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ORIGINAL PAPER

Two forms of long-term depression in a polysynaptic pathway in the leech CNS: one NMDA receptor-dependent and the other cannabinoid-dependent

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Abstract Although long-term depression (LTD) is a well-studied form of synaptic plasticity, it is clear that multiple cellular mechanisms are involved in its induction. In the leech, LTD is observed in a polysynaptic connection between touch mechanosensory neurons (T cells) and the S interneuron following low frequency stimulation. LTD elicited by 450 s low frequency stimulation was blocked by *N*-methyl-D-aspartic acid (NMDA) receptor antagonists. However, LTD elicited by 900 s low frequency stimulation was insensitive to NMDA receptor antagonists and was instead dependent on cannabinoid signaling. This LTD was blocked by both a cannabinoid receptor antagonist and by inhibition of diacylglycerol lipase, which is necessary for the synthesis of the cannabinoid transmitter 2-arachidonyl glycerol (2-AG). Bath application of 2-AG or the cannabinoid receptor agonist CP55 940 also induced LTD at this synapse. These results indicate that two forms of LTD coexist at the leech T-to-S polysynaptic pathway: one that is NMDA receptor-dependent and another that is cannabinoid-dependent and that activation of either form of LTD is dependent on the level of activity in this circuit.

Keywords Long-term depression · NMDA receptor · Cannabinoid · Leech · Synaptic plasticity

Abbreviations

2-AG	2-Arachidonyl glycerol
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AM251	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide
ANOVA	Analysis of variance
AP5	2-Amino-5-phosphonopentanoic acid
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
EPSP	Excitatory post-synaptic potential
LFS	Low frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
mGluR	Metabotropic glutamate receptor
MK801	(5R,10S)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine
NMDAR	*N*-methyl-D-aspartic acid receptor

Introduction

Activity-dependent changes in synaptic transmission, such as long-term potentiation (LTP) and long-term depression (LTD), are critical mechanisms for modifying neural circuits during experience-dependent changes in behavior (Bear and Abraham 1996; Linden 1994). A complicating factor in studying the functional role of LTP and LTD is that both are mediated by a variety of different cellular mechanisms (Malenka and Bear 2004; Anwyl 2006). Given that invertebrates are particularly amenable to linking changes in cellular processes to changes in behavior, studies of LTP and LTD in such animals may greatly facilitate our understanding of the functional role of activity-dependent synaptic plasticity.

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The leech (*Hirudo*) has a well-characterized central nervous system (CNS) that permits recording from the same, identifiable neurons from one preparation to the next, making it useful for studying the mechanisms and functions of neuroplasticity. The polysynaptic connection between the mechanosensory touch (T) cells and the S interneuron (the "S" refers to the large action potential or spike this interneuron generates; Frank et al. 1975) exhibits a number of interesting forms of synaptic plasticity including a homosynaptic form of LTP that is metabotropic glutamate receptor (mGluR)-dependent and a heterosynaptic form of LTD that is NMDA receptor (NMDAR)-dependent (Burrell and Sahley 2004; Burrell and Li 2008). The properties of homosynaptic LTD, however, are not known. Understanding the mechanisms regulating T-to-S synaptic plasticity is of interest because the S cell is thought to be critical for behavioral arousal and certain types of learning in the leech (Sahley et al. 1994; Modney et al. 1997; Burrell et al. 2003; Kristan et al. 2005).

In this study, we examined whether homosynaptic LTD could be induced using a low frequency stimulation protocol that is frequently used in vertebrate studies of LTD. Two distinct forms of LTD were observed at the T-to-S connection: one that is NMDAR-dependent and another that is cannabinoid-dependent. Which LTD-inducing cellular mechanism is activated appears to depend on the level of synaptic activity; LTD elicited by 450 s low frequency stimulation is NMDAR-dependent and LTD elicited by 900 s low frequency stimulation is cannabinoid-dependent.

Materials and methods

Animal preparation

Leeches (3 g) were obtained from a commercial supplier (Leeches USA, Westbury, NY) and kept in pond water [0.52 g/l H_2O Hirudo salt (Leeches USA Ltd.)] on a 12 h light/dark daily cycle at 18°C. During dissection, individual midbody ganglia were removed from the animal and placed in a recording chamber (1.5 ml) with constant perfusion (~1.5 ml/min). The dissections and recordings were carried out in leech saline containing (in mM): 115 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES (pH = 7.4). Stock solutions of 2-amino-5-phosphonopentanoic acid (AP5; Sigma) and (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]cyclohepten-5,10-imine (MK801; Tocris) were made in water. 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-pyrazole-3-carboxamide (AM251), 2 arachidononoyl glycerol (2-AG), CP55 940, okadaic acid and cyclosporin A (Tocris) were dissolved in dimethyl sulfoxide (DMSO). Final concentrations were prepared in saline and made just prior to the start of individual experiments. Final DMSO concentrations for AM251, 2-AG, CP55 940, okadaic acid and cyclosporin A were 0.01, 0.03, 0.01, 0.005, and 0.005%, respectively.

Intracellular recording

Dual intracellular recordings were made by impaling neurons with glass microelectrodes using a micropositioner (Model 1480; Siskiyou Inc., Grants Pass, OR). Microelectrodes were pulled from borosilicate capillary tubing (1.0 mm OD, 0.75 mm ID; FHC, Bowdoinham, ME) to a resistance of 25–35 MΩ (Sutter Instruments P-97; Novato, CA) and filled with 3 M potassium acetate. Current pulses were delivered using a two-channel stimulator with stimulus isolation units (S88 and SIU5, respectively; Astromed-Grass, West Warwick, RI). Signals were amplified with a bridge amplifier (BA-1S; NPI, Tamm, Germany) and then digitally converted (Digidata 1322A A/D converter) for viewing and subsequent analysis (Axoscope; Molecular Devices, Sunnyvale, CA).

T and S cells were identified based on their position within the ganglion, cell size and action potential shape. The T-to-S excitatory post-synaptic potential (EPSP) consists of both a polysynaptic chemical component and a "monosynaptic" electrical component (Muller and Scott 1981). A T cell action potential elicits a short latency 1–2 mV electrically driven EPSP (referred to as the "electrical EPSP") followed by a 4–6 mV chemically driven EPSP (referred to as the "chemical EPSP"; see Fig. 1a). However, the T and S cells do not physically contact each other and nearly all synaptic input from the T onto the S cell is passively routed through a second interneuron, the coupling (C) cell (Muller and Scott 1981). The C cell is located in the interior of the ganglion neuropil and is prohibitively difficult to record from on the ventral side of the ganglion (Crisp and Muller 2006). The S and C cells are linked by a non-rectifying electrical synapse and the coupling between the S and C cells is so strong that EPSPs elicited in the C cell will propagate to the S cell with minimal delay or attenuation, essentially mimicking a monosynaptic connection (Muller and Scott 1981). The additional interneurons that mediate the polysynaptic, chemical component of the T-to-S synapse are unknown. α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonists block the chemical EPSP, indicating that at least one connection in the polysynaptic pathway is glutamatergic (Li and Burrell 2008). Although the electrical and chemical components overlap, recordings in high Mg^{2+} (15 mM) saline demonstrate that the peak of the chemical component corresponds with the falling phase of the electrical component (Fig. 1a).

Low frequency stimulation of the T-to-S synapse was used to induce LTD. Two different, well-established low frequency stimulation protocols were used: one in which the

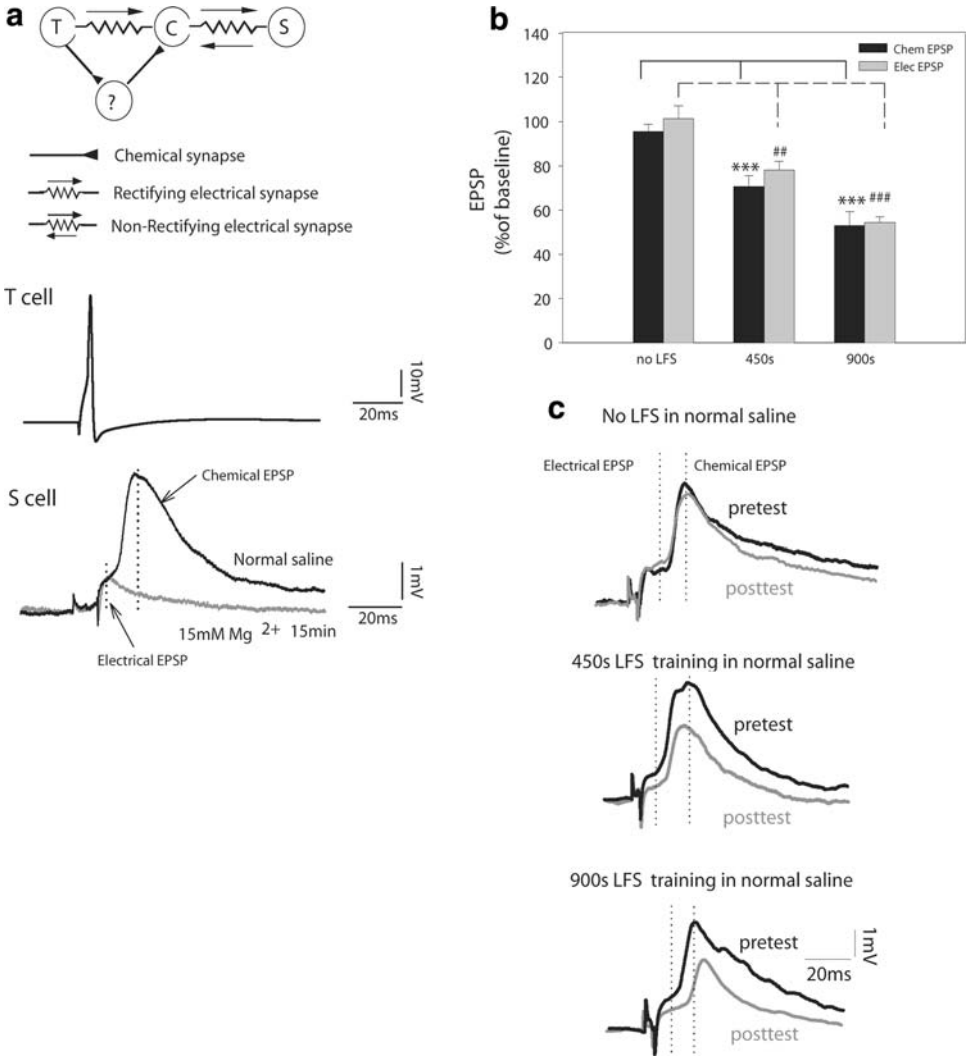

Fig. 1 Homosynaptic LTD at the T-to-S synapse. **a** *Top* The T-to-S synapse consists of a "monosynaptic" electrical component and a polysynaptic chemical component. *Bottom* Representative traces showing a T cell action potential corresponding electrical and chemical components of the EPSP in the S cell. The chemical component has a considerable delay relative to the electrical component, as revealed by comparing EPSP recordings in normal saline and high (15 mM) $MgCl_2$ saline. *Dotted lines* indicate where measurements of electrical and chemical EPSP amplitude were made. **b** *Bar graph* showing that both the 450 s ($n = 5$) and 900 s low frequency stimulation (abbreviated as "LFS" in the figures; $n = 5$) protocols induce LTD compared to synapses that received no low frequency stimulation ($n = 6$). Both the chemical (*black bars*; one-way ANOVA $F = 6.76$, $P < 0.001$) and electrical synapse (*gray bars*; $F = 5.61$, $P < 0.001$) underwent significant depression and post hoc analysis (Newman–Keuls') confirmed significant depression in both the 450 s and 900 s groups relative to the no low frequency stimulation control group (*** $P < 0.001$; $^{\#\#\#}$ $P < 0.001$; $^{\#\#}$ $P < 0.01$). **c** Representative traces from the no low frequency stimulation group (*top*), the 450 s group (*middle*) and the 900 s group (*bottom*); *gray traces* represent pretest and *black traces* posttest EPSPs

synapse was stimulated 450 times at 1 Hz (450 s low frequency stimulation) and a second procedure in which the synapse was stimulated 900 times at 1 Hz (900 s low frequency stimulation; Anwyl 2006). For all experiments, measurements were made of both the electrical and chemical components of the EPSP amplitude following a single T cell action potential prior to and 40 min following low frequency stimulation (referred to as the pretest and posttest, respectively). To minimize any bias in how EPSPs were quantified, the pre- and posttest EPSPs were temporally aligned based on the peak of the T cell action potentials that elicited the EPSP. The peak amplitude of chemical component was measured from the pretest recordings and that same time point was used for measurements of the posttest chemical EPSP amplitude. As for the electrical component, the pretest measurement was made at the time point corresponding to the middle of when the electrical EPSP plateaus and that same time point was used for measurements of the posttest electrical EPSP amplitude. The time point at which the chemical EPSP was measured includes only a small and decaying

component of the electrical EPSP (Fig. 1a) that accounted for approximately 10% of the EPSP amplitude. Pharmacological treatments were applied during the low frequency stimulation treatment only. Control experiments with the same concentration and duration of pharmacological treatment, but without low frequency stimulation, were performed to confirm that these drugs had no non-specific effects on the T-to-S EPSP. Since a decrease in input resistance can cause an apparent decrease in synaptic signaling, we measured input resistance throughout each experiment by injecting negative current pulses (0.5 nA, 500 ms).

It is not possible to record from the S cell throughout the pre- and post-low frequency stimulation periods given that such chronic recording causes a progressive decline in S cell EPSP amplitude and input resistance (Burrell and Sahley 2004). This is likely attributable to the S cell becoming damaged by the recording process due to its small size (≈ 15 μm), movement/elasticity of the leech ganglion and other, unknown processes (c.f., the disruptive effect of intracellular recording on synaptic plasticity in *Aplysia* neurons described by Eliot et al. 1994). Attempts have been made to make long-term recordings of the S cell, such as by lightly fixing the ganglion (to reduce movement of the ganglion), but such fixation dramatically reduces the size of the EPSP (data not shown). As a result, it is necessary to impale the S cell twice, once during the pretest and then during the posttest, to measure the T-to-S EPSP. These repeated impalements have the potential to damage the S cell, causing a decrease in EPSP amplitude that is due to a decrease in input resistance and not to any changes at the synaptic level. Therefore, considerable care was taken to make sure that S cell input resistance was unchanged between the pre- and posttest recordings during each LTD experiment. Only experiments with stable input resistance data were included for analysis; across all experiments, pretest input resistance was 13.04 ± 0.33 MΩ and posttest input resistance was 12.19 ± 0.65 MΩ.

Statistics

EPSP measurements were normalized to their initial values (% of baseline) and presented as means ± SE. Statistical analyses were performed using one-way analysis of variance (ANOVA), two-way ANOVA and Newman–Keuls' post-hoc test with Statistica analysis software (Statsoft).

Results

NMDAR-dependent and -independent forms of LTD at the T-to-S synapse

T-to-S LTD was induced using low frequency stimulation protocols at two different durations: 450 stimuli at 1 Hz (450 s low frequency stimulation) or 900 stimuli at 1 Hz (900 s low frequency stimulation). Both low frequency stimulation protocols induced significant depression of the chemical component of the T-to-S synapse (Fig. 1b, c) with the 900 s low frequency stimulation producing significantly greater depression compared to the 450 s low frequency stimulation protocol. Significant depression of the electrical synapse was also observed following 450 s or 900 s low frequency stimulation (Fig. 1b, c).

The effects of NMDAR antagonists AP5 and MK-801 were tested during both 450 and 900 s low frequency stimulation training. In synapses that underwent 450 s low frequency stimulation, LTD of the chemical component was inhibited by treatment with 100 μM AP5 or 40 μM MK801 (Fig. 2a–c). Surprisingly, LTD of the chemical component was not affected by either NMDA receptor antagonist following 900 s low frequency stimulation. The difference in the effectiveness of the NMDAR antagonist was not due to the length of drug treatment given that LTD following 450 s low frequency stimulation was still inhibited by AP5 applied for 900 s (see Supplementary Fig. 1). These data indicate that there are two forms of LTD of the chemical component of the T-to-S synapse: one that is NMDAR-dependent (elicited by the 450 s low frequency stimulation) and one that NMDAR-independent (elicited by the 900 s low frequency stimulation).

Depression of the electrical component of the T-to-S synapse by 450 s low frequency stimulation training was also inhibited by AP5 or MK801 (Fig. 2a–c). In contrast to the chemical synapse, both AP5 and MK801 partially blocked the depression of the electrical component induced by 900 s low frequency stimulation. That is, the electrical synapse of drug-treated groups following 900 s low frequency stimulation was significantly depressed compared to the no low frequency stimulation control group, but were also significantly elevated compared to synapses that underwent 900 s low frequency stimulation in normal saline. Therefore, it was concluded that LTD of the electrical component of the T-to-S synapse following 450 s low frequency stimulation was mediated by NMDAR-dependent processes, whereas LTD following 900 s low frequency stimulation was mediated by NMDAR-dependent and -independent mechanisms.

Many forms of NMDAR-dependent LTD also require activation of protein phosphatases (Anwyl 2006). To test the involvement of protein phosphatases in T-to-S NMDAR-dependent LTD, 450 s low frequency stimulation was carried out in the presence of either 1 μM okadaic acid, a specific inhibitor of protein phosphatases 1/2A or 1 μM cyclosporine A, a phosphatase 2B inhibitor. Both inhibitors prevented LTD normally induced by 450 and 900 s low frequency stimulation in the chemical (Fig. 2d) and electrical (data not shown) components of the T-to-S EPSP.

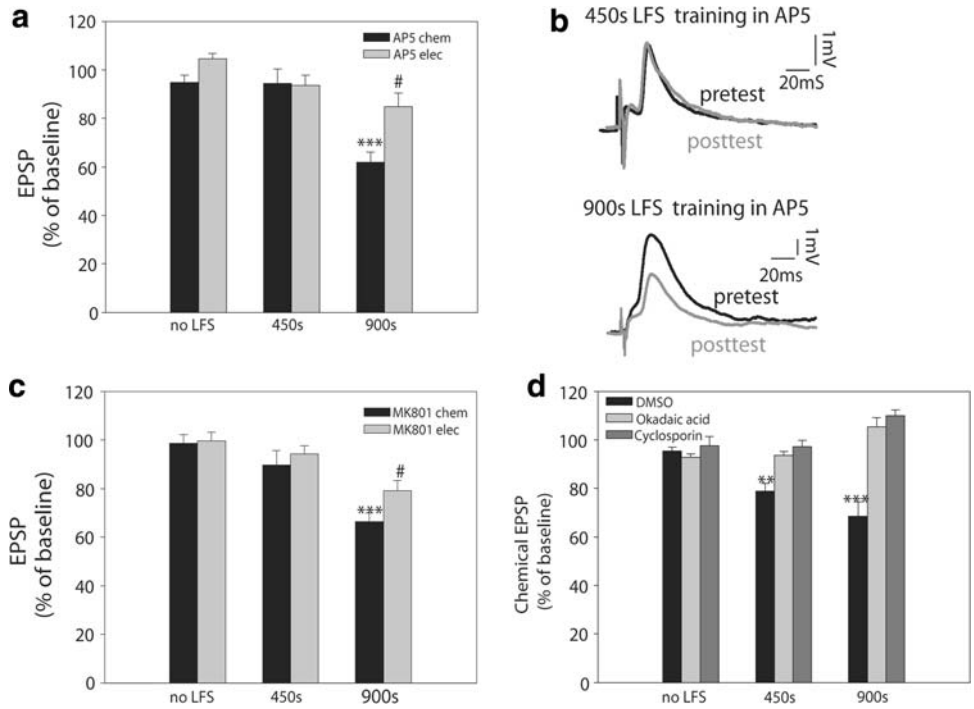

Fig. 2 Different durations of low frequency stimulation produce LTD with differing sensitivities to NMDA receptor antagonists. **a** AP5 blocked 450 s low frequency stimulation induced-LTD of the T-to-S chemical synapse (*black bars* $n = 5$ in both AP5 450 s and AP5 no low frequency stimulation group; one-way ANOVA, $F = 4.33$, $P < 0.01$, Newman–Keuls' test: AP5 450 s versus AP5 no low frequency stimulation, not significant [ns]). Depression of the chemical synapse following 900 s low frequency stimulation was not affected by AP5 treatment ($n = 8$; AP5 900 s versus AP5 no low frequency stimulation, *** $P < 0.001$). Depression of the electrical synapse following 450 s low frequency stimulation was blocked by AP5 (*gray bars* one-way ANOVA, $F = 3.38$, $P < 0.05$, Newman–Keuls' test: AP5 450 s versus AP5 no low frequency stimulation, ns), but AP5 did not block depression of the electrical synapse following 900 s low frequency stimulation (AP5 900 s versus AP5 no low frequency stimulation, $^{\#}P < 0.05$). **b** Representative traces showing effects of AP5 plus 450 s low frequency stimulation (*top traces*) and AP5 plus 900 s low frequency stimulation (*bottom traces*). **c** MK801 blocked LTD of the chemical synapse (*black bars*) following 450 s low frequency ($n = 5$ in both MK801 450 s and MK801 no low frequency stimulation group; one-way ANOVA, $F = 4.69$, $P < 0.01$; Newman–Keuls' test: MK801 450 s versus no low frequency stimulation, ns), but did not affect LTD following 900 s low frequency stimulation ($n = 8$, Newman–Keuls' test: *** $P < 0.001$). Depression of the electrical synapse (*gray bars*) was partially blocked by MK801 bath application (Newman–Keuls' test, $^{\#}P < 0.05$). **d** Okadaic acid (*light gray bars*) or cyclosporine A (*dark gray bars*) blocked LTD of the chemical synapse following 450 s low frequency stimulation (two-way ANOVA analysis $F = 5.20$, $P < 0.01$, Newman–Keuls' test: both 450 s drug groups versus control group, ns) and following 900 s low frequency stimulation (Newman–Keuls' test: both 900 s drug groups versus no drug group, both ns). 450 s or 900 s low frequency stimulation in saline with DMSO (*black bars*) still elicited significant LTD (** $P < 0.01$, *** $P < 0.001$, respectively)

The 900 s low frequency stimulation-induced LTD is cannabinoid-dependent

The cannabinoid system has emerged as an important signaling mechanism for mediating activity-dependent short-term and long-term synaptic depression at various types of synapses throughout vertebrate brain (Mackie 2006). Since there is evidence of an active cannabinoid system in the leech CNS (Stefano et al. 1997; Matias et al. 2001), the potential involvement of the cannabinoid system during the NMDAR-independent LTD elicited by 900 s low frequency stimulation was examined. Bath-application of AM251 (10 μM), a cannabinoid receptor antagonist, during 900 s low frequency stimulation training completely blocked LTD of the chemical synapse, but did not affect LTD elicited by the 450 s low frequency stimulation protocol (Fig. 3). Again, the length of drug treatment did not play a role in the effectiveness of AM251 given that AM251 treatment for 900 s still failed to block LTD induced by 450 s low frequency stimulation (see Supplementary Fig. 1). The cannabinoid receptor antagonist blocked LTD of the electrical component of the T-to-S synapse following both 900 and 450 s low frequency stimulation (Fig. 3a–c). These results indicate that LTD of the chemical synapse elicited by 900 s low frequency stimulation was dependent on activation of cannabinoid receptors, but LTD elicited by 450 s low frequency stimulation was not. For the electrical synapse, both NMDAR- and cannabinoid-dependent processes contributed to LTD following 450 s and 900 s low frequency stimulations. To test the involvement of protein phosphatases in cannabinoid-dependent LTD, 900 s low frequency stimulation was also carried out in the presence

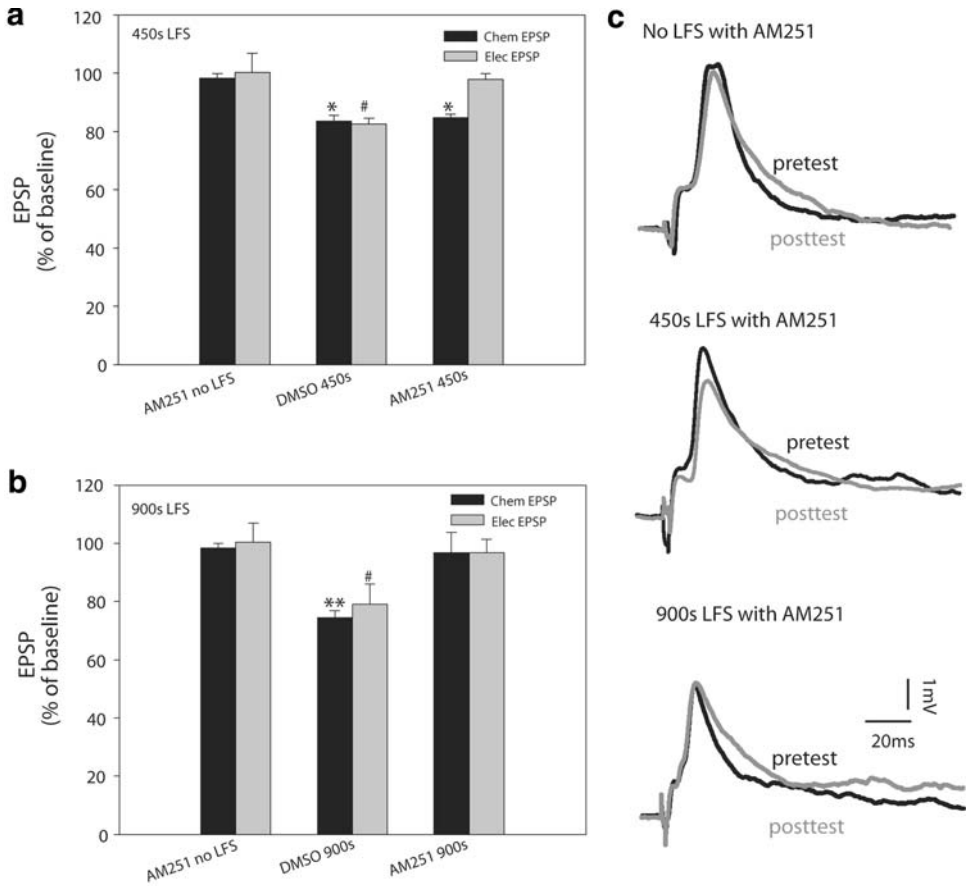

Fig. 3 The 900 s low frequency stimulation induced LTD is cannabinoid-dependent. **a** Significant depression of the chemical synapse (*black bars*) was observed in both the AM251-treated ($n = 5$) and DMSO ($n = 5$) groups following 450 s low frequency stimulation compared to the AM251 group that received no low frequency stimulation ($n = 4$; one-way ANOVA $F = 5.01$, $P < 0.001$; Newman–Keuls' test, * $P < 0.05$). LTD of the electrical synapse (*gray bars*) following 450 s low frequency stimulation was blocked by AM251 (one-way ANOVA, $F = 4.35$, $P < 0.05$; Newman–Keuls' test: DMSO with 450 s versus AM251 no low frequency stimulation, $^{\#}P < 0.05$; AM251 450 s versus AM251 no low frequency stimulation, ns). **b** AM251 blocked LTD of the chemical synapse following 900 s low frequency stimulation ($n = 5$ in all three groups; one-way ANOVA, $F = 5.57$, $P < 0.001$; Newman–Keuls' test: DMSO with 900 s versus AM251 no low frequency stimulation, ** $P < 0.01$; AM251 900 s versus AM251 no low frequency stimulation, ns). Depression of the electrical synapse following 900 s low frequency stimulation was also blocked by AM251 (one-way ANOVA, $F = 3.72$, $P < 0.01$; Newman–Keuls' test: DMSO with 900 s versus AM251 no low frequency stimulation, $^{\#}P < 0.05$; AM251 with 900 s and AM251 no low frequency stimulation, ns). **c** Representative traces showing effects of AM251 without low frequency stimulation (*top*), AM251 plus 450 s low frequency stimulation (*middle*), and AM251 plus 900 s low frequency stimulation (*bottom*)

of different protein inhibitors. Both 1 μM okadaic acid and 1 μM cyclosporine A were effective in blocking cannabinoid-dependent LTD of the chemical (Fig. 2d) and electrical (data not shown) components of the T-to-S EPSP.

2-Arachidonoylglycerol (2-AG) has been found to be the most abundant cannabinoid transmitter in the leech CNS (Matias et al. 2001) and synthesis of 2-AG is mediated by diacylglycerol lipase which can be inhibited by RHC80267 (Di Marzo et al. 1999; Leung et al. 2008). Because cannabinoid transmitters are lipids synthesized ''on demand'' by the activated cells (Hashimotodani et al. 2007), it was hypothesized that inhibition of 2-AG synthesis during low frequency stimulation would prevent this putative cannabinoid-dependent LTD. Bath-application of 100 μM RHC80267 during 900 s low frequency stimulation blocked LTD of the chemical and electrical components of the T-to-S synapse (Fig. 4a). These results show that, like application of the of the cannabinoid receptor antagonist AM251, inhibition of 2-AG synthesis blocks LTD elicited by the 900 s low frequency stimulation.

The ability of an exogenously applied cannabinoid receptor ligand to depress T-to-S synaptic transmission was also examined. Persistent depression of the T-to-S synapse was observed following a 15 min bath-application of 60 μM 2-AG (the post-treatment EPSP measurement was conducted 40 min after washout of 2-AG) and this depression was prevented by co-application of 10 μM AM251 (Fig. 4b). 10 μM CP55 940, a synthetic non-selective

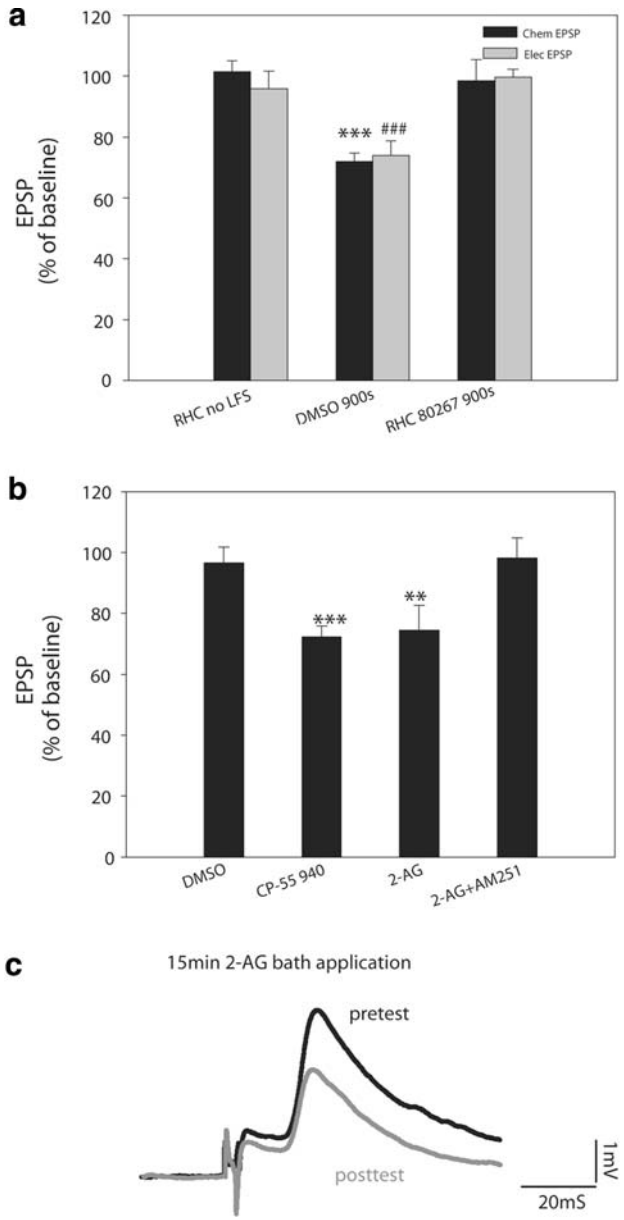

Fig. 4 Cannabinoid-dependent LTD in the T-to-S connection. **a** RHC80267 blocked LTD of the chemical synapse (*black bars*; one-way ANOVA $F = 28.7$, $P < 0.001$; Newman–Keuls *** $P < 0.001$) and the electrical synapse (*gray bars* one-way ANOVA $F = 7.29$, $P < 0.001$; Newman–Keuls ### $P < 0.001$) following 900 s low frequency stimulation. **b** CP55 940 or 2-AG depressed chemical transmission at the T-to-S synapse (one-way ANOVA, $F = 3.57$, $P < 0.05$, Newman–Keuls' test, *** $P < 0.01$; ** $P < 0.05$). Co-application of AM251 with 2-AG prevent the 2-AG induced LTD (Newman–Keuls' test between 2AG+AM251 and DMSO groups, ns). Post-treatment EPSP measurements were made following a 40 min washout period. **c** Representative traces showing bath-application of 2-AG mimicked the induction of LTD at the T-to-S synapse

cannabinoid receptor agonist, also induced depression in the T-to-S chemical (Fig. 4b, c) and electrical (data not shown) synapse. These results show that exogenous application of cannabinoid receptor ligands can produce persistent depression that is similar to what is observed during 900 s low frequency stimulation LTD.

Discussion

Two forms of LTD at a mixed chemical/electrical synapse

These results demonstrate that the mixed chemical/electrical synapse mediating the T-to-S polysynaptic connection utilizes two distinct forms of LTD and that the type of LTD elicited depends on the duration of activity. For the chemical component of the EPSP, the 450 s low frequency stimulation produced LTD that was NMDAR-dependent, whereas 900 s low frequency stimulation produced LTD that was NMDAR-independent and instead required the activation of the cannabinoid system. LTD of the electrical component also involved both NMDAR-dependent and cannabinoid-dependent processes, but the pattern differed from that of the chemical component in that the cannabinoid system and NMDARs made a significant contribution to LTD of the electrical component following both low frequency stimulation durations. Interestingly, both NMDAR- and cannabinoid-dependent LTD at the T-to-S connection were inhibited by treatment with okadaic acid (a protein phosphatase 1/2A inhibitor) or cyclosporine A (a protein phosphatase 2B inhibitor). This is consistent with a requirement for protein phosphatase activation for both NMDAR and cannabinoid-dependent forms of LTD (Anwyl 2006; Heifets et al. 2008).

An obvious limitation of this study is that the chemical component is a polysynaptic connection in which the intervening neuron(s) is unknown. Therefore, it is not possible at this time to determine the exact loci of depression or whether depression is the result of synaptic changes or changes in excitability by one or more of the unknown neurons. Nevertheless, the ultimate outcome of repetitive stimulation of this synaptic pathway is a persistent decrease in the T-to-S EPSP. Understanding the properties of synaptic plasticity in this pathway is of interest given that the S cell may contribute to learning and/or arousal in the leech (Sahley et al. 1994; Modney et al. 1997; Burrell et al. 2003; Kristan et al. 2005). The S cell receives afferent input from both photo- and mechanosensory neurons and has synaptic input to a variety of targets within the leech CNS including motoneurons, the serotonergic Retzius cells and a swim-gating interneuron (Magni and Pellegrino 1978; Friesen 1981; Muller and Scott 1981; Weeks 1982; Peterson 1984; Baccus et al. 2000; Crisp and Muller 2006). Previous work with the T-to-S pathway has shown that this circuit is capable of both LTP and LTD (Burrell and Sahley 2004; Burrell and Li 2008).

It is unknown why the electrical and chemical components of the T-to-S EPSP differ in terms of the cannabinoid

contribution to LTD following 450 s low frequency stimulation. It is possible that the gap junctions that mediate the electrical synapse are either more sensitive to cannabinoid transmitters or are in closer proximity to the source of cannabinoid transmitters compared to the elements mediating the chemical synapse. It is interesting that both the electrical and chemical components depressed in parallel, suggesting a degree of coordination in the plasticity of these two synaptic components. In the leech T-to-T synapse, which is also a mixed electrical/chemical connection, low frequency stimulation induced LTD in both the monosynaptic electrical and polysynaptic chemical component (Li and Burrell 2008). Coordinated potentiation of a mixed electrical and chemical synapse has been observed in the goldfish Mauthner cell synapse (Smith and Pereda 2003). This synapse also undergoes cannabinoid-mediated modulation, although cannabinoid receptor activation surprisingly elicits potentiation of the electrical and chemical synapse and not depression (Cachope et al. 2007). One possible explanation for this coordination of electrical and chemical synaptic plasticity is that the gap junctions are clustered in proximity to the postsynaptic density allowing for interaction between the neurotransmitter receptors in the postsynaptic density and gap junction proteins via either by short-range intercellular signaling or direct protein-to-protein contact (Sotelo and Korn 1978; Rash et al. 2000; Lynn et al. 2001; Zoidl et al. 2007). Treatments that inhibit receptor trafficking prevented LTD of chemical component in the T-to-T synapse and also blocked LTD of the electrical component (Li and Burrell 2008), which is consistent with the hypothesis of an interaction between receptors and gap junctions.

Evidence for NMDAR-dependent LTD in an invertebrate synapse has also been observed in behavioral experiments using the *Aplysia* gill withdrawal reflex (Ezzeddine and Glanzman 2003; Jami et al. 2007) and in the neuromuscular junction of *Drosophila* and the crayfish (Gavrila et al. 2005; Guo and Zhong 2006). Invertebrate NMDARs have been cloned in the molluscs *Aplysia* and *Lymnaea*, the insects *Drosophila* and the honeybee, and in the nematode *Caenorhabditis elegans* (Ha et al. 2006; Ultsch et al. 1993; Xia et al. 2005; Zannat et al. 2006; Brockie et al. 2001). Although evidence of leech NMDARs in this study is based solely on pharmacology, recently a partial sequence of the NR1 subunit of a leech NMDAR has been published (*Hir*NR1; Grey et al. 2009). Together, these findings indicate that there is substantial conservation between vertebrate and invertebrate nervous systems in the cellular processes of NMDAR-dependent LTD.

Cannabinoids in the invertebrate CNS

This is the first report of cannabinoid-dependent LTD in an invertebrate, although cannabinoid-dependent short-term synaptic inhibition has been observed in the mollusk, *Helix* (Lemak et al. 2007). The involvement of the cannabinoid system in T-to-S LTD was based on a number of observations. LTD was blocked by application of either a cannabinoid receptor antagonist (AM251) or by an inhibitor of diacylglycerol lipase (RHC80267) that is necessary for the synthesis of 2-AG, the cannabinoid transmitter that is present at the highest concentration in the leech CNS (Matias et al. 2001; Basavarajappa 2007). Depression of the T-to-S EPSP was mimicked by treatment with 2-AG and this 2-AG mediated depression was blocked by co-application of the cannabinoid receptor antagonist, AM251. Lastly, CP55 940, a persistent agonist of cannabinoid receptors (Wiley et al. 1995), also mimicked T-to-S LTD. There is considerable evidence that protostomal invertebrates, such as the leech, have an active cannabinoid system (see reviews by Salzet and Stefano 2002; McPartland 2004; Elphick and Egertova 2005). A number of different endocannabinoids including anandamide, 2-AG, *N*-palmitoylethanolamine and *N*-linolenoylethanolamine have been detected in the leech CNS (Matias et al. 2001). The leech CNS also possesses at least one enzyme involved in cannabinoid metabolism, fatty acid amide hydrolase, which hydrolyzes anandamide (Matias et al. 2001). Although diacylglycerol lipase has not been directly observed in the leech CNS, it is known to be present in *Drosophila* (Elphick and Egertova 2005; Leung et al. 2008).

The presence of cannabinoid receptors in the leech, or for that matter other protostomal invertebrates, is less clear. A cannabinoid receptor that is an orthologue of both the vertebrate cannabinoid 1 and 2 receptors has been identified in the deuterostomal invertebrate *Ciona intestinalis* (Elphick et al. 2003; Egertova and Elphick 2007). In protostomal invertebrates, a number of pharmacological and antibody studies have identified cannabinoid-like receptors (Stefano et al. 1997; De Petrocellis et al. 1999; Matias et al. 2001; McPartland et al. 2006; Lemak et al. 2007; Rawls et al. 2006, 2007) and a partial (480 bp) sequence encoding a putative cannabinoid receptor was isolated from the leech CNS (Stefano et al. 1997). However, this latter finding has been questioned (Elphick 1998; Elphick and Egertova 2005) and an analysis of genome databases for the protostomal invertebrates *Drosophila* and *C. elegans* and from the sea urchin, another deuterostomal invertebrate, have shown no evidence of orthologues to the vertebrate cannabinoid 1 and 2 receptors (Elphick and Egertová 2001; McPartland et al. 2001; Burke et al. 2006).

How then does one explain the consistent ability of cannabinoid-specific agonists and antagonists to bind to protostomal invertebrate tissue and have physiological effects consistent with modulation of cannabinoid-dependent functions, such as depolarization-induced inhibition in *Helix* (Lemak et al. 2007) and LTD in the leech? One explanation

is that the leech and other protostomal invertebrates possess a unique cannabinoid receptor that is sensitive to vertebrate cannabinoid receptor agonists and antagonists. A similar situation is observed with vertebrate and invertebrate gap junction proteins (connexins and innexins, respectively), which are encoded by unrelated genes but show a remarkable degree of functional and pharmacological similarity (Phelan 2005). Alternatively, there may be an additional cannabinoid receptor conserved between vertebrates and invertebrates that is unrelated to the cannabinoid 1 and 2 receptors but still responds to cannabinoid receptor pharmacological agents (Elphick and Egertova 2005). There is evidence for additional cannabinoid receptor types in cannabinoid 1 receptor knockout mice, but this putative cannabinoid receptor has not been identified (Breivogel et al. 2001; Hajos et al. 2001).

Co-expression of multiple forms of LTD in other synapses

The ability of synapses to express two distinct forms of LTD is not unique. Two forms of LTD also coexist in hippocampal pyramidal neurons (Oliet et al. 1997). However, while one form of LTD (NMDAR-dependent) was induced by synaptic activity, the other was (mGluR-dependent) was chemically induced following bath-application of an mGluR agonist. In the leech T-to-S synapse, the two different forms of LTD were elicited by two different durations of low frequency stimulation. It is not known how low frequency stimulation of different durations induce LTD with such distinct cellular mechanisms in the T-to-S synapse, but there is clearly a shift from NMDAR-dependent LTD to an LTD that is cannabinoid-dependent and NMDAR-independent. One possibility is that once NMDAR-dependent LTD has been elicited, further synaptic activation causes some sort of NMDAR-dependent reversal of this process, possibly a de-depression (potentiation of a depressed synapse) which can be mediated by NMDARs (Morishita and Malenka 2008). Another possibility is that activation of cannabinoid receptors inhibits NMDAR-dependent signaling pathways that mediate LTD so that only the cannabinoid-dependent processes contribute to LTD.

The functional significance of having two distinct forms of LTD is also unknown. It is unlikely that these two forms of LTD represent redundant mechanisms for inducing LTD given that each seems to be selectively induced by different levels (durations) of synaptic activity. One possibility is that these two forms of LTD represent two different systems for detecting the coincidence of pre- and postsynaptic activation. NMDAR-dependent synaptic plasticity (LTP or LTD) is usually induced by coincident pre- and postsynaptic activity that operates on a time scale of milliseconds (Bi and Poo 2001), whereas cannabinoid-dependent LTD may be induced by the coincident pre- and postsynaptic activity over longer time scales (Heifets et al. 2008).

The cannabinoid system has attracted considerable interest given that cannabinoid receptors can mediate both short- and long-term forms of synaptic depression that may contribute to learning and memory as well as having a neuroprotective role against pathologic conditions involving hyperexcitability, such as seizures and strokes (Chevaleyre et al. 2007; Hashimotodani et al. 2007; Lemak et al. 2007). An alternative role for both NMDAR-dependent and cannabinoid receptor-dependent forms of LTD at the T-to-S synapse might be to attenuate or filter incoming afferent input produced by repetitive or redundant mechanosensory stimulation, similar to the role that has been ascribed to conduction block in leech mechanosensory cells (Macagno et al. 1987; Baccus 1998). Regardless of the function, one of the major findings presented here is that a synaptic pathway can support multiple forms of synaptic plasticity mediated by very different cellular mechanisms. This capacity of synapses to utilize multiple forms of plasticity has only recently been recognized (Burrell and Sahley 2004; Bender et al. 2006; Sjöström et al. 2007; Tzounopoulos et al. 2007; Burrell and Li 2008) and represents a critical element in understanding how neural circuits are reorganized in an activity-dependent manner.

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