Dopamine D₁/D₅ Receptor Activation Reverses NMDA Receptor-Dependent Long-Term Depression in Rat Hippocampus

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Activation of dopamine D₁/D₅ receptors (D₁/D₅Rs) in area CA1 of the rat hippocampus modulates the expression of synaptic plasticity in a manner that is dependent on the timing of the D₁/D₅R activation. Here, we measured field EPSPs in rat hippocampal slices to examine the modulation of long-term depression (LTD) in CA1 by D₁/D₅Rs when activated immediately after the induction of LTD by low-frequency stimulation (LFS) or bath application of NMDA or the metabotropic glutamate receptor agonist DHPG [(RS)-3,5-dihydroxyphenylglycine]. Activation of D₁/D₅Rs by SKF 38393 [(±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrobromide] completely reversed a moderate LFS-induced LTD in a time-dependent manner, presumably through an adenylate cyclase/cAMP cascade. In support of this, general adenylate cyclase activation by forskolin ([3*R*-(3α,4αβ,5β,6β,6α,10α,10aβ,10bα)]-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1*H*-naphtho[2,1-*b*]pyran-1-one) immediately, but not 60 min, after LFS also reversed the LTD. β-Adrenergic receptor activation by isoproterenol failed to reverse the LTD, indicating that reversal is specific to D₁/D₅R-mediated increased cAMP production. SKF 38393 only partially reversed a more robust LFS-induced LTD, indicating that some components of consolidated LTD are resistant to reversal. LTD induced by bath application of NMDA, but not DHPG, was also reversed by SKF 38393. Western blot analysis of postsynaptic density fractions after NMDA-induced LTD revealed that the LTD was attributable to dephosphorylation of the AMPA receptor subunit glutamate receptor 1 (GluR1) at serine 845, without a change in total GluR content. Reversal of the LTD by SKF 38393 was associated with rephosphorylation of this same residue. Together, these findings demonstrate a new role for dopamine in the neuromodulation of hippocampal LTD.

Key words: LTD; dopamine; D₁/D₅R; hippocampus; NMDA; DHPG

Introduction

Information storage in the brain is thought to be achieved through synaptic plasticity involving both long-term potentiation (LTP) and long-term depression (LTD) of glutamatergic excitatory synaptic transmission. The principal responses are mediated by glutamate binding to postsynaptic ionotropic AMPA receptors (AMPA) and NMDA receptors (NMDARs), whereas synaptic plasticity is modulated by the activity-dependent release of other neurotransmitters, such as dopamine and noradrenaline (Frey et al., 1991; Swanson-Park et al., 1999; Vanhoose and Winder, 2003).

Dopamine plays a central role in facilitating LTP induction and maintenance in many areas of the brain, such as the basal ganglia (Kerr and Wickens, 2001) and prefrontal cortex (Otani et al., 2003), in which both the timing of dopamine receptor activation and agonist concentration are critically important. In the hippocampus, dopamine D₁/D₅ receptors (D₁/D₅Rs) agonists play a vital role in the consolidation of late-phase LTP in CA1

(Swanson-Park et al., 1999; Sajikumar and Frey, 2004) via up-regulation of the adenylate cyclase/cAMP second-messenger system (Mockett et al., 2004). LTP can also be consolidated behaviorally by pretetanus exposure to a range of novel objects, an effect that is blocked by the D₁/D₅R antagonist SCH 23390 [*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride] and mimicked by the agonist SKF 38393 [(±)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrobromide] (Li et al., 2003). Furthermore, D₁/D₅R antagonists block the late phase of hippocampal LTP both *in vitro* and *in vivo* (Frey et al., 1991; Huang and Kandel, 1995; Swanson-Park et al., 1999). In accord with these findings, intrahippocampal injection of dopamine agonists enhanced memory performance in eight-arm radial maze tasks (Packard and White, 1991).

Surprisingly, relatively little is known about the role of D₁/D₅Rs in the induction of hippocampal LTD, its consolidation, or in depotentiation of established LTP. Dopamine administration has, however, been shown to both induce LTP or LTD, depending on the concentration of agonist applied (Sajikumar and Frey, 2004), and enhance low-frequency stimulation (LFS)-induced LTD (Chen et al., 1995). Activation of D₁/D₅R has also been shown to inhibit depotentiation by LFS in area CA1 and the dentate gyrus (Otmakhova and Lisman, 1998; Kulla and

Received Feb. 23, 2006; revised Dec. 31, 2006; accepted Jan. 18, 2007.

This work was supported by a grant from the New Zealand Health Research Council.

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DOI:10.1523/JNEUROSCI.0838-06.2007

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Manahan-Vaughan, 2000) and to consolidate a transient LFS-induced LTD through a protein kinase A (PKA)-dependent process in prefrontal cortex (Huang et al., 2004). Collectively, these studies serve to reinforce the importance of dopamine as a neuromodulator of synaptic plasticity and suggest that the recent history of glutamatergic neuronal activity is critical in determining the effect of D₁/D₅R activation.

The purpose of the present study was to determine whether D₁/D₅ agonists facilitate the persistence of LTD, in a similar manner to LTP (Swanson-Park et al., 1999). In contrast to the LTP effect, we found that LTD was in fact reversed by this treatment and that both the LTD and its reversal involve modulation of the glutamate receptor subunit 1 (GluR1) serine 845 phosphorylation state in the postsynaptic density.

Materials and Methods

Slice preparation and recording. Transverse hippocampal slices (400 μ m) were prepared from male Sprague Dawley rats (42–50 d, 180–300 g), as described previously (Mockett et al., 2004). All experimental procedures were performed in accordance with regulations laid down by the Animal Ethics Committee of the University of Otago.

Rats were anesthetized with ketamine (100 mg/kg, i.p.) and decapitated, and slices were prepared. Slices were then transferred to an incubation chamber in which they were submerged and held for at least 2 h to equilibrate. An artificial CSF (aCSF) [in mM: 124 NaCl, 3.2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 2.5 CaCl₂, 1.3 MgCl₂, and 10 D-glucose (equilibrated with carbogen, 95% O₂–5% CO₂)] was perfused continuously through the chamber at a rate of 2 ml/min and maintained at a temperature of 32.5°C. Baseline field EPSP (fEPSPs) were elicited in area CA1 by stimulation of the Schaffer collateral–commissural pathway at 0.017 Hz (diphasic pulses, 0.1 ms half-wave duration) using Teflon-coated 50 μ m tungsten wire monopolar electrodes (A-M Systems, Carlsborg, WA). Evoked responses were recorded with glass microelectrodes filled with 2 M NaCl (1–2 M Ω) and placed in stratum radiatum of area CA1. During periods of baseline recording, the stimulation intensity was adjusted to elicit an fEPSP amplitude of 0.5 mV in which LFS was used to induce LTD or 1 mV for protocols using pharmacological agents to induce LTD. Baseline stability was visually assessed immediately before bath perfusion of the drugs and by linear regression at the conclusion of each experiment (Raymond et al., 2000). Group averages included only those slices whose baseline slope, as determined by linear regression, varied by less than 10% over the 30 min baseline. LTD was induced by LFS consisting of a 3 Hz, 1200 pulse train delivered to the Schaffer collateral–commissural pathway at a stimulus intensity evoking a 1 mV fEPSP.

fEPSP data analysis. The initial slopes of the fEPSPs were measured and expressed as a percentage change from the baseline level, calculated from an average of the last 15 min of the baseline recording period (Mockett et al., 2002). The degree of LTD for each experiment was measured as the average of the last 5 min of the post-LFS or postdrug recording period, whereas the group means were expressed as the percentage $\pm$ SEM change. Group means were compared statistically by two-tailed independent Student's *t* tests with statistical significance set at *p* < 0.05.

Drugs and reagents. All salts for electrophysiology were obtained from BDH Chemicals (Poole, UK). NMDA and D(-)-2-amino-phosphonovaleric acid (APV) were purchased from Tocris Cookson (Bristol, UK) and dissolved in double-distilled water as stock solutions. SKF 38393, DHPG [(R)-3,5-dihydroxyphenylglycine], and forskolin [(3R-(3 α ,4 α ,5 β ,6 β ,6 α ,10 α ,10 β ,10 α)]-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1H-naphtho[2,1-b]pyran-1-one were also purchased from Tocris Cookson but dissolved in DMSO as stock solutions. Isoproterenol was purchased from Sigma (St. Louis, MO) and dissolved in double-distilled water. Stock solutions were diluted 1:1000 with aCSF for the final working concentration.

Tissue preparation for biochemical analysis. Tissue was dissected and slices were cut in a manner similar to that already described for the electrophysiological experiments, except that the slices were further dissected to contain predominantly area CA1. These CA1 mini-slices were

placed in tissue culture dishes (35 mm, four to six slices per dish) containing 1 ml of aCSF and transferred to the upper compartment of an incubation chamber. Water in the lower compartment was gradually heated to 32°C over 2 h via a heating coil attached to a temperature control unit, and carbogen (95% O₂–5% CO₂) was bubbled through the water to provide a humidified and oxygen-rich environment in the chamber. At the conclusion of the 2 h equilibration, the aCSF bathing solution was replaced with aCSF containing 20 μ M NMDA for 2 min. This treatment was repeated 45 min later and thus replicated the protocol used in the electrophysiological experiments to induce a chemical LTD. Other drug treatments were bath applied in fresh oxygenated and warmed aCSF. To account for possible effects associated with repeated disturbance of the slices during drug-carrying aCSF exchanges, control slices that received no LTD-inducing NMDA or other drug treatments were subjected to identical aCSF exchanges at the same times as treated groups. At the conclusion of the experiment, slices were transferred to 1.5 ml Eppendorf tubes and processed according to the protocols described below. All processed slices were stored at –80°C until assayed for relative AMPA receptor subunit content and phosphorylation state by quantitative Western blot analysis.

Preparation of protein extracts from CA1 mini-slices. Whole extracts were prepared essentially according to Williams et al. (1998) by homogenization and sonication of four to five hippocampal CA1 mini-slices in ice-cold extraction buffer A [20 mM Tris, pH 7.6, 1 mM EDTA, 2 mM DTT, 10 mM CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate), 0.5% SDS, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mM NaF, and 10 mM Na₂P₂O₇], supplemented with 20 μ M H89 (N-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide 2HCl) (Biomol, Plymouth Meeting, PA), 20 μ M KN62 {1-[N, O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine} (Tocris Cookson), 20 μ M chelerythrine chloride (1,2dimethoxy-12methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride), 1 μ M okadaic acid (Tocris Cookson), 10 μ M cyclosporin A (Tocris Cookson), and Complete protease inhibitor (Roche Diagnostics, Auckland, New Zealand). After centrifugation (14,000 $\times$ g, 30 min, 4°C), supernatants were stored at –80°C before estimation of protein concentration (BCA assay using BSA protein standards Sigma) and quantitative Western blot analysis.

Isolation of the postsynaptic density-enriched fraction from CA1 mini-slice synaptoneuroosomes. Synaptoneuroosomes were prepared from CA1 mini-slices essentially according to the method of Hollingsworth et al. (1985), as reported by Williams et al. (2003). Briefly, four to six CA1 mini-slices were homogenized in HEPES buffer [50 mM HEPES, 124 mM NaCl, 3.2 mM KCl, 1.06 mM KH₂PO₄, 2.5 mM CaCl₂, 1.3 mM MgCl₂, 26 mM NaHCO₃, 10 mM glucose, Complete protease inhibitor, and 0.7 mM chloramphenicol (Sigma)] supplemented with 10 μ M KN62, H89, and chelerythrine chloride. Protein concentration was estimated using the Bradford Assay (Bio-Rad, Hercules, CA), and postsynaptic density (PSD)-enriched fractions were prepared by incubation (ice, 15 min) of equal amounts of synaptoneuroosomes in extraction buffer [20 mM Tris, pH 7.6, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 25 mM NaF, 10 mM Na₂P₂O₇, 1 mM sodium orthovanadate (Sigma), and 1% Triton X-100], supplemented with 20 μ M H89 (Biomol), 20 μ M KN62 (Tocris Cookson), 20 μ M chelerythrine chloride, and Complete protease inhibitor, according to the method of Strack et al. (1997). The PSD-enriched fraction was precipitated by centrifugation (14,000 $\times$ g, 30 min, 4°C) and resuspended by sonication in extraction buffer plus 1% SDS. Samples were stored at –80°C before quantitative Western blot analysis.

Quantitative Western blot analysis. Western blotting was performed as described previously (Williams et al., 1998). Samples (50 μ g) were separated through 9% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH). After blocking with 3% non-fat dried milk powder/PBS/0.1% Tween 20 (1 h at room temperature), membranes were incubated overnight at 4°C with antibodies to GluR1, phospho-GluR1_{S831}, phospho-GluR1_{S845} (Upstate Biotechnology, Lake Placid, NY), or GluR3 (Zymed, San Francisco, CA). Antibody binding was detected using HRP-conjugated anti-rabbit secondary antibodies (Sigma) and enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL). Resulting x-ray films (Agfa Gavaert, Mortsel, Belgium) were scanned using a Bio-Rad imaging densitometer and quanti-

fied using Molecular Analyst software (Bio-Rad). Data (mean $\pm$ SEM absorbance reading) are reported as a ratio of each drug-treated group relative to the no-drug controls. To evaluate differences between groups, ANOVA [one-way ANOVA with Bonferroni's *post hoc* test was performed using Prism version 4.00 (GraphPad Software, San Diego, CA)], and independent Student's *t* tests were performed.

Results

Dopamine D₁/D₅, but not β -adrenergic, receptor activation reverses synaptically induced LTD

The initial experiments were designed to investigate the effect of D₁/D₅R activation on LTD induced by a previous LFS in CA1. Control LTD of moderate magnitude was induced by a single bout of LFS consisting of 1200 pulses at 1 mV and applied at 3 Hz ($-16.5 \pm 4.7\%$ measured 2 h after LFS; $n = 7$) (Fig. 1A). Application of the dopamine D₁/D₅R agonist SKF 38393 (100 μ M, 20 min) immediately after the LFS did not alter the expression of the early phase of the depression, but, after 15 min, the depression began to decline and had completely reversed back to baseline within 40 min. The EPSP slope then remained at baseline levels for the remaining 80 min of the recording period ($1.4 \pm 4.4\%$; $n = 8$) (Fig. 1B). SKF 38393, applied by itself without a preceding LFS, had no immediate effect on baseline recording levels, although a gradual decline over the duration of the experiment was observed ($-11.6 \pm 2.5\%$; $n = 4$) (Fig. 1C) as reported previously (Mockett et al., 2004). To test whether the reversal of LTD is a general phenomenon of adenylate cyclase activation through G-protein-linked receptors, we investigated the effect of β -adrenergic receptor activation on the reversal of LFS-induced LTD. Previously, we determined that activation of β -adrenergic receptors by 0.5 μ M isoproterenol facilitated LTP induction in CA1 (Cohen et al., 1999) and that 1 μ M caused a twofold increase in cAMP production, similar to that induced by SKF 38393 (S. C. Webb, W. C. Abraham, and W. P. Tate, unpublished observations). We therefore chose 1 μ M as an effective concentration for the present experiment. Isoproterenol, applied immediately after the LFS, did not significantly reverse the LTD ($-12.2 \pm 3.4\%$; $n = 6$) (Fig. 1D). This suggests that the reversal of synaptically induced LTD is specific to D₁/D₅R activation.

To examine further the involvement of the adenylate cyclase/cAMP cascade in LTD reversal, we applied the general adenylate cyclase activator forskolin (10 μ M, 20 min) to induce a global rise in intracellular cAMP levels. This concentration causes a more than fivefold increase in cAMP production in CA1 (Webb, Abraham, and Tate, unpublished observations). When applied immediately after the LFS, forskolin initiated a rapid reversal of the LTD, which was complete within the period of forskolin perfusion and persisted after forskolin washout ($1.3 \pm 3.2\%$; $n = 5$) (Fig. 2A). However, when application of forskolin was delayed for 60 min after the LFS, the recovery back to baseline, although rapid, was only transient and returned to levels indistinguishable from LFS controls by the end of the experiment ($-15.8 \pm 3.6\%$; $n = 9$) (Fig. 2B). To determine whether the D₁/D₅R-mediated reversal of LTD was also time dependent, we repeated the protocol and replaced forskolin with SKF 38393. LFS again produced a significant LTD 60 min after LFS ($-18.2 \pm 4.1\%$; $n = 4$) that was not reversed by SKF 38393 ($-21.8 \pm 10.8\%$, measured 40 min after drug washout) (Fig. 2C). These findings demonstrate a time-dependent consolidation of LTD that renders it resistant to reversal by D₁/D₅R receptor activation and cAMP accumulation.

Having established that D₁/D₅R and adenylate cyclase activation can completely reverse a moderate LTD, we next sought to determine whether a similar reversal would occur after the induc-

Figure 1. Selective activation of dopamine D₁/D₅Rs reverses LTD. **A**, A single bout of LFS (1200 pulses, 3 Hz; $n = 7$) induced LTD lasting at least 120 min. **B**, Application of the D₁/D₅R agonist SKF 38393 (100 μ M, 20 min; $n = 8$) immediately after the LFS completely reversed the LTD within 40 min. **C**, SKF 38393 applied in the absence of LFS did not significantly change baseline transmission levels ($n = 4$). Pauses in recording correspond to periods of LFS delivery in this and other experiments. **D**, Stimulation of β -adrenergic receptors using isoproterenol (1 μ M, 20 min; $n = 6$) immediately after the LFS did not reverse the LTD. Sample fEPSP recordings are shown to the right of the figure. Each numbered recording is the average of 10 traces taken at the time indicated by the corresponding number on the adjacent plot. Calibration: 0.5 mV, 5 ms.

tion of a strong LTD. A stronger, more persistent LTD was induced by LFS consisting of 1200 pulses (1 mV, 3 Hz) delivered twice with a 5 min interval ($-25.2 \pm 2.8\%$, measured 120 min after LFS; $n = 7$) (Fig. 3A). This LTD was confirmed to be NMDAR dependent, because administration of D-APV (50 μ M) during the LFS protocol blocked the LTD ($-5.9 \pm 3.9\%$, 60 min after LFS; $n = 5$) (Fig. 3C). A second period of LFS administration after APV washout successfully established LTD ($-33.2 \pm 2.7\%$; $p < 0.001$) (Fig. 3C). Application of SKF 38393 (100 μ M, 20 min) immediately after two bouts of 1200 pulses appeared to cause a partial reversal after 120 min, but this did not reach statistical

Figure 2. Time-dependent reversal of LTD by forskolin. **A**, Forskolin (10 μ M, 20 min; $n = 5$), applied immediately after the delivery of an LFS, rapidly reversed the LTD. **B**, Forskolin, applied 60 min after the LFS, caused only a transient reversal of the depression, which then returned to control LTD levels ($n = 9$). **C**, SKF 38393 (100 μ M, 20 min; $n = 4$), applied 60 min after the LFS, did not reverse the LFS-induced LTD. Sample waveforms are as in Figure 1.

significance ($-14.3 \pm 4.1\%$; $n = 10$; $p = 0.062$) (Fig. 3B). Delaying the application of the SKF 38393 by 30 min was also ineffective in reversing the LTD ($-19.8 \pm 5.5\%$; $n = 5$; data not shown). In a final attempt to induce the reversal of strong LTD, we also examined the effects of isoproterenol (1 μ M, 20 min; $n = 5$) and forskolin (10 μ M, 20 min; $n = 5$) applied immediately after the second LFS. LTD levels measured 60 min after the second bout of LFS in SKF 38393, isoproterenol, and forskolin groups were not significantly different from the LTD observed in the control group (Fig. 3D).

One consideration regarding the resistance to reversal by strong LTD is that the 5 min delay between bouts of LFS may have provided sufficient time for intracellular events to consolidate the LTD before the D₁/D₅R activation. To test this possibility, we applied the strong LTD-inducing protocol in one continuous uninterrupted bout (2400 pulses, 3 Hz, 1 mV). This protocol produced robust LTD, measured 60 min after LFS, that was very similar to that observed at the same time point in the previous experiment ($-23.6 \pm 2.3\%$, $n = 9$, and $-21.7 \pm 3.3\%$, $n = 11$, respectively) (Fig. 4A). When SKF 38393 (100 μ M, 20 min) was applied immediately after the uninterrupted LFS, a partial, but significant, reversal of the LTD was observed ($-12.7 \pm 2.1\%$; $n = 7$; $p < 0.01$ relative to control slices) (Fig. 4B). Together, these experiments demonstrate that, although moderate LTD is completely reversible by D₁/D₅R activation and global intracellular

Figure 3. Strong LTD induced by two bouts of LFS is resistant to reversal. **A**, LTD induced by two bouts of LFS (1200 pulses, 3 Hz; $n = 7$) each separated by 5 min. **B**, D₁/D₅R receptor activation by SKF 38393 (100 μ M, 20 min; $n = 10$), applied immediately after the second bout of LFS, failed to significantly reverse the LTD after 120 min ($p = 0.06$). **C**, LFS-induced LTD is NMDAR dependent. NMDAR blockade by APV (50 μ M; $n = 5$) during LFS blocked LTD induction. After APV washout, LTD could again be induced by LFS. **D**, Histogram comparing LTD recorded in control slices 60 min after two bouts of LFS with that recorded in slices treated with APV (50 μ M), SKF 38393 (SKF; 100 μ M, 20 min), isoproterenol (ISO; 1 μ M, 20 min; $n = 5$), or forskolin (FSK; 10 μ M, 20 min; $n = 5$). No significant reversal of LTD was observed in any group. Waveforms are as in Figure 1.

adenylate cyclase activation, a stronger LTD-inducing protocol generates a rapidly consolidating LTD that renders it only partially susceptible to cAMP-linked mechanisms of de-depression within 5 min and irreversible by these mechanisms by 60 min.

Pharmacological induction of LTD by NMDA is also reversed by D₁/D₅R activation

To understand the generality of the reversal effect, we asked whether pharmacologically induced LTD was also sensitive to D₁/D₅R-mediated reversal. Although several protocols for inducing LTD by bath-applied NMDA have been published (Lee et al.,

Figure 4. D_1/D_5R activation partially reverses strong LTD induced by uninterrupted LFS. **A**, LTD induced by one continuous bout of LFS (2400 pulses, 3 Hz; $n = 11$). **B**, D_1/D_5R activation by SKF 38393 ($100 \mu\text{M}$, 20 min; $n = 7$) immediately after continuous LFS partially reversed the LTD ($p < 0.05$). Waveforms are as in Figure 1.

1998; Kamal et al., 1999; van Dam et al., 2002; Li et al., 2004), these have not reliably induced LTD in our hands. Consequently, we developed a priming paradigm that consistently induced LTD in CA1 of adult hippocampal slices (Fig. 5). NMDA ($20 \mu\text{M}$, 2 min), when applied once, produced no long-lasting effect on the fEPSPs ($-4.6 \pm 1.1\%$; $n = 5$). However, when a second identical administration of NMDA was made 45 min later, an LTD of $-18.3 \pm 6.3\%$ was generated that persisted for at least 100 min after the second NMDA application (Fig. 5A). This LTD was prevented by the application of SKF 38393 ($100 \mu\text{M}$, 40 min) when applied immediately after the second NMDA application ($1.8 \pm 3.9\%$; $n = 6$; $p < 0.05$) (Fig. 5B). When the application of SKF 38393 was delayed by 60 min, however, no reversal occurred ($-21.8 \pm 7.7\%$, measured 40 min after drug washout; $n = 4$) (Fig. 5C). In contrast to the LTD-reversing effect of early D_1/D_5R activation, β -adrenergic receptor activation by isoproterenol ($1 \mu\text{M}$, 40 min) failed to significantly modulate LTD ($-13.7 \pm 5.7\%$; $n = 3$) (Fig. 5D). Together with the findings from LFS-induced LTD, these data indicate that D_1/D_5R , but not β -adrenergic receptor, activation reverses the NMDAR-induced mechanisms that lead to the induction and maintenance of LTD.

LTD induced by group 1 metabotropic glutamate receptor activation is not reversed by D_1/D_5R activation

To examine the specificity of D_1/D_5R -mediated LTD reversal, we tested whether a non-NMDAR-dependent form of LTD is also reversible by a D_1/D_5R agonist. Activation of G-protein-linked group 1 metabotropic glutamate receptors (mGluR) by the agonist DHPG rapidly induces an LTD that is reported to be mechanistically distinct from NMDAR-dependent LTD (Oliet et al., 1997; Harris et al., 2004). To be comparable with the NMDA-dependent LTD experiments, we selected a DHPG paradigm that induced moderate levels of LTD, similar to that induced by a single bout of LFS or by the NMDA paradigm, which our previous experiments demonstrated could be reversed by SKF 38393 (Figs. 1, 5). DHPG ($50 \mu\text{M}$, 10 min) induced a moderate LTD that persisted for at least 140 min after DHPG washout ($-16.8 \pm$

Figure 5. Pharmacological induction of LTD by NMDA is reversed by D_1/D_5R activation. **A**, NMDA ($20 \mu\text{M}$, 2 min; $n = 5$) induced a response that was typified by a near-complete depression of synaptic transmission followed by a rapid recovery to a transient potentiated state. When applied twice at 45 min intervals, the NMDA treatment led to a stable LTD after the second application. **B**, Application of SKF 38393 ($100 \mu\text{M}$, 40 min; $n = 6$) immediately after the second NMDA application completely prevented the development of LTD from the potentiated state, with synaptic transmission returning only to baseline levels. **C**, Delayed application of SKF 38393 ($100 \mu\text{M}$, 40 min; $n = 4$) 60 min after the second NMDA application did not reverse the LTD. **D**, Isoproterenol ($1 \mu\text{M}$, 40 min; $n = 3$) delivered immediately after the second NMDA application did not prevent the development of LTD or return it to baseline transmission levels. Waveforms are as in Figure 1.

2.0% ; $n = 4$) (Fig. 6A). SKF 38393 ($100 \mu\text{M}$, 20 min), applied immediately after DHPG, failed to reverse this LTD ($-17.1 \pm 4.5\%$; $n = 6$) (Fig. 6B). This finding indicates that LTD reversal by D_1/D_5R activation is not a feature of all forms of LTD but is specific to NMDAR-mediated LTD.

NMDA-induced LTD is expressed through dephosphorylation of synaptic GluR1 serine 845

One of the early events identified in the development of NMDAR-dependent LTD is the dephosphorylation of the PKA-

Figure 6. mGluR-mediated LTD is not reversed by D₁/D₅R activation. **A**, Application of the mGluR group 1 agonist DHPG (50 μM, 10 min; $n = 4$) produced a small, slowly decaying LTD similar in magnitude to that produced in previous experiments using a mild LFS protocol. **B**, DHPG-induced LTD was not reversed by SKF 38393 (100 μM, 20 min; $n = 6$) applied immediately after the DHPG perfusion ($-17.1 \pm 4.5\%$; $n = 6$). Waveforms are as in Figure 1.

targeted serine 845 residue of the AMPA receptor GluR1 subunit (Kameyama et al., 1998; Lee et al., 1998, 2000). We therefore investigated whether the NMDAR-dependent LTD in our hands showed a similar dephosphorylation correlate and, if so, whether D₁/D₅R or β -adrenergic receptor activation would reverse the effect. For this study, we chose the chemical LTD approach, using two NMDA applications, to maximize the number of synapses exhibiting the LTD effect in the slice. Consistent with the paradigm for reversal of pharmacological LTD, SKF 38393 (100 μM) was applied for 40 min either immediately or 60 min after the second NMDA application, whereas isoproterenol (1 μM) was applied only at the earlier time point. Control slices received SKF 38393 or isoproterenol alone.

Quantitative Western blot analysis was used to determine the phosphorylation state of the GluR1 serine 845 and 831 residues in NMDA-treated CA1 mini-slices (see Materials and Methods). All tissue was collected at a time equivalent to that at which LTD levels were determined in the electrophysiological experiments. Initial experiments examined these phosphorylation states in whole homogenates made from the mini-slices. Neither 2× NMDA nor SKF 38393, either alone or together, had a significant effect on serine 831 phosphorylation in this preparation, measured 60 min after drug application (data not shown). In contrast, SKF 38393, by itself or in combination with NMDA, induced a twofold increase in serine 845 phosphorylation (2.18 ± 0.41 , $n = 4$, $p < 0.05$ and 2.31 ± 0.48 , $n = 7$, $p < 0.05$, respectively). This effect appears to be entirely attributable to D₁/D₅R activation because NMDA by itself had no significant effect (1.15 ± 0.13 ; $n = 7$). In no condition was the total amount of GluR1 altered from control levels.

The lack of an effect by NMDA on GluR1 serine 845 phosphorylation levels was unexpected because previous studies had reported a dephosphorylation of this site during NMDA receptor-dependent LTD (Lee et al., 1998). Because previous studies had used a crude membrane preparation, we therefore

refined our whole-cell extract to a PSD-enriched fraction (see Materials and Methods), using mini-slices collected in an additional experiment and with each data point normalized to total GluR1 levels. Using this preparation, NMDA caused a significant reduction in serine 845 phosphorylation (0.48 ± 0.10 ; $p < 0.001$; $n = 6$) (Fig. 7A,B), without affecting the serine 831 phosphorylation state (1.18 ± 0.12 ; $n = 4$) (Fig. 7A,D). D₁/D₅R activation by SKF 38393 immediately after the second NMDA application reversed the NMDA-induced dephosphorylation of serine 845 (1.40 ± 0.22 ; $n = 6$; $p < 0.01$), but was ineffective when applied after 60 min (0.72 ± 0.09 ; $n = 6$) (Fig. 7A,B). SKF 38393 by itself had no significant effect on the phosphorylation state of serine 845 (1.24 ± 0.18 ; $n = 5$), implying that the phosphorylation response observed to the drug in whole homogenates primarily occurred in nonsynaptic receptors.

Isoproterenol caused a large increase in serine 845 phosphorylation when applied alone ($1.47 \pm 0.16\%$; $n = 6$) and significantly increased these levels above NMDA-treated levels when applied immediately after NMDA treatment (NMDA plus isoproterenol, $0.90 \pm 0.09\%$; NMDA, $0.48 \pm 0.10\%$; $n = 6$; $p < 0.01$); however, this remained significantly less than isoproterenol applied alone ($p < 0.01$) (Fig. 7A,B). We considered that, because neither SKF 38393 (Fig. 1) nor isoproterenol (data not shown) by themselves caused an increase in basal synaptic transmission, then any change in serine 845 phosphorylation levels induced by these agonists alone was unlikely to be involved in the modulation of synaptic transmission after LTD induction. On this basis, we subtracted the mean change in serine 845 phosphorylation levels induced by the agonists alone from that of any NMDA treatment group in which the agonist was applied. As can be seen in Figure 7C, this analysis demonstrates that only SKF 38393 applied immediately after NMDA treatment was effective in fully reversing the induced dephosphorylation, whereas neither SKF 38393 applied 60 min after NMDA treatment nor isoproterenol applied immediately after NMDA treatment could rescue the serine 845 dephosphorylation effect.

Because NMDAR-dependent LTD might be expected to be mediated in part by a reduction in PSD-associated AMPA receptors (Beattie et al., 2000; Ehlers 2000), we also examined whether NMDA treatment caused a reduction in GluR1- or GluR3-containing AMPA receptors in the same PSD extracts as used for the phosphorylation analysis. No significant change in either GluR1 or GluR3 protein was found in the PSD fraction after NMDA or its reversal by SKF 38393 (e.g., NMDA treatment group: GluR1, 1.02 ± 0.05 ; GluR3, 1.18 ± 0.22 ; $n = 6$). Together, our findings suggest that the reversal of NMDA-induced LTD by D₁/D₅R activation is mediated, at least in part, by either preventing or reversing the dephosphorylation of the synaptic GluR1 serine 845 residue without changing overall levels of synaptic AMPA receptors.

Discussion

We have shown in the present experiments that LTD can be rapidly and completely reversed both by activation of D₁/D₅R and by global activation of adenylate cyclase immediately after its induction. Partial reversal of a stronger LTD could also be achieved. These new findings add to the growing understanding of the importance of timing in glutamate–dopamine interactions, particularly because D₁/D₅R activation during LFS promotes the persistence of LTD (Sajikumar and Frey, 2004). The reversal of LTD is specific to D₁/D₅R activation because alternative adenylate cyclase activation by β -adrenergic receptor stimulation with isoproterenol, at a dose that gives similar cAMP accu-

mulation to that obtained with SKF 38393, did not significantly affect the LTD, although there was a trend for a small reversal effect. This result is intriguing given that isoproterenol is a potent activator of adenylate cyclase activity and cAMP production and increases GluR1 serine 845 phosphorylation levels (Vanhoose and Winder, 2003), all of which are normally associated with LTD reversal (Lee et al., 1998; Kameyama et al., 1998; Esteban et al., 2003). These data support the view that there are specific and separable roles of β -adrenergic and D₁/D₅R activation in CA1 synaptic plasticity (Swanson-Park et al., 1999), possibly attributable to spatial segregation of the receptor subtypes.

Mechanism of NMDAR-mediated LTD and reversal

Previously, it has been demonstrated that NMDA-induced LTD has many similarities to electrical stimulation-induced LTD and appears to share common induction and expression mechanisms (Lee et al., 1998; Beattie et al., 2000). We therefore developed a chemical LTD protocol that allowed electrophysiological demonstrations of NMDA-induced LTD and its reversal by activation of D₁/D₅Rs, so that parallel biochemical experiments could be undertaken to investigate GluR1 phosphorylation levels. Using a PSD-enriched fraction, we found in agreement with others (Lee et al., 1998, 2000; Snyder et al., 2003; Vanhoose and Winder, 2003) that NMDA caused a significant and long-lasting reduction in synaptic GluR1 serine 845 phosphorylation and that D₁/D₅R, but not β -adrenergic receptor, activation reversed this effect. In contrast, neither NMDA nor SKF 38393 affected the phosphorylation state of GluR1 serine 831, an observation also in agreement with previous studies (Lee et al., 1998; Snyder et al., 2000; Chao et al., 2002) (but see Vanhoose and Winder, 2003). Interestingly, overall levels of GluR1 or GluR3 in the PSD were not affected by NMDA or SKF 38393, suggesting that global changes in AMPA receptor levels in the PSD did not underlie the induction of LTD or its reversal. This result has a correlate in the failure to observe a change in AMPAR surface expression during LTP in CA1 slices (Grosshans et al., 2002). The lack of an effect on total PSD-associated AMPAR subunits suggests that LTD and its reversal are more related to changing the kinetics of the AMPA receptor/channel than to changes in the synaptic expression of AMPA receptors, at least in adult tissue. Because phosphorylation of serine 845 by PKA has been shown to increase the peak open probability of the AMPAR (Banke et al., 2000), it is conceivable that NMDAR activation in adult tissue induces LTD by decreasing open channel probability and that this effect is reversed by D₁/D₅R-mediated activation of PKA and subsequent phosphorylation of GluR1 serine 845. These findings do not exclude the possibility, however, that the AMPAR content of synapses may decrease at later times after LTD induction (Heynen et al., 2000).

Figure 7. NMDA-induced LTD and its reversal are correlated with the phosphorylation state of GluR1 serine 845 in PSD-enriched fractions prepared from synaptoneurosomes. CA1 mini-slices were subjected to the same experimental paradigm as shown in Figure 5. Changes in GluR1 phosphorylation states were determined by extracting PSD-enriched fractions from CA1 mini-slice synaptoneurosomes followed by Western blot analysis. **A**, Representative Western blots of PSD-enriched fractions probed with an antibody recognizing GluR1_{ser845}, GluR1_{ser831}, and GluR1. Total GluR1 was not affected by any manipulation, and thus all phosphorylation state data in **B–D** were normalized against total GluR1. SKF, SKF 38393; Iso, isoproterenol. **B**, Summary graph showing that NMDA application (N; $n = 6$) caused a significant reduction in GluR1_{ser845} phosphorylation relative to the no-drug controls (C; $n = 6$). SKF 38393 (S; $n = 5$) applied by itself did not significantly affect GluR1_{ser845} phosphorylation but completely reversed the NMDA effect (N+S) when applied early (E; 0–40 min; $n = 6$) but not late (L; 60–100 min; $n = 6$) after the second NMDA administration. Isoproterenol (I; $n = 6$) applied by itself resulted in a significant increase in GluR1_{ser845} phosphorylation, which was significantly reduced when applied in combination with NMDA (N+I; $n = 6$) at the early time point. RAU, Relative absorbance units. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tailed independent t test. **C**, Summary graph showing data from **B**, corrected for effect of SKF 38393 and isoproterenol alone by subtracting the mean change in response to SKF 38393 or isoproterenol alone from the corresponding value when the drug was applied with NMDA. The NMDA-induced reduction in GluR1_{ser845} phosphorylation was reversed by early but not late application of SKF 38393 or by isoproterenol (** $p < 0.01$, one-way ANOVA with Bonferroni's *post hoc* test). **D**, GluR1 serine 831 phosphorylation ($n = 4$) was not significantly altered by NMDA, D₁/D₅R, or β -adrenergic receptor activation.

Recent reports suggest that D₁/D₅R activation may also modulate plasticity through the synthesis and trafficking of GluR1 subunits. In nucleus accumbens cultured neurons, D₁/D₅R activation increased the rate of AMPAR insertion (Wolf et al., 2004), whereas Sun et al. (2005) observed that D₁/D₅R activation induced PKA-dependent trafficking of GluR1 subunits from intracellular pools to the extrasynaptic membrane in prefrontal neurons and that subsequent NMDAR activation was required for translocation into the synapse. Similarly, D₁/D₅R activation increases the synthesis of GluR1 subunits in the soma and dendrites of hippocampal neurons (Smith et al., 2005). Our findings do not rule out a role for increased externalization of GluR1 in LTD reversal, although we found no evidence for an increase in GluR1 in either the total pool or the PSD fraction after D₁/D₅R activation. It should be noted, however, that our techniques would not have been able to detect any receptors that may have relocated within the PSD itself.

Time dependence of LTD reversal

The reversal of LTD was time dependent, because delayed application of either SKF 38393 or forskolin failed to reverse LTD. We

suggest that the expression of LTD immediately after the LFS involves posttranslational modification of ionotropic glutamate receptors such as dephosphorylation of serine 845 of the AMPA GluR1 subunit and that this is readily reversed by activation of specific receptors that mediate cAMP-induced PKA activation. LTD consolidation and resistance to reversal that occurred after 60 min may involve processes not directly mediated by PKA such as protein synthesis (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000; Sajikumar and Frey, 2003). This interpretation would also explain why DHPG-induced LTD, which is protein synthesis dependent (Huber et al., 2001), was not reversed by D₁/D₅R agonist. The difference in reversibility between NMDA- and DHPG-induced LTD supports the view that these two forms of LTD have differential dependence on the regulation of GluR1 serine 845 phosphorylation (Harris et al., 2004). Indeed, DHPG-induced LTD contrasts with NMDA-induced LTD by inducing an increase in GluR1 serine 845 phosphorylation (Delgado and O'Dell, 2005).

D₁/D₅R activation alone does not induce an activity-independent LTP

Previous reports demonstrating an activity-independent induction of LTP by D₁/D₅R agonists (Huang and Kandel, 1995; Yang, 2000) or cAMP analogs (Frey et al., 1993) have been controversial because other laboratories (Otmakhova and Lisman, 1996, 1998; Kameyama et al., 1998; Blond et al., 2002), including our own (Swanson-Park et al., 1999; Mockett et al., 2004), have not been able to replicate this effect. The present study confirms and extends our previous findings. We found that, in addition to SKF 38393 failing to induce LTP, it also failed to alter phosphorylation of GluR1 at serine 845 or serine 831 in the PSD-enriched fraction, although an increase in phosphorylated serine 845 occurred in the whole-cell extract. This finding reflects the observation that GluR1 serine 845 phosphorylation is necessary but not sufficient for delivery of GluR1 subunits to the synapse in adult tissue (Kameyama et al., 1998; Esteban et al., 2003). Conversely, others have shown that D₁/D₅R receptor stimulation can lead to presentation of GluR1-containing receptors to the cell surface of cultured neurons (Sun et al., 2005; Oh et al., 2006). It is possible that, in our studies, the large degree of serine 845 phosphorylation generated by SKF 38393 in total homogenates may also have been associated with delivery of AMPA receptors to the cell surface. However, the lack of an increase in total receptor number in the PSDs indicates that, if such delivery does occur, it is confined to extrasynaptic sites.

Recent work by Otmakhov et al. (2004) suggests that activity-independent LTP after PKA activation may occur in some hippocampal slice preparations in which a reduced state of inhibition leads to a greater level of NMDAR activity, and indeed this has been shown to be the case in a number of *in vitro* studies (Huang and Kandel, 1995; Barco et al., 2002; Otmakhov et al., 2004; Smith et al., 2005; Sun et al., 2005). Thus, preparations in which slices inherently exhibit a higher level of NMDAR responsiveness to test stimuli may exhibit synaptic potentiation in response to D₁/D₅R activation.

Behavioral significance of D₁/D₅R-mediated regulation of LTD

Studies examining the behavioral significance of D₁/D₅R activation during learning have demonstrated that the timing of activation is critical for the successful acquisition of the learning task or expression of plasticity. Baldwin et al. (2002) and Smith-Roe and Kelley (2000) have shown, for example, that coincident

NMDAR and D₁/D₅R activation is necessary for appetitive instrumental learning in the nucleus accumbens and medial prefrontal cortex, whereas Li et al. (2003) demonstrated that previous activation of D₁/D₅Rs by exposure to a novel environment is required to consolidate a decaying LTP induced 5 min later by a weak tetanus. A delay of 20 min abolished this effect. Our own findings mirror this latter finding with activation of adenylate cyclase failing to reverse LTD if delayed for 60 min after induction. This requirement for immediate activation after LTD may serve to upregulate acutely depressed synapses to facilitate the immediate acquisition of new learning strategies in novel environments (Lisman and Grace, 2005).

Conclusion

We have shown that dopamine D₁/D₅R have a novel neuromodulatory role in regulating hippocampal synaptic plasticity, i.e., time-dependent reversal of NMDAR-dependent LTD. Given its established role in consolidating weak LTP and inhibiting depotentiation in the hippocampus, these new findings indicate that one overall function of D₁/D₅Rs is to maintain hippocampal synapses in a potentiated state and to moderate the effects of depression-inducing stimuli. This work expands the range of actions for dopamine in the brain and further demonstrates its complex integration into the neuromodulatory control of hippocampal function.

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