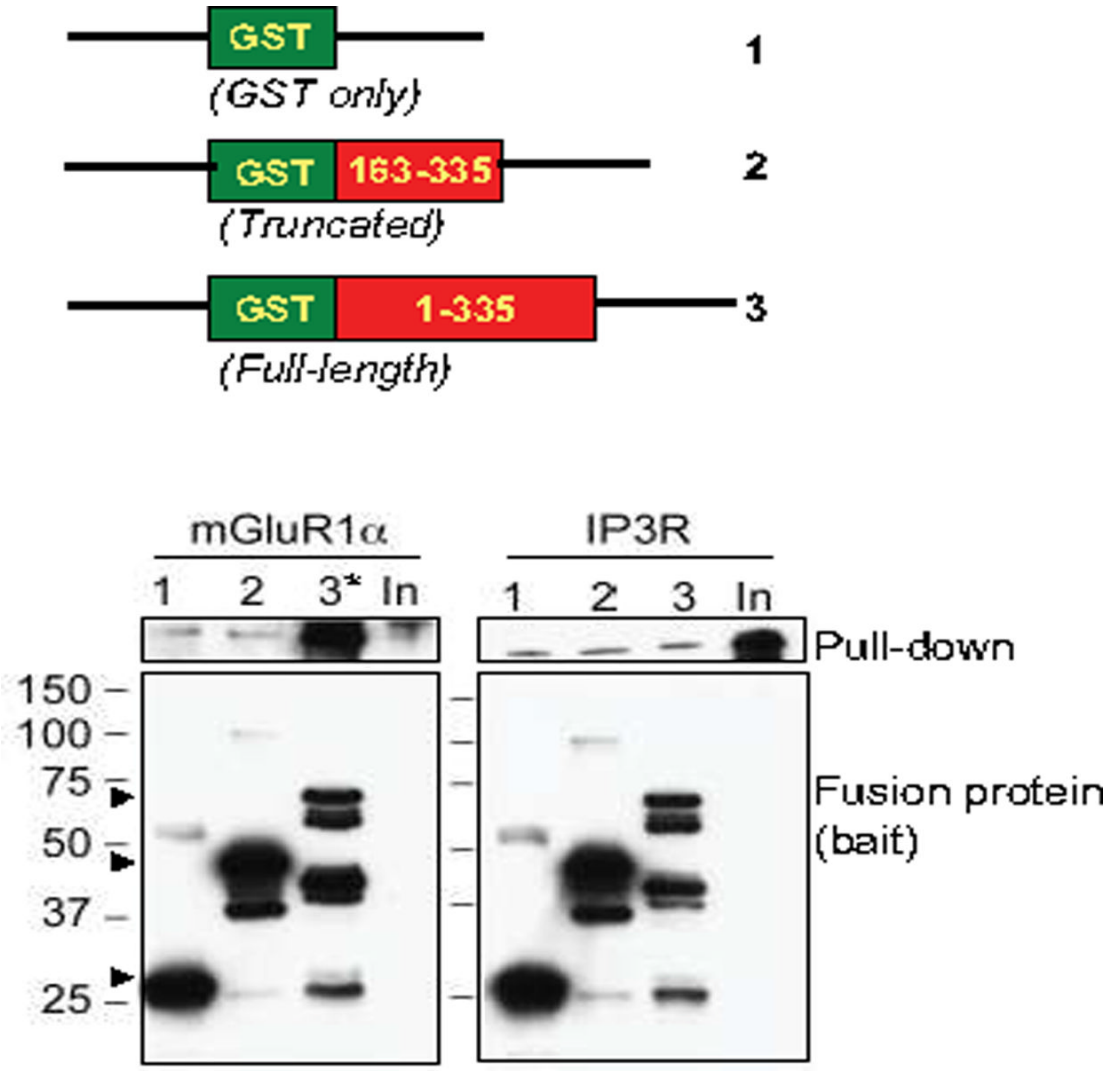

Figure 7. ApHomer co-precipitates rat mGluR 1α

Top: GST (1), or GST fusion proteins with either amino acids 163–335 (2) or the first 335 amino acids of ApHomer (3), were bacterially expressed and purified for a GST-pull-down with rat cerebellar extract. **Bottom:** Western blot for mGluR1α and IP_3R of pull-downs. Lane numbers represent the matching number beside constructs used as bait. In = input. Full-length GST-ApHomer co-precipitates mGluR1α (Western blot left panel, upper portion, lane 3), while GST alone (lane 1) and a GST fusion protein containing the C-terminal portion of ApHomer (lane 2) do not. In contrast, an interaction between ApHomer and rat IP_3R could not be conclusively demonstrated due to a non-specific signal observed between all constructs and IP_3R. Upper panels left and right show Western blot for mGluR1 and IP_3R, while lower panels show the same blots stripped and re-probed with an anti-GST antibody. The presence of GST or GST fusion proteins is indicated by arrowheads at left.

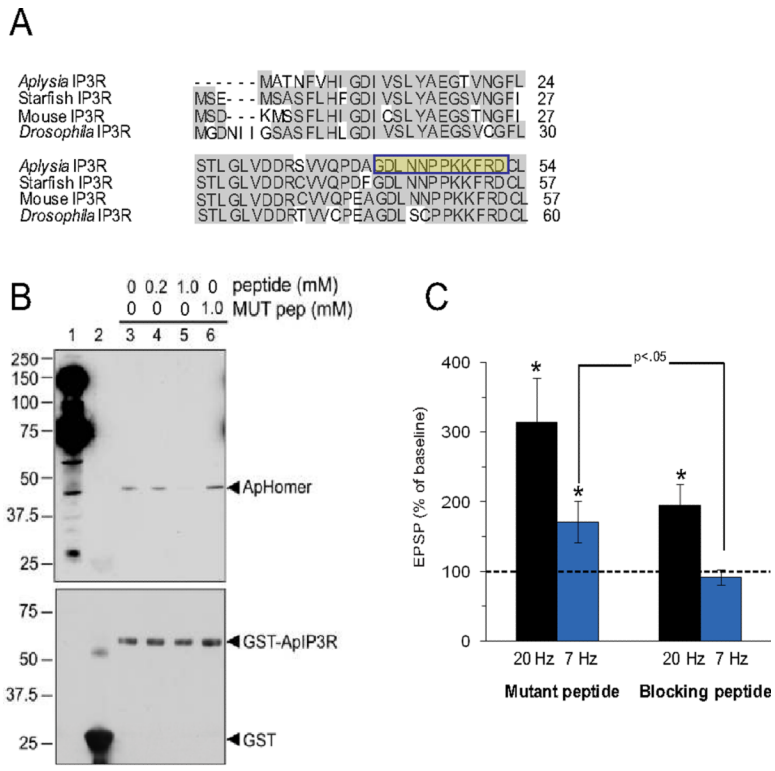

Figure 8. Interaction between ApHomer and ApIP$_3$R is required for PTP sharing

A, Alignment of the first 54 amino acids of ApIP$_3$R with starfish, mouse, and *Drosophila*. Conserved amino acids are shaded. A synthetic peptide including the Homer binding consensus sequence is boxed in blue. ***B,*** GST pull-down in which GST-ApIP$_3$R was used to isolate ApHomer from *Aplysia* CNS extract. Lane 1, Input; lane 2, pull-down with GST as bait; lane 3, ApIP$_3$R pull-down without any peptide included; lane 4, pull-down including low concentration peptide; lane 5, pull-down including high concentration peptide; lane 6, pull-down including high concentration mutant peptide. Blots for ApHomer (top) were stripped and reprobed for GST, shown at bottom. ***C,*** IP$_3$R peptide (N=6) or mutant peptide (N=5) was injected into an MN prior to induction of PTP and sharing. Mutant peptide has no effect, whereas the blocking peptide completely prevents sharing.

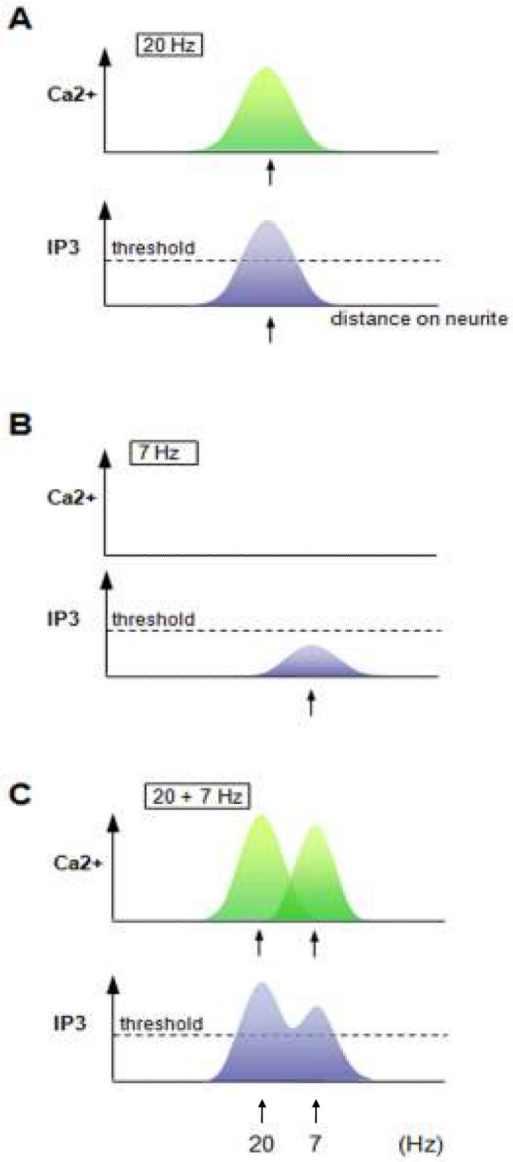

Figure 9. Molecular model for sharing of short-term synaptic plasticity

Model for molecular coincidence detection: dotted line represents IP_3 threshold for intracellular Ca^{2+} release sufficient to induce PTP. Abscissa indicates Ca^{2+}/IP_3 spread within a neurite. ***A,*** 20 Hz delivered to an SN is sufficient for local threshold IP_3 generation (blue) thus leading to intracellular Ca^{2+} release (green). ***B,*** However, 7 Hz alone does not achieve the IP_3 threshold required for Ca^{2+} release. ***C,*** In contrast, when neighboring synapses are coactivated at 7 and 20 Hz within a narrow temporal window, summation of local IP_3 signals surpass threshold for Ca^{2+} release and thus give rise to potentiation of the 7 Hz SN synapse, resulting in sharing of synaptic enhancement.