Co-Induction of LTP and LTD and its Regulation by Protein Kinases and Phosphatases

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ABSTRACT

The cellular properties of long-term potentiation (LTP) following pairing of pre- and postsynaptic activity were examined at a known glutamatergic synapse in the leech, specifically between the pressure (P) mechanosensory and anterior pagoda (AP) neurons. Stimulation of the presynaptic P cell (25Hz) concurrent with a 2nA depolarization of the postsynaptic AP cell significantly potentiated the P-to-AP EPSP, in an NMDA receptor (NMDAR)-dependent manner based on inhibitory effects of the NMDAR antagonist MK801 and inhibition of the NMDAR glycine binding site by 7-Chlorokynurenic acid (7-Cl-KYNA). Long-term potentiation was blocked by injection of BAPTA into the postsynaptic (AP) cell, indicating a requirement for postsynaptic elevation of intracellular Ca\(^{2+}\). Autocamtide-2-related inhibitory peptide (AIP), a specific inhibitor of Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII), and Rp-cAMP, an inhibitor of PKA, also blocked pairing-induced potentiation, indicating a requirement for activation of CaMKII and PKA. Interestingly, application of AIP during pairing resulted in significantly depressed synaptic transmission. Co-application of AIP with the protein phosphatase inhibitor okadaic acid restored synaptic transmission to baseline levels, suggesting an interaction between CaMKII and protein phosphatases during induction of activity-dependent synaptic plasticity. When postsynaptic activity preceded presynaptic activity, NMDAR-dependent long term depression (LTD) was observed that was blocked by okadaic acid. Postsynaptic (AP cell) injection of botulinum toxin blocked P-to-AP potentiation while postsynaptic injection of SVKI, an inhibitor of AMPA receptor endocytosis, inhibited LTD, supporting the hypothesis that glutamate receptor trafficking contributes to both LTP and LTD at the P-to-AP synapse in the leech.
INTRODUCTION

NMDAR-dependent long-term potentiation (LTP) and long-term depression (LTD) have been extensively studied in the mammalian brain as a result of the central role that LTP and LTD play in modifying neural circuits in the context of neural development, sensory processing and learning and memory (Massey and Bashir 2007; Feldman 2009). NMDARs are also present in a wide range of invertebrates including *C. elegans*, *Aplysia*, the honey bee, and the leech (Brockie et al. 2001; Ha et al. 2006; Zannat et al. 2006; Grey et al. 2009), and NMDAR-dependent LTP and LTD have been reported in the mollusk *Aplysia* and the leech *Hirudo* (Lin and Glanzman 1994; Murphy and Glanzman 1997, 1999; Burrell and Sahley 2004; Grey and Burrell 2008; Grey et al. 2009; Li and Burrell 2009). In vertebrates, many forms of LTP and LTD require activation of NMDARs and increases in postsynaptic Ca\(^{2+}\). In the case of LTP, this increase in Ca\(^{2+}\) activates PKA and CaMKII, kinases which are thought to stimulate the insertion of additional of glutamate receptors to the postsynaptic surface (Nayak et al. 1998; Kessels and Malinow 2009). In the case of LTD, increases in Ca\(^{2+}\) activate protein phosphatases in hippocampal culture, which ultimately results in the removal of glutamate receptors from the postsynaptic surface (Beattie et al. 2000).

Evidence from *Aplysia* indicate that LTP in invertebrates also depends on increases in postsynaptic Ca\(^{2+}\) (Lin and Glanzman 1996; Murphy and Glanzman 1996), but it is not known whether invertebrate LTP and LTD utilizes the same cellular signaling processes, e.g. Ca\(^{2+}\)/calmodulin kinase or protein phosphatases, as in vertebrates. Understanding the mechanisms that support NMDAR-dependent synaptic plasticity in invertebrates is important given the utility of these animals (especially in regards to their well characterized nervous systems and/or ease of genetic manipulation) in studying fundamental processes in nervous
system function, including learning and memory. The leech provides a useful model system for examining the biochemical pathways that mediate pairing-induced synaptic potentiation. The leech possesses a distributed CNS with each ganglion containing approximately 400 neurons, many of which can be readily identified, making it possible to test synaptic transmission between that same pair of cells across multiple preparations. In addition, removing individual ganglia for electrophysiological recordings preserves all the synaptic connections between neurons, an advantage not seen in slice preparations used in many vertebrate brain studies.

In this study, activity-dependent plasticity is examined at the synapse formed by the pressure-sensitive (P) neuron onto the anterior pagoda (AP) neuron. This P-to-AP synapse is glutamatergic (Wessel et al. 1999) and has both monosynaptic and polysynaptic components (Gu 1991). In addition, NR1, the obligate NMDA subunit, transcript is present in the postsynaptic (AP) neuron (Grey et al. 2009). Previously, NMDAR-dependent potentiation of the P-to-AP synapse was observed following paired activation of the pre- and postsynaptic neurons (Grey et al. 2009) or following forskolin application (“chemical LTP”; Grey and Burrell 2008). Here, the intracellular signaling mechanisms mediating pairing-dependent synaptic plasticity are examined in more detail. LTP was observed when pre- and postsynaptic activity coincided and this potentiation required postsynaptic increases in intracellular Ca\(^{2+}\) and activation of PKA and CaMKII. LTD was observed when postsynaptic activity preceded presynaptic activity and required activation of protein phosphatases. Interestingly, when protein kinase activity was blocked during LTP induction, LTP was prevented and LTD was observed. This “uncovered” form of LTD could itself be blocked by application of the protein phosphatase inhibitor okadaic acid, indicating that LTP-inducing protocols can initiate both LTP and LTD signal cascades that interact with each other.
MATERIALS AND METHODS

Leeches, *Hirudo verbana* (Siddall et al. 2007), weighing 3g were obtained from a commercial supplier (Leeches USA, Westbury, N.Y. or Niagra Medicinal Leeches, Niagra Falls, Canada) and kept in pond water (0.52 g/L $\text{H}_2\text{O}$ *Hirudo* salt [Leeches USA Ltd]) at 15°C, under a 12 hour light/dark cycle. Individual ganglia were dissected and placed in a recording chamber (1mL) with constant superfusion (~1 ml/min). Dissections and recordings were carried out in leech saline containing (mM): 115 NaCl, 4 KCl, 1.8 $\text{CaCl}_2$, 1 MgCl$_2$, and 10 HEPES.

Dual intracellular recordings were made by impaling individual neurons with a glass microelectrode using a micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR). Electrodes were pulled from borosilicate capillary tubing (1.0mm outer diameter, 0.75mm inner diameter; FHC Bowdoinham, ME) to a resistance of 25-35 MΩ and filled with 3M potassium acetate. Signals were amplified with a bridge amplifier (BA-1S; National Precision Instruments, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for viewing and subsequent analysis (Axoscope; Molecular Devices, Sunnyvale, CA). Individual neurons were identified based on their position, size, and action potential shape. Current pulses were delivered to individual neurons using a programmable stimulator (MultiChannel Systems STG 1004). Excitatory postsynaptic potentials (EPSPs) in the AP cell were elicited by brief, 1.5nA, 10 ms current injections into a contralateral P-cell. To prevent the initiation of action potentials, the AP neuron was hyperpolarized to the same membrane potential during both the pre- and posttests (-75mV). Input resistance of the postsynaptic AP cell was measured throughout each experiment by injecting negative currents (0.5nA, 500msec). Typically, 4-6 EPSPs and 7-9 input resistance measurements were averaged per recording.
In all experiments, baseline EPSP amplitude and input resistance measurements were taken in normal saline. NMDAR-dependent synaptic plasticity requires the co-agonist glycine, therefore 1µM glycine was superfused in the bath during pairing sessions (Burrell and Sahley 2004). The pairing protocol was based on studies by Lin and Glanzman (1997) and consisted of 25Hz P cell stimulation (1.5nA for 10ms, 10 pulses) and a simultaneous depolarization of the AP cell (2nA for 500 msec). Paired activation of the P and AP cells was repeated 5 times with an intertrial interval of 2 minutes (0ms ISI, 2min ITI). The pairing protocol was followed by a 45 minute consolidation period in normal saline, followed by a posttest of the P-to-AP EPSP and AP input resistance. A no stimulation control group consisted of 10 minute superfusion of saline + 1µM glycine followed by a 45 minute consolidation period in normal saline, and posttest of the P-to-AP EPSP. Additional control experiments, such as presynaptic stimulation alone and postsynaptic stimulation alone, were performed and reported in an earlier study (Grey et al. 2009). Bath-applied drugs were delivered via superfusion during pairing. Iontophoretically-injected drugs were delivered using 5nA, 100ms negative current pulses for 5 minutes prior to induction of the pairing protocol. For both types of drug treatments, control experiments were performed in the absence of the drug. Across all experiments (350 P-to-AP synapses), the average AP resting potential was approximately -40mV, and the average initial input resistance was 12 ± 0.1 MΩ. EPSP amplitude and input resistance measurements were taken at the conclusion of each 60 min experiment, normalized to their initial values (% of baseline), and presented as the mean ± SE. Cells were excluded if input resistance changed >30% from baseline. In addition, only cells with initial EPSPs <7mV were studied, as synapses >7mV did not potentiate, consistent with observations in both the leech and vertebrates that synapses with
an especially large EPSP amplitude do not potentiate (Burrell and Sahley 2004; Grey and Burrell 2008; Bi and Poo 1998; Montgomery et al. 2001).

Solutions

Bath-applied drugs were dissolved in dH₂O except where noted and superfused in leech saline + 1µM glycine at the following concentrations: autacamide-2-related inhibitory peptide (AIP; 1µM, Tocris, Ellisville, MO); CPA (20µM, Tocris) dissolved in DMSO; 7-Chloro-kynurenic acid (7-Cl-KYNA, 20µM, Tocris); MK-801 (40µM , Sigma); nimodipine (10µM, Tocris) dissolved in methanol; okadaic acid (OkA, 1µM, Sigma) dissolved in DMSO; Rp-cAMP (50µM, Sigma); ryanodine (20µM, Tocris) dissolved in ethanol; TMB-8 (100µM, Sigma) dissolved in DMSO. The following drugs were dissolved in 3M KAc and iontophoresed directly into the cell: BAPTA (1mM, Sigma); botulinum neurotoxin type B light chain (0.5µM, List Biological Laboratories, Campbell, CA); EGTA (1µM-0.5mM, Sigma); SVKI (100µM, Tocris)

Statistics

Statistical tests were conducted using Statistica analysis software (Statsoft). Statistical significance (p < 0.05) was determined using a one-way ANOVA and Tukey’s HSD post hoc comparison for all experiments unless otherwise indicated.
RESULTS

Pairing-induced potentiation requires NMDAR activation

Administration of paired P and AP cell activation resulted in significant potentiation of the P-to-AP EPSP (Pairing, 163 ± 12%, n = 15; Figure 1B and C) compared to no stimulation control synapses (No stimulation, 106 ± 8%, n = 10; p < 0.05). An earlier study of LTP at this connection also demonstrated that no potentiation was observed when only the P or AP cell was activated (Grey et al. 2009). Previous studies have found that both pairing-dependent LTP and forskolin-induced chemical LTP at the P-to-AP synapse were blocked by the competitive NMDAR antagonist AP5 (Grey and Burrell 2008; Grey et al. 2009). To confirm that pairing-induced LTP required NMDAR activation, experiments were carried out in the presence of the NMDAR open channel blocker MK801 (40µM). The amino acids required for MK801 binding are conserved in the leech (Grey et al. 2009), and MK801 has been shown to block currents in C. elegans (Brockie et al. 2001). MK801 blocked the potentiation when applied during pairing (Pairing + MK801, 108 ± 8%, n = 6; p < 0.05; Figure 1C) without an effect on baseline synaptic transmission when applied without pairing (MK801 control, 96 ± 8%, n = 9; F(3,27) = 11.598, p < 0.001; Figure 1C). No change in input resistance was observed between pairing and no pairing synapses (p > 0.05).

Glycine is an obligatory co-agonist for NMDAR function and testing glycine-dependence of P-to-AP LTP would provide further support for NMDAR involvement. In previous experiments at the P-to-S synapse, glycine was required during induction of LTP (Burrell and Sahley 2004). Therefore to test whether P-to-AP plasticity showed the same glycine dependence, glycine was omitted from the saline superfusion during the P + AP pairing protocol, and this resulted in no observed potentiation (Pairing, no glycine, 104 ± 5%, n = 5; p < 0.05;
Figure 1D). In a second set of experiments, the pairing protocol (with 1µM glycine) was administered in the presence of 7-Chlorokynurenic acid (7-Cl-KYNA, 20µM), an inhibitor of the NMDAR glycine-binding site. 7-Cl-KYNA blocked pairing-induced potentiation (Pairing + 7-CL KYNA, 97 ± 9%; n = 8; p < 0.05), but did not affect basal synaptic transmission when 7-Cl-KYNA was applied without P + AP pairing (7-Cl-KYNA control, 105 ± 12%, n = 11; F(4,30) = 8.034, p < 0.001; Figure 1D; 87 total experiments for Figure 1). Input resistance was not significantly different between treatments (p > 0.05).

Pairing-induced potentiation requires an increase in postsynaptic intracellular Ca$^{2+}$

In both mammals and the marine mollusc *Aplysia*, increases in postsynaptic Ca$^{2+}$ are necessary for the expression of LTP (Lynch et al. 1983; Murphy and Glanzman 1996), presumably due to Ca$^{2+}$ influx through NMDARs. To determine whether pairing-induced potentiation requires postsynaptic increases in intracellular Ca$^{2+}$ in the leech, the Ca$^{2+}$ chelator BAPTA (1mM) was iontophoretically injected into the AP (postsynaptic) cell for 5 minutes prior to the administration of the pairing protocol. Previous studies indicate that BAPTA reaches synaptic regions of leech neurons within this period of time (Grey and Burrell 2008). Injection of BAPTA into the postsynaptic AP cell before pairing blocked LTP (AP BAPTA + Pairing, 85 ± 9%, n = 5; p < 0.05; Figure 2A), indicating that this potentiation depends on an increase in postsynaptic intracellular Ca$^{2+}$. Injection of BAPTA into the postsynaptic cell alone did not alter baseline synaptic transmission (AP BATPA control, 93 ± 7%, n = 5, p < 0.05; F(2,16) = 30.156, p < 0.001) and there was no significant change in input resistance for any of the BAPTA groups (p > 0.05).
The role of presynaptic Ca\textsuperscript{2+} during pairing-induced LTP was also examined. Another Ca\textsuperscript{2+} chelator, EGTA, was used to assess Ca\textsuperscript{2+} increases in the P cell, since this chelator has slower kinetics than BAPTA (Nevian and Sakmann 2006) and therefore would be less likely to disrupt synaptic transmission. Nevertheless, presynaptic EGTA did substantially reduce basal P-to-AP synaptic transmission, even at concentrations as low as 1µM (Figure 2B; P-to-AP EPSP tested 5 minutes following EGTA injection), consistent with observations by Ivanov and Calabrese (2006). Despite this reduction in synaptic transmission, significant potentiation was observed in synapses that underwent pairing after EGTA injection compared to synapses that were treated with EGTA but did not undergo pairing (EGTA + Pairing, 124 ± 10%, n = 4; EGTA control, 84 ± 10%, n = 7; p < 0.05; F(1,9) = 6.882, p = 0.028; Figure 2C) even though the level of potentiation in the EGTA-treated synapses was reduced compared to LTP in untreated ganglia (compare black bars in Figure 2A and 2C). One interpretation of these results is that EGTA reduced basal synaptic transmission, but LTP was unaffected and the reduction of potentiation is simply a consequence of EGTA’s effect on synaptic transmission. Support for this explanation comes from a previous study of the P-to-AP synapse in which NMDAR-dependent LTP was induced by forskolin application and not by activation of P and AP neurons. In those experiments, presynaptic injection of Ca\textsuperscript{2+} chelators did not affect this chemical LTP, supporting the idea that potentiation did not involve increases in presynaptic Ca\textsuperscript{2+} levels (Grey and Burrell 2008).

In addition to NMDARs, other sources of Ca\textsuperscript{2+} may also contribute to the increase in postsynaptic Ca\textsuperscript{2+} seen in this pairing-induced potentiation, such as voltage-gated Ca\textsuperscript{2+} channels (VGCCs) and release of Ca\textsuperscript{2+} from internal stores, the latter potentially mediated by either IP\textsubscript{3} or ryanodine receptors (Kapur et al. 1998; Balschun et al. 1999; Vargas et al. 2007). To test
whether LTP requires VGCC activation, nimodipine (10µM) was superfused during administration of the pairing protocol. Dihydropyridines such as nimodipine and nifedipine, have been reported to be effective in invertebrates (Elliot et al. 1993; Dierkes et al. 2004; but see Kleinhaus & Angstadt 1995). Since VGCCs are located both pre- and postsynaptically, application of nimodipine could potentially interfere with synaptic transmission during administration of the pairing protocol. In order to test this possibility, the P-to-AP EPSP was tested directly after 10 minutes of nimodipine application and was not found to affect the P-to-AP EPSP (100 ± 3%, n = 3, data not shown). Application of nimodipine during pairing blocked potentiation without affecting baseline synaptic transmission or input resistance (Nimodipine + Pairing, 88 ± 2%, n = 5; Nimodipine control, 93 ± 6%, n = 6; p < 0.05; input resistance p > 0.05; Figure 2D). Nimodipine was dissolved in methanol, yielding a final methanol concentration of 0.1% v/v. As a vehicle control for nimodipine, 0.1% v/v methanol in saline was applied during pairing, but was not found to disrupt LTP in this synapse (0.1% MeOH + Pairing, 164 ± 11%, n = 11; p < 0.05; F(3,24) = 17.043, p < 0.001; Figure 2D).

The role of Ca\(^{2+}\) release from intracellular stores during LTP was tested by three different drugs. First, cyclopiazonic acid (CPA) inhibits the Ca\(^{2+}\)-pump, thereby depleting internal Ca\(^{2+}\) stores and has been shown to be effective in both Hirudo and Helix neurons (Beck et al. 2001; Willoughby et al. 2001). Superfusion of CPA (20µM) during P + AP pairing blocked LTP (CPA + Pairing, 106 ± 9%, n = 8; p < 0.05), while CPA application in the absence of pairing did not affect baseline synaptic transmission (CPA control, 93 ± 7%, n = 5; p < 0.05; Figure 2E).

Second, to test the role of IP\(_3\)-receptor mediated store-released Ca\(^{2+}\), the pairing protocol was administered in the presence of TMB-8, which has been shown to inhibit IP\(_3\)-induced Ca\(^{2+}\) release in sea urchin eggs (Clapper and Lee 1985). Application of TMB-8 (100µM) blocked
pairing-induced potentiation without affecting baseline synaptic transmission (TMB-8 + Pairing, 102 ± 8%, n = 8; TMB-8 control, 98 ± 9%, n = 5; p < 0.05; Figure 2E). Third, the role of ryanodine receptor (RyR)–mediated release from intracellular Ca\textsuperscript{2+} stores was also examined. Ryanodine, at micromolar concentrations (Sutko et al. 1997), is a selective inhibitor for RyRs and has been shown to be effective in a variety of invertebrates, including *Aplysia*, *Hirudo* and *Drosophila* (Xu et al. 2000; Trueta et al. 2004, Geiger and Magoski 2008). Application of ryanodine (50µM) in conjunction with the pairing protocol blocked LTP (Ryanodine + Pairing, 102 ± 9%, n = 10; Ryanodine control, 93 ± 9%, n = 11; p < 0.05; F(8,68) = 10.898, p < 0.001; Figure 2E) indicating that RyR-mediated Ca\textsuperscript{2+} release is necessary for pairing-induced potentiation of the P-to-AP synapse. Neither DMSO (0.2% v/v), which was used as a solvent for both CPA and TMB-8, nor ethanol (0.5% v/v), the solvent for ryanodine, significantly affected LTP (saline (Pairing), 163 ± 12%, n = 15; DMSO + Pairing, 153 ± 8%, n = 16; Ethanol + Pairing, 153 ± 7%, n = 9; both vehicle controls p > 0.05 compared to saline (Pairing); Figure 2E). There was no significant change in input resistance for any of the Ca\textsuperscript{2+} drug groups (p > 0.05; n = 146 total experiments shown in Figure 2). These results indicate that IP\textsubscript{3}-receptor and RyR-mediated release of Ca\textsuperscript{2+} from intracellular stores contribute to pairing-dependent LTP in the leech. It is possible that these processes contribute to postsynaptic increases in intracellular Ca\textsuperscript{2+}, but additional experiments using injectable, membrane-impermeant blockers of these receptors are needed to confirm this conclusion.

The role of CaMKII and PKA during pairing-induced LTP

Given the requirement for Ca\textsuperscript{2+} signaling during P-to-AP LTP, the role of Ca\textsuperscript{2+}-activated biochemical pathways known to mediate LTP in vertebrates, specifically calmodulin kinase II
(CaMKII) and protein kinase A (PKA), was investigated (Blitzer et al. 1998; Miyamoto 2006; Yang et al. 2004; Zheng & Keifer 2009). The specific CaMKII inhibitor, AIP, has been shown to block both vertebrate LTP (Yang et al. 2004) and forskolin-induced chemical LTP in the leech (Grey and Burrell 2008). Application of AIP (0.1µM) during the pairing protocol blocked pairing-induced potentiation in the P-to-AP synapse (Figures 3A and 3B). Interestingly, AIP treatment during pairing resulted in significant depression of the P-to-AP EPSP (AIP + Pairing, 57 ± 5%, n = 6, p < 0.05; Figure 3B). This was observed only when AIP and pairing were combined and was not the result of non-specific effects of AIP on P-to-AP synaptic transmission. The AIP + pairing group was significantly different from both the pairing group in normal saline and control groups in which AIP was applied but pairing was omitted (AIP control, 101 ± 5%, n = 7, p < 0.05; F(4,29) = 22.751, p < 0.001; Figure 3B). In addition, no changes were observed in input resistance as a result of AIP treatment (p > 0.05). This apparent unmasking of synaptic depression in the pairing + AIP group was also seen when AIP was applied during forskolin-induced chemical LTP in the leech (Grey and Burrell 2008).

What is the explanation for this observed depression in AIP + pairing experiments? One possibility is that the molecular signaling pathways associated with both synaptic potentiation and depression are activated by the pairing protocol, but functional expression of synaptic depression is suppressed by CaMKII activity. Protein phosphatases are known to mediate NMDAR-dependent LTD in vertebrates (Anwyl 2006) and are also required for NMDAR-dependent LTD in synapses made by the leech T-to-S synapse (Li and Burrell 2009). In addition, modeling studies by Pi and Lisman (2008) have shown that interactions between protein phosphatase 2A and CaMKII can play a critical role in determining whether synapses express LTP or LTD. Therefore it is possible that the depression observed AIP + pairing
synapses could be prevented by blocking the activity of protein phosphatases. To test this hypothesis, okadaic acid (1µM; OkA), which inhibits protein phosphatase 1 and 2A, was co-applied with AIP during pairing. The biochemical properties of protein phosphatases have been characterized in *Aplysia*, showing similar characteristics to vertebrate protein phosphatases (Endo et al. 1992). Furthermore, OkA has been used to block NMDAR-dependent long-term habituation in *Aplysia* and LTD at the leech T-to-S synapse (Ezzeddine and Glanzman 2003; Li and Burrell 2009). Co-application of OkA with AIP during pairing blocked the depression observed in AIP + pairing synapses (AIP + OkA + Pairing, 123 ± 8%, n = 8; AIP + OkA control: 100 ± 11%, n = 7; p < 0.05; F(4,29) = 22.751, p < 0.001; Figure 3B) with no change in input resistance (p > 0.05). While a modest level of potentiation was observed in AIP + OkA + pairing synapses, the percent change in EPSP was not statistically different from control preparations that did not undergo pairing.

The potential role of PKA in LTP in the leech was examined using Rp-cAMP, a competitive inhibitor of PKA previously used to block PKA-dependent neuromodulation both in *Aplysia* and the leech (Hochner and Kandel 1992; Burrell and Sahley 2005; Grey and Burrell 2008). Application of Rp-cAMP (50µM) during pairing (Rp-cAMP + pairing) blocked LTP and produced depression of the P-to-AP EPSP, similar to the AIP + pairing experiments (Figure 3B and 3C). The level of depression was not statistically significant compared to Rp-cAMP control synapses when analyzed by a one-way ANOVA (Rp-cAMP + Pairing, 69 ± 6%, n = 15; p = 0.128; Figure 3C), but a significant difference was observed between the Rp-cAMP control and Rp-cAMP + pairing groups when analyzed by an independent t-test (p = 0.008, t = 2.864, d.f. = 25). Rp-cAMP alone did not alter P-to-AP synaptic transmission (Rp-cAMP control, 95 ± 6%, n = 12; p < 0.05), indicating that the depression observed in the Rp-cAMP + pairing group was not
due to Rp-cAMP acting on basal synaptic transmission. Since the observed depression is similar to the results observed when CaMKII inhibition was combined with pairing (Figure 3B), the effects of combining OkA with Rp-cAMP + pairing were examined. Co-application of OkA and Rp-cAMP during pairing produced synaptic transmission similar to baseline levels (Rp-cAMP + OkA + Pairing, 96 ± 9%, n = 9; non-significant trend by ANOVA; Figure 3B and 3C). When the differences between the Rp-cAMP + pairing and Rp-cAMP + OkA + pairing groups were analyzed by an independent t-test, this revealed an apparent reversal of the LTD observed in the Rp-cAMP + pairing by OkA (p < 0.05). Rp-cAMP + OkA did not affect baseline synaptic transmission (Rp-cAMP + OkA control, 100 ± 10%, n = 14; p < 0.05; F(4,51) = 12.252, P < 0.001; Figure 3C). No changes in input resistance were observed between drug-treated and control groups (p > 0.05; 93 total experiments for Figure 3). These results suggest pairing-induced potentiation requires activation of the CaMKII and PKA signaling pathways and that these pathways interact with and likely suppress protein phosphatase activity.

**Negative pairing can produce NMDAR-dependent LTD**

In all of the experiments described so far, LTP has been induced by the simultaneous activation of the pre- (P cell) and postsynaptic (AP cell) neurons. Since alterations in the temporal order of pre- and postsynaptic stimulation can change the polarity of synaptic plasticity (Bi and Poo 1998), the effects of different intervals between the presynaptic and postsynaptic stimulation during pairing were examined. Specifically, experiments were conducted in which presynaptic stimulation preceded postsynaptic stimulation by 500 ms, 1 second, and 10 seconds, and postsynaptic preceded presynaptic stimulation by 500 ms, 1 second, and 10 seconds (referred to as negative intervals). No significant changes in the P-to-AP EPSP were observed at any of
the additional intervals tested except when postsynaptic (AP) stimulation preceded presynaptic
(P) stimulation by 1 second, which produced significant depression of synaptic transmission (-1 second, post-before-pre, 58 ± 3%, n = 9; p < 0.05 compared to the no stimulation control (not shown in figure); F(7,52) = 7.255, p < 0.001; Figure 4A and 4B). To investigate whether this form of depression was NMDAR-dependent, MK801 (40µM) was applied during administration of the -1 second pairing protocol. MK801 blocked -1 second pairing-induced LTD, demonstrating this form of synaptic depression is NMDAR-dependent (MK801 + -1 sec ISI, 93 ± 6%, n = 6; p < 0.05; F(3,30) = 10.233, p < 0.001; Figure 4C). To assess whether protein phosphatase activation is required for LTD, OkA was applied during -1 second pairing. OkA (1µM) blocked -1 second pairing-induced LTD (OkA + -1 sec ISI, 96 ± 6%, n = 7; p < 0.05), despite significantly depressing baseline synaptic transmission (OkA control, 80 ± 7%, n = 7; F(3,29) = 11.592, p < 0.001; Figure 4D).

**Glutamate receptor trafficking**

Insertion of AMPA-type glutamate receptors into the postsynaptic membrane is thought to be a critical component of LTP expression (Kessels and Malinow 2009). There are no known antibodies that recognize AMPA receptors in the leech; therefore, an alternative approach was employed. Botulinum toxin type B (BTX-B) cleaves SNARE proteins necessary for exocytosis (Montecucco and Schiavo 1995), including those in the leech (Bruns et al. 1997). BTX-B injections inhibit synaptic potentiation, presumably by blocking insertion of glutamate receptors into the postsynaptic membrane and BTX-B injections have been used to inhibit synaptic potentiation in both vertebrates and invertebrates (Chitwood et al. 2001; Jin & Hawkins 2003; Li et al. 2005; Antonov et al. 2007; Frey et al. 2009). Iontophoresis of BTX-B into the postsynaptic
AP-cell prevented pairing-induced LTP (BTX-B + Pairing, 104 ± 6%, n = 6; p < 0.05; Figure 5), while postsynaptic BTX-B treatment by itself did not affect synaptic transmission (BTX-B control, 109 ± 9%, n = 5; p < 0.05; F(3,23) = 11.37, p<0.001) or input resistance (p > 0.05; n = 26 total experiments shown in Figure 5). These results are consistent with the hypothesis that pairing-induced potentiation in the glutamatergic P-to-AP synapse requires the postsynaptic insertion of glutamate receptors, although the possibility that BTX-B is blocking an alternative postsynaptic exocytotic event necessary for LTP cannot be excluded.

Removal of glutamate receptors from the postsynaptic membrane is thought to underlie LTD (Lüscher et al. 1999; Beattie et al. 2000). In order to test whether inhibition of glutamate receptor removal would block LTD in the leech, -1 second pairing was conducted in the presence of SVKI, a peptide that inhibits the endocytosis of AMPA receptors by blocking interactions between the GluR2 subunit and scaffolding proteins (Daw et al. 2000). Invertebrate AMPA-type glutamate receptors do contain PDZ-binding domains that are responsible for interactions with scaffolding proteins and the mechanisms involved in glutamate receptor trafficking appear to be conserved between vertebrates and invertebrates (Chang and Rongo, 2005; Walker et al., 2006). Furthermore, SVKI inhibited synaptic depression in leech T-to-T synapses (Li and Burrell 2008).

Iontophoresis of SVKI (100µM) into the postsynaptic AP cell during -1 second pairing blocked LTD (SVKI + Pairing, 97 ± 8%, n = 5; p < 0.05; Figure 5), but did not affect basal synaptic transmission (SVKI control, 100 ± 7%, n = 5; p < 0.05; F(3,25) = 12.44, p < 0.001; Figure 5) or input resistance (p > 0.05; n = 102 total experiments shown in Figure 5). These results are consistent with the hypothesis that LTD in the P-to-AP synapse requires the removal of glutamate receptors from the postsynaptic membrane.
Paired activation of presynaptic and postsynaptic spike trains can elicit NMDAR-dependent, bidirectional plasticity in a leech glutamatergic synapse. The pattern of plasticity in the present experiments is similar to what others have observed following pairing of pre- and postsynaptic trains of action potentials in both vertebrates and invertebrates (Lin and Glanzman 1997; Butts et al. 2007). Specifically, pairing of bursts resulted in potentiation only when pre- and post stimulation occurred simultaneously (0ms ISI); when pre- and post bursts were desynchronized, little or no synaptic plasticity was observed. One difference from these earlier findings is that in the present experiments post-before-pre pairing (that is, AP before P) of one second elicits robust synaptic depression.

P-to-AP LTP was blocked by the NMDAR antagonist MK801 and by inhibition of the NMDAR glycine binding site, either through application of 7-Cl-KYNA, which blocks the NMDAR glycine binding site, or by omitting glycine during pairing. These findings confirm earlier reports that LTP in the leech P-to-S and P-to-AP synapses was NMDAR-dependent, based on the inhibitory effect of the NMDAR competitive antagonist AP5 (Burrell and Sahley 2004; Grey et al. 2009). LTP in the leech required postsynaptic increases in intracellular Ca$^{2+}$, given that postsynaptic injection of BAPTA prevented pairing-induced potentiation. The results of the presynaptic (P cell) EGTA injections suggest that increases in presynaptic Ca$^{2+}$ are not required for pairing-induced LTP, consistent with previous experiments in which increases in presynaptic Ca$^{2+}$ were not required for NMDAR-dependent LTP induced by forskolin (Grey and Burrell 2009). In addition to NMDARs, increases in intracellular Ca$^{2+}$ appear to require VGCC activation; release of Ca$^{2+}$ from internal stores, mediated by both ryanodine and IP$_3$ receptors may contribute to LTP induction as well. PKA and CaMKII activity is also required for LTP at this synapse, as is the insertion of additional glutamate receptors into the postsynaptic
membrane. A schematic of the cellular mediators of burst-pairing LTP and LTD explored here is summarized in Figure 6.

LTD at the P-to-AP synapse was elicited when postsynaptic activity preceded presynaptic activity by 1 second. In addition, P-to-AP LTD was NMDAR-dependent, required protein phosphatase activation and endocytosis of postsynaptic glutamate receptors. These properties have been observed in LTD at other synapses in the leech CNS, indicating that this form of synaptic depression is not limited to the P-to-AP synapse (Burrell and Sahley 2004; Li and Burrell 2008, 2009). These findings indicate that synapses in the leech have the capacity for bidirectional synaptic plasticity.

The evidence for glutamate receptor trafficking during LTP and LTD in the P-to-AP synapse is, admittedly, indirect given that there are no tools to directly label leech glutamate receptors. The ability of BTX-B to block LTP has been used as evidence for glutamate receptor insertion in both vertebrates and invertebrates (Chitwood et al. 2001; Jin & Hawkins 2003; Li et al. 2005; Antonov et al. 2007; Frey et al. 2009). However, it is also possible that BTX-B prevents potentiation via alternative processes, such as blocking the postsynaptic release of a retrograde transmitter necessary for LTP, although no evidence of such a retrograde signaling mechanism has yet been reported. Glutamate receptor removal from the postsynaptic membrane contributes to P-to-AP LTD, as iontophoresis of SVKI blocked depression following -1 second pairing of P and AP activity. SVKI selectively inhibits endocytosis of AMPA receptors, and has been shown to prevent LTD in vertebrate synapses, by disrupting the GluR2 subunit interaction with glutamate receptor interacting protein (GRIP), AMPA receptor binding protein (ABP), and protein interacting with C kinase (PICK1) at the GluR2 PDZ-binding domain. Although it is not known precisely what AMPA receptor subtype is trafficked in these leech synapses, the SVKI
target sequence is found in invertebrate glutamate receptors and the mechanisms involved in glutamate receptor trafficking appear to be largely conserved between vertebrates and invertebrates (Chang and Rongo 2005; Walker et al. 2006). In addition, both SVKI and general inhibitors of endocytosis (dyanmin inhibitory peptide and concanavalin A) have been found to inhibit LTD in the synapse between touch mechanosensory neurons in the leech (Li and Burrell 2008).

One of the most interesting findings from these experiments is that blocking a signaling pathway that mediates LTP (CaMKII) unmasked LTD in this synapse. These results address a fundamental issue concerning NMDAR-mediated synaptic plasticity. How are NMDAR/ Ca\(^{2+}\) signals able to generate both synaptic potentiation and depression? Recent studies have suggested that this bidirectional plasticity relies on cellular switches that change the balance between potentiating and depressing processes to determine the polarity of synaptic plasticity (Nishiyama et al. 2000; Graupner and Brunel 2007; Van Woerden et al. 2009). Specifically, changes in cytosolic Ca\(^{2+}\) levels, mediated by NMDARs and intracellular Ca\(^{2+}\) stores, are sufficient to control both the strength and direction of synaptic plasticity (Bi and Poo 1998). These different cytosolic Ca\(^{2+}\) levels activate kinases (which contribute to LTP) and phosphatases (which contribute to LTD) to differing degrees and current modeling studies indicate that the signaling molecule that is more strongly activated inhibits the less-activated molecule (Zhabotinski 2000; Graupner and Brunel 2007; Pi and Lisman 2008). For example, phosphatases that mediate LTD are more sensitive to Ca\(^{2+}\) than kinases that mediate LTP (such as CaMKII), thus lower levels of intracellular Ca\(^{2+}\) would preferentially activate protein phosphates, which would in turn actively inhibit protein kinases leading to LTD. When intracellular Ca\(^{2+}\) levels are sufficiently high, both the kinases that mediate LTP and the
phosphatases that mediate LTD are presumably activated, yet LTP ‘wins out’ because the
kinases that mediate potentiation also actively inhibit the phosphatases/LTD pathway.

Phosphorylation, particularly autophosphorylation, of CaMKII inhibits protein phosphatase
activation and has been shown to be critical to initiate LTP (Giese et al. 1998; Fukunaga et al.
2000). Conversely, active protein phosphatases can dephosphorylate CaMKII as well as initiate
processes necessary for LTD (Mulkey et al. 1993; Yoshimura et al. 1999). In addition to
CaMKII, PKA also inhibits protein phosphatases, specifically protein phosphatase 1, and by this
mechanism PKA is said to “gate” the induction of LTP (Blitzer et al. 1998). The data from both
the CaMKII (AIP) and the PKA (Rp-cAMP) experiments presented in this study support this
hypothesis. Inhibition of CaMKII or PKA during pairing not only blocked potentiation, but also
revealed a form synaptic depression in the P-to-AP connection. This unmasked depression was
blocked when the protein phosphatase inhibitor okadaic acid was applied.

The findings from this study have a number of important implications in terms of
evolution and phylogenetic relationship of synaptic function and plasticity between vertebrates
and invertebrates. It is well established that invertebrate nervous systems possess many of the
molecules necessary for activity-dependent synaptic plasticity in vertebrates (e.g. NMDAR
subunits, CaMKII, protein phosphatases; also see Moroz et al. 2006), but there have been
relatively few detailed studies of the cellular properties of invertebrate LTP and LTD.
Furthermore, bioinformatic studies comparing the complement of synaptic proteins in vertebrates
and invertebrates have suggested a reduced capacity for synaptic plasticity in invertebrates (Ryan
et al. 2008; Ryan and Grant 2009). The present results demonstrate that, as in vertebrates,
invertebrate synapses are capable of bidirectional modification governed by the relative
activation of the same compliment of protein kinases and phosphatases that regulate bidirectional
synaptic plasticity in vertebrates. These findings indicate that a number of fundamental
dprocesses of activity-dependent synaptic plasticity are conserved between in invertebrates and
are likely to play a functional role in processes of learning and memory, development and
sensory processing.

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**FIGURE LEGENDS**

**Figure 1.** Paired pre- and postsynaptic activity induces potentiation at the P-to-AP synapse. 

A: Schematic of the pairing protocol. Pre- and posttest EPSP measurements in the postsynaptic AP cell were obtained by eliciting a single presynaptic (P cell) action potential. Pairing consisted of a 10 pulse train (25 Hz, 10msec) applied to the P-cell coinciding with a 2nA step depolarization (500 msec) of the AP cell. Posttest EPSP measurements were completed after a 45 minute consolidation period. 

B: Representative EPSP traces prior to (pre) and following pairing (post) from synapses that underwent pairing in normal saline (top pair of traces) or in MK801 (bottom pair of traces). The gray trace denotes the pretest EPSP and the black trace denotes the posttest EPSP. 

C: Effects of the NMDAR antagonist, MK801, on LTP. Pairing-induced LTP was blocked by the application of the NMDAR antagonist MK801 (Pairing + MK801). No stimulation and MK801 control groups are significantly different than the pairing group, indicating that potentiation only occurs following coordinated activation of the pre- postsynaptic neurons. 

D: Role of glycine during LTP. Elimination of glycine from the bath during the training protocol (Pairing, no glycine) or application of 7-Chlorokynurenic acid (Pairing + 7-Cl KYNA), which blocks the NMDAR glycine binding site, prevented pairing-induced potentiation. Administration of 7-Cl KYNA alone (7-Cl-KYNA control) did not significantly affect baseline synaptic transmission. * indicates statistically significant difference relative to the pairing group.

**Figure 2.** Role of multiple sources of postsynaptic Ca\(^{2+}\) during pairing-induced LTP. 

A: Effects of postsynaptic BAPTA injection on LTP. No potentiation was observed when BAPTA was injected into the postsynaptic (AP) cell before administration of the pairing protocol (AP
BAPTA + Pairing). Postsynaptic injection of BAPTA alone did not affect baseline synaptic transmission (AP BAPTA control). B: Effects of presynaptic EGTA injection on the P-to-AP EPSP. After 5 minutes of iontophoretically injected EGTA into the presynaptic (P) cell, evoked P-to-AP synaptic transmission decreased, on average, 50% from pre-EGTA treatment levels. (Top) Representative EPSP traces from a P-to-AP synapse treated with 1µM EGTA. The posttest (Post, gray trace) was conducted 1 hour after the pretest (Pre, black trace). (Bottom) Averaged change in EPSP size following presynaptic EGTA treatment for concentrations between 1µM to 0.5mM. C: Effects of presynaptic EGTA injection on LTP. Iontophoresis of 0.5mM EGTA into the presynaptic (P cell) before administration of the pairing protocol produced potentiation relative to the EGTA control (EGTA treatment, but no pairing). D: Effects of nimodipine of LTP. Nimodipine blocked pairing-induced potentiation (Nimodipine + Pairing). Methanol, the vehicle control for nimodipine, did not affect pairing-induced potentiation (0.1% v/v Methanol + Pairing), and nimodipine administration alone did not affect baseline synaptic transmission (Nimodipine control). E: Effects of inhibition of release from intracellular Ca²⁺ stores on LTP. Inhibition of Ca²⁺ release from intracellular stores also contributed to pairing-induced LTP, which was blocked by depletion of Ca²⁺ stores by CPA, inhibition of IP₃ receptors by TMB-8, or by inhibition of ryanodine receptors by ryanodine. The vehicle controls, DMSO for CPA and TMB-8 and ethanol for ryanodine, did not affect potentiation. * indicates statistically significant difference relative to the pairing group.

Figure 3. Effects of CaMKII and PKA on pairing-induced LTP. A: Representative traces showing synaptic depression in the AIP + pairing group (left). No depression was observed when okadaic acid (OkA) was applied in addition to AIP + pairing (AIP + OkA + pairing; right).
Application of the CaMKII inhibitor, AIP, in conjunction with the pairing protocol significantly depressed the P-to-AP EPSP, as measured by a one-way ANOVA (see Results), compared to the AIP control group where no change in EPSP amplitude was observed. Application of OkA in conjunction with AIP + pairing prevented this depression (AIP + OkA + Pairing). Application of the PKA inhibitor, Rp-cAMP, induced depression (#, p < 0.05) compared to Rp-cAMP controls, as analyzed by an independent t-test. Similar to experiments involving inhibition of CaMKII, OkA combined with Rp-cAMP and pairing (Rp-cAMP + OkA + pairing) prevented this depression. * indicates statistically significant difference relative to the pairing group.

**Figure 4.** Effects of the alteration of temporal order of paired P and AP cell activation on synaptic plasticity. A: Shifting the relative onset between pre- and postsynaptic stimulation produces two windows of plasticity, one at the 0ms ISI time point that resulted in potentiation and a second at the -1 sec ISI that resulted in depression, relative to the no stimulation control group (not shown). B: Representative pre- and postsynaptic EPSP traces from synapses that have undergone coincident pairing (0ms ISI) resulting in LTP and from synapses in which AP activity preceded P cell activity (-1sec ISI) resulting in LTD. C: The LTD produced by -1 sec ISI protocol was blocked by the NMDAR antagonist MK801. D: Application of OkA during negative pairing blocked depression. OkA by itself yields a slightly depressed baseline compared to no stimulation controls. * indicate statistically significant difference relative to the no stimulation control group.
Figure 5. Effects of inhibition of postsynaptic receptor trafficking on pairing-induced plasticity. Iontophoresis of BTX-B, which is thought to prevent exocytosis of glutamate receptors into the postsynaptic membrane, into the postsynaptic (AP) cell prior to training completely blocked pairing-induced potentiation. BTX-B iontophoresis into the AP cell by itself did not affect baseline synaptic transmission. Iontophoresis of SVKI, which inhibits endocytosis of AMPA-type glutamate receptors, into the postsynaptic cell prior to administration of the negative pairing protocol blocked pairing-induced LTD. Postsynaptic SVKI treatment without pairing did not affect synaptic transmission.

Figure 6. Model of cellular events in pairing-induced LTP and LTD in the leech. This schematic represents the mechanisms of pairing-induced NMDAR-dependent LTP and LTD examined in this paper. Solid lines represent pathways of pharmacological manipulation reported in this paper, and dashed pathways represent likely events based on evidence reported in the literature. Arrows indicate an activation or increase of the molecule, and a T-junction indicates inhibition of the molecule. Activation of the NMDAR allows for Ca$^{2+}$ into the postsynaptic cell, which activates PKA, CaMKII, and protein phosphatases. CaMKII and PPs mutually inhibit each other, and PKA can inhibit PPs. Activation of CaMKII and protein phosphatases have been shown to promote AMPA receptor trafficking, correspondingly inserting and removing receptors from the postsynaptic membrane (Kakegawa et al. 2004).