Development/Plasticity/Repair

Hyperdopaminergic Tone Erodes Prefrontal Long-Term Potential via a D_2 Receptor-Operated Protein Phosphatase Gate

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Dopamine (DA) plays crucial roles in the cognitive functioning of the prefrontal cortex (PFC), which, to a large degree, depends on lasting neural traces formed in prefrontal networks. The establishment of these permanent traces requires changes in cortical synaptic efficacy. DA, via the D_1-class receptors, is thought to gate or facilitate synaptic plasticity in the PFC, with little role recognized for the D_2-class receptors. Here we show that, when significantly elevated, DA erodes, rather than facilitates, the induction of long-term potentiation (LTP) in the PFC by acting at the far less abundant cortical D_2-class receptors through a dominant coupling to the protein phosphatase 1 (PP1) activity in postsynaptic neurons. In mice with persistently elevated extracellular DA, resulting from inactivation of the DA transporter (DAT) gene, LTP in layer V PFC pyramidal neurons cannot be established, regardless of induction protocols. Acute increase of dopaminergic transmission by DAT blockers or overstimulation of D_2 receptors in normal mice have similar LTP shutoff effects. LTP in mutant mice can be rescued by a single *in vivo* administration of D_2-class antagonists. Suppression of postsynaptic PP1 mimics and occludes the D_2-mediated rescue of LTP in mutant mice and prevents the acute erosion of LTP by D_2 agonists in normal mice. Our studies reveal a mechanistically unique heterosynaptic PP1 gate that is constitutively driven by background DA to influence LTP induction. By blocking prefrontal synaptic plasticity, excessive DA may prevent storage of lasting memory traces in PFC networks and impair executive functions.

Introduction

The prefrontal cortex (PFC) may store lasting memories and use this mnemonic information to guide behavior, thought, and emotion (Fuster, 1993; Goldman-Rakic, 1995; Seamans and Yang, 2004). Much of prefrontal functions, most notably working memory mediated by the dorsolateral PFC in primates (Goldman-Rakic, 1995) and the medial PFC (mPFC) in rodents (Seamans and Yang, 2004), depend on the mesocortical dopamine (DA) system. DA action is mediated by two classes of receptors. The D_1-class receptors (D_1 and D_5) positively couple to the cAMP/protein kinase A (PKA) signaling that leads to phosphorylation of inhibitor 1 (I-1) and inhibition of the protein phosphatase 1 (PP1) (Greengard et al., 1999). The D_2-class receptors, including D_2, D_3, and D_4, negatively regulate this cAMP/PKA/I-1/PP1 cascade. In the PFC, dopaminergic and glutamatergic afferents converge onto the same dendritic spines of deep layer pyramidal neurons, forming "synaptic triads" (Goldman-Rakic et al., 1989; Carr and Sesack, 1996). Both classes of receptors are localized in distal spines in the PFC, with the D_1 receptors being far more abundant than D_2 receptors (Lidow et al., 1991; Gaspar et al., 1995). Through this heterosynaptic architecture, DA profoundly influences the transmission and plasticity of cortical glutamatergic systems.

The establishment of memory traces that underlie PFC functions are believed to depend on changes in synaptic strength, e.g., long-term potentiation (LTP), in cortical network (McClelland et al., 1995; Squire and Alvarez, 1995). As in most central synapses, prefrontal LTP requires activation of the NMDA receptor (NMDAR) (Hirsch and Crepel, 1991; Jay et al., 1995; Vickery et al., 1997; Zhao et al., 2005) and elevation of postsynaptic Ca^{2+} concentrations (Hirsch and Crepel, 1992). Limited studies have investigated the intracellular signaling mechanisms downstream to NMDAR activation and Ca^{2+} influx that trigger LTP in the PFC. Although it appears that calmodulin (CaM)-stimulated adenylyl cyclases (AC1 and AC8), PKA, and Ca^{2+}/CaM-dependent protein kinase IV (CaMKIV) (Jay et al., 1998; Zhuo, 2008) mediate certain aspects of prefrontal LTP, the molecular and signaling details underlying the induction, expression, maintenance, and modulation of LTP in the PFC remain less explored (Otani et al.,

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2003; Zhuo, 2008) compared with other memory systems (Malenka and Bear, 2004; Kauer and Malenka, 2007).

DA is thought to facilitate LTP induction in the PFC. In both deep layer PFC synapses and hippocampal-PFC synapses, D_1-class agonists facilitate, whereas antagonists impair, NMDAR-dependent LTP via cAMP-dependent mechanisms (Gurden et al., 2000; Huang et al., 2004). Conversely, little contribution has been recognized for the D_2 class in LTP induction (Gurden et al., 2000; Li et al., 2003; Huang et al., 2004; Lemon and Manahan-Vaughan, 2006). However, a low concentration of DA has been shown to convert an NMDAR-independent form of long-term depression to LTP that requires both D_1- and D_2-class receptors (Matsuda et al., 2006), suggesting a "priming" role for background DA in PFC plasticity.

Here, we identify a prefrontal synaptic modification process that is sensitive to D_2 dysregulation. Mice lacking the DA transporter (DAT), which controls DA transmission by reuptaking DA into presynaptic terminals, display a persistently elevated extracellular DA level and several other alterations in the DA system (Giros et al., 1996; Jones et al., 1998). We show that LTP in the mPFC, considered to be homologous to the primate dorsolateral PFC (Kolb and Cioe, 2004), is absent in this hyperdopaminergia mouse model, revealing an unexpected eroding role for DA in LTP. This erosion can be mimicked in normal mice by acute elevation of DA levels using pharmacological agents and is mediated by the far less abundant D_2-class receptors and a downstream postsynaptic phosphatase gating mechanism. Because elevated DA signaling is implicated in several neuropsychiatric disorders, such as schizophrenia, attention-deficit hyperactivity disorder (ADHD), and stress-induced prefrontal impairments (Seeman, 1987; Goldman-Rakic, 1995; Castellanos and Tannock, 2002; Arnsten, 2009), our studies are relevant to the understanding of the cellular and molecular basis of these disorders.

Materials and Methods

Slice preparation. All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol from the Harvard Medical Area Standing Committee on Animals. Cogenic C57BL/6 DAT knock-out (KO) (postnatal day 35–60) and their wild-type (WT) littermates were killed, and their brains were rapidly removed. In cases in which drugs were given *in vivo* via intraperitoneal or subcutaneous injections, mice were killed 30 min after the last dose. Coronal cortical slices (300 μm) containing the anterior cingulated cortex (ACC) and/or the prelimbic (PrL) cortex were cut using a vibratome. Slices were superfused with an ice-cold artificial CSF (ACSF) that contained the following (in mM): 126 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4, and 25 D-glucose. ACSF was saturated with 95% O_2 and 5% CO_2. Slices were incubated in ACSF for at least 1 h at room temperature (21–23°C) before transferring to a recording chamber continuously perfused with oxygenated ACSF.

Electrophysiology. Whole-cell voltage- and current-clamp recordings were performed on individual pyramidal neurons under infrared differential interference contrast microscopy using an Axoclamp 2B amplifier (Molecular Devices). Pyramidal neurons were identified by their morphology and, in some cases, by their characteristic adaptive firing patterns in response to constant current injections. Cortical recordings were made from layer V neurons while presynaptic stimuli (0.033 Hz, 200 μs) were delivered with a concentric bipolar electrode (FHC) placed at layer II/III of the ACC or PrL. For current clamping, electrodes were filled with the following (in mM): 142 KCl, 8 NaCl, 10 HEPES, 0.4 EGTA, 2 Mg-ATP, and 0.25 GTP-Tris, pH 7.25. For evoked EPSC recordings, neurons were voltage clamped at −60 mV unless indicated otherwise. Picrotoxin at 50 μM was present in the superfusion medium to block $GABA_A$ receptor-mediated synaptic responses. Tetrodotoxin (1 μM) was added during recordings of miniature EPSCs (mEPSCs). Recording pipettes (4.5–5.5 MΩ) were filled with solution containing the following (in mM): 142 Cs-gluconate, 8 NaCl, 10 HEPES, 0.4 EGTA, 2.5 QX-314 [*N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide], 2 Mg-ATP, and 0.25 GTP-Tris, pH 7.25 (with CsOH). Series resistance was monitored throughout whole-cell recordings, and data were discarded if the resistance changed by >15%. All recordings were made at 32°C with a temperature controller (Warner Instruments). Drugs were delivered to the bath with a gravity-driven perfusion system (Harvard Apparatus). For intracellular dialysis experiments, we waited for at least 10 min after the patch rupture to allow diffusion of the inhibitors.

After obtaining stable EPSCs for 10 min, three different protocols were used to induce LTP. Protocol I involved theta-burst stimulation (TBS) (five trains of burst with four pulses at 100 Hz, 200 ms interval; repeated four times at intervals of 10 s). Protocol II involved tetanus stimulation (tetanus; 100 pulses at 100 Hz). Protocol III involved pairing presynaptic stimulation of 80 pulses at 2 Hz with depolarization of postsynaptic cells at +30 mV. The magnitude of LTP was quantified as the ratio of the averages of 20 EPSCs between 50 and 60 min after LTP induction to the 20 EPSCs collected during the 10 min baseline recording.

We used two methods to measure NMDA/AMPA ratios. In the kinetically based method, five EPSCs were recorded first at −60 mV, followed by five EPSC recordings at +40 mV. Average EPSCs were computed for each holding potential. The NMDA/AMPA ratio is defined as the amplitude of the NMDAR component 80 ms after stimulation at +40 mV divided by the peak AMPAR component at −60 mV. In the pharmacologically based method, EPSCs were recorded at +40 mV in the absence (to derive total EPSC) and then the presence (to derive $EPSC_{AMPA}$) of the NMDAR antagonist D-2-amino-5-phosphonopentanoic acid (AP-5) (50 μM). An average of five EPSCs were collected and averaged for each EPSC type. $EPSC_{NMDA}$ was isolated by subtracting $EPSC_{AMPA}$ from the total EPSCs. The NMDA/AMPA ratio was then computed.

A personal computer in conjunction with Digidata 1322A and pClamp software (version 9.2; Molecular Devices) were used for data acquisition and analysis. Signals were filtered at 1 kHz and digitized at 10–20 kHz. mEPSCs were analyzed by Mini Analysis 6 (Synaptosoft).

In vivo *microdialysis in freely moving mice. In vivo* microdialysis experiments were performed as described previously for striatum in mice (Gainetdinov et al., 2003) with modifications related to PFC analysis (Ihalainen et al., 1999). Briefly, 3-month-old mice were anesthetized with ketamine–xylazine mixture and placed in a stereotaxic frame, and a dialysis probe (2 mm membrane length, 0.24 mm outer diameter, Cuprophane, 6 kDa cutoff; CMA-11; CMA/Microdialysis) was implanted into the right PFC. The stereotaxic coordinates for implantation were as follows (measured at the probe tip; in mm): anteroposterior, 1.9; dorsoventral, −3.2; lateral, 0.5 relative to bregma (Paxinos and Franklin, 2001). Placement of the probe was verified by histological examination subsequent to the experiments. After surgery, animals were returned to their home cages with *ad libitum* access of food and water. Twenty-four hours after insertion of the probe, a quantitative "low perfusion rate" microdialysis experiment (Smith et al., 1992; Gainetdinov et al., 2003; Cyr et al., 2006) was conducted in freely moving mice for determination of basal extracellular DA levels in the PFC. The dialysis probe was connected to a syringe pump and perfused at 100 nl/min with an artificial CSF (CMA/Microdialysis) composed of the following (in mM): 147 NaCl, 2.7 KCl, 1.2 $CaCl_2$, and 0.85 $MgCl_2$. After a minimum 1 h equilibration period, at least three samples were collected every 60 min to a tube containing 2 μl of 0.5 M HCl. Dialysates were analyzed for levels of DA by using HPLC with electrochemical detection (Alexis 100; Antec Leyden). DA was separated on a reverse-phase column (ALB-105, 3 μm, 50 × 1 mm) with a mobile phase consisting of 50 mM phosphate buffer, 8 mM KCl, 500 mg/L octyl sodium sulfate, 0.1 mM EDTA, and 3% methanol, pH 6.0, at a flow rate of 50 μl/min. DA was detected by a Decade II electrochemical detector equipped with two micro VT-03 electrochemical flow cells and 0.7-mm-diameter glassy carbon electrode (Alexis 100; Antec Leyden). The volume of injection was 5 μl.

Western blot analysis. Mouse (2–3 months old) cortices were rapidly dissected on ice and immediately frozen in liquid nitrogen. Acute cortical

slices (300 μm) were stimulated with NMDA (200 μM) or vehicle for 5 min, followed by 30 min incubation in oxygenated ACSF. PFC containing ACC and PrL were carefully dissected and snap frozen in liquid nitrogen, and tissues from individual mice were pooled. Frozen tissues were homogenized in ice-cold buffer containing 320 mM sucrose, 10 mM Tris-HCl, and 5 mM EDTA, pH 7.4, and centrifuged at 2200 rpm for 10 min to remove large cellular fragments. Membranes were sedimented by centrifugation (15,000 rpm, 30 min, 4°C), resuspended, sonicated in TE buffer (10 mM Tris-HCl and 5 mM EDTA, pH 7.4), and solubilized in a 1% deoxycholate (DOC) buffer (50 mM Tris-HCl, pH 9.0, and 1% sodium deoxycholate). Samples were neutralized in buffer containing 0.1% Triton X-100, 0.1% DOC, 50 mM Tris-HCl, pH 9.0, and centrifuged at 15,000 rpm for 1 h at 4°C. The supernatant was collected and stored at −80°C until use. Protein kinase inhibitor cocktail (Roche) was present in all buffers. For the detection of phospho-α-CaMKII, a protein phosphatase inhibitor cocktail (Sigma) was present in buffers.

Subcellular fractionation was performed as described previously (Gardoni et al., 2006). Briefly, cortical tissues were homogenized in 0.32 M ice-cold sucrose containing 1 mM HEPES, 1 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors at pH 7.4 and centrifuged at 1000 × *g* for 10 min. The resulting supernatant (S1) was centrifuged at 3000 × *g* for 15 min to obtain crude membrane (P2) and cytosolic (S2) fractions. The pellet (P2) was resuspended in 1 mM HEPES and centrifuged at 100,000 × *g* (1 h). The pellet (P3) was resuspended in 75 mM KCl containing 1% Triton X-100 and centrifuged at 100,000 × *g* (1 h). The supernatant (S4) was stored and referred as Triton X-100-soluble fraction. The insoluble pellet (P4) was homogenized in a glass–glass potter in 20 mM HEPES, glycerol added, giving rise to the Triton X-100-insoluble fraction (TIF). TIF has been shown to be enriched in postsynaptic densities (PSDs) (Gardoni et al., 2001, 2006) and was used instead of the classical PSD because of limited amount of the starting material.

Proteins were separated by 10% SDS-PAGE (10–50 μg/lane) and transferred to polyvinylidene difluoride membranes. Blots were immunostained with primary antibodies against NR1, NR2A, NR2B, NR2A/2B, GluR1, GluR2/3, actin, α-CaMKII (all from Millipore), or phosphor-Thr286-specific α-CaMKII (Promega), followed by incubation with peroxidase-conjugated goat secondary antibodies. Immunoreactive signals were detected with an ECL-based LAS-3000 image system (Fujifilm). Densitometric analysis was performed within linear range using ImageGauge (Fujifilm). For each quantification, actin was used as loading controls in which the band densities for the protein to be measured was normalized to the actin band densities from the same loading lanes.

Surface biotinylation. Procedures for biotinylation and analysis of cell surface proteins have been described previously (Zhang et al., 2007; Huang et al., 2009). Acute PFC slices (300 μm) were washed in ice-cold ACSF and incubated in 1 mg/ml NHS-SS-biotin (Pierce) at 4°C for 30 min (Huang et al., 2009). After a wash in ACSF containing 1 mM lysine, slices were homogenized and sonicated in lysis buffer (20 mM Tris, 50 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1 mM EGTA, pH 7.4) containing proteinase and phosphatase inhibitors. After mixing at 4°C for 30 min, the homogenates were centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants were quantified by a Micro BCATM Protein Assay (Pierce) and divided into two aliquots. One aliquot was used to determine the total NMDAR subunits by Western blotting. The second aliquot was incubated at 4°C overnight with Neutravidin-linked beads (Pierce) to capture biotinylated surface proteins. After three washes with lysis buffer, the surface proteins were eluted with protein sample buffer containing DTT and subjected to Western blotting.

Drugs. Drugs used and their sources were as follows: ALX5407 [(*R*)-(*N*-[3-(4′-fluorophenyl)-3-(4′-phenylphenoxy)propyl])sarcosine] (Sigma), AP-5 (Sigma), clozapine (Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Sigma), D-amphetamine (Sigma), fostriecin (Tocris Bioscience), GBR12909 (1-[2-[bis(4-fluorophenyl)-methoxy]ethyl]-4-[3-phenylpropyl]piperazine) (Sigma), haloperidol (Fujisama), KN-93 (2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) (Tocris Bioscience), microsystin LR (Calbiochem), picrotoxin (Sigma), PKI(6–22) (PKA inhibitor 6–22 amide) (Calbiochem), quinpirole (Sigma), QX-314 (Sigma), raclopride (Sigma), SCH23390 [*R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride] (Tocris Bioscience), SKF81297 (6-chloro-2,3,4,5-tetrahydro-1-phenyl-1*H*-3-benzazepine hydrobromide) (Sigma), and tetrodotoxin (Sigma).

The concentrations for drugs directly applied in bath were chosen based on their specificity (IC_{50} or EC_{50}) with related references indicated in Results. The doses for the various DA agonists and antagonists were determined based on published dose–response studies on behaviors (e.g., locomotor activity, prepulse inhibition, etc.), gene expression (e.g., c-*fos* activation), and/or signal transduction events (e.g., protein phosphorylation) in mice. The drug/literature specifics are as follows: amphetamine (Ralph et al., 1999), acute and chronic clozapine (Leveque et al., 2000; MacDonald et al., 2005), acute and chronic haloperidol (Usiello et al., 2000; MacDonald et al., 2005), GBR12909 (Salahpour et al., 2008), quinpirole (Halberda et al., 1997), raclopride (Ralph et al., 2001), SCH23390 (Usiello et al., 2000; Ralph et al., 2001), and SKF81297 (Usiello et al., 2000; Gainetdinov et al., 2003).

Statistics. All data are expressed as mean ± SEM. Statistical analysis was performed using Student's tests, one-way ANOVA followed by *post hoc* Tukey–Kramer tests, or Kolmogorov–Smirnov test, as specified in individual figures.

Results

Prefrontal LTP is absent in DAT KO mice

We examined LTP in layer V pyramidal neurons in slices prepared from mouse mPFC. These neurons receive convergent dopaminergic and glutamatergic inputs, are targets of the hippocampus (Carr and Sesack, 1996), and give rise to massive cortical and subcortical projections (Sesack et al., 1989), thus representing a core element of PFC executive circuits (Williams and Goldman-Rakic, 1995; Seamans and Yang, 2004). These neurons also bear the majority of DA receptors in rodent cortex (Gaspar et al., 1995). We performed whole-cell patch-clamp recordings from visually identified pyramidal neurons in the anterior cingulate or prelimbic cortices, while delivering extracellular stimuli at layer II/III (Fig. 1*A*).

We identified pyramidal neurons by morphological features or their adaptive firing patterns in response to current injections (Fig. 1*B*). The EPSCs at these synapses were mediated by glutamate receptors, because they were completely abolished by the AMPAR antagonist CNQX (20 μM) and the NMDAR antagonist AP-5 (50 μM) (data not shown). To induce LTP, we used the TBS protocol (Tsvetkov et al., 2004; Zhao et al., 2005). TBS consistently produced a robust, long-lasting potentiation of EPSCs in WT (157.1 ± 7.2% of baseline at 50–60 min) (Fig. 1*C*,*E*). This LTP depended on the activation of NMDARs and CaMKII, because it was completely abolished by AP-5 (50 μM) and the CaMKII inhibitor KN-93 (20 μM) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). In contrast to WT, no LTP could be elicited in slices prepared from DAT KO mice (102.3 ± 3.9%) (Fig. 1*D*,*E*).

To examine whether the inability to induce LTP in DAT KO was attributable to the specific LTP induction paradigm used, we tested two additional protocols. First, the classical tetanus protocol (100 pulses at 100 Hz) produced a significant, long-lasting potentiation of EPSCs in WT (166.3 ± 11.7%) (Fig. 1*F*,*H*) but failed to elicit potentiation in mutant mice (117.2 ± 5.7%; $p < 0.001$ vs WT) (Fig. 1*G*,*H*). Second, pairing low-frequency (2 Hz) presynaptic stimulation with postsynaptic depolarization (+30 mV) produced robust and sustained LTP in WT (155.8 ± 12.1%) (Fig. 1*I*,*K*), but this LTP was absent in KO slices (111.1 ± 4.0%; $p < 0.001$ vs WT) (Fig. 1*J*,*K*). The pairing protocol bypasses the requirements of intact presynaptic terminal release and spine

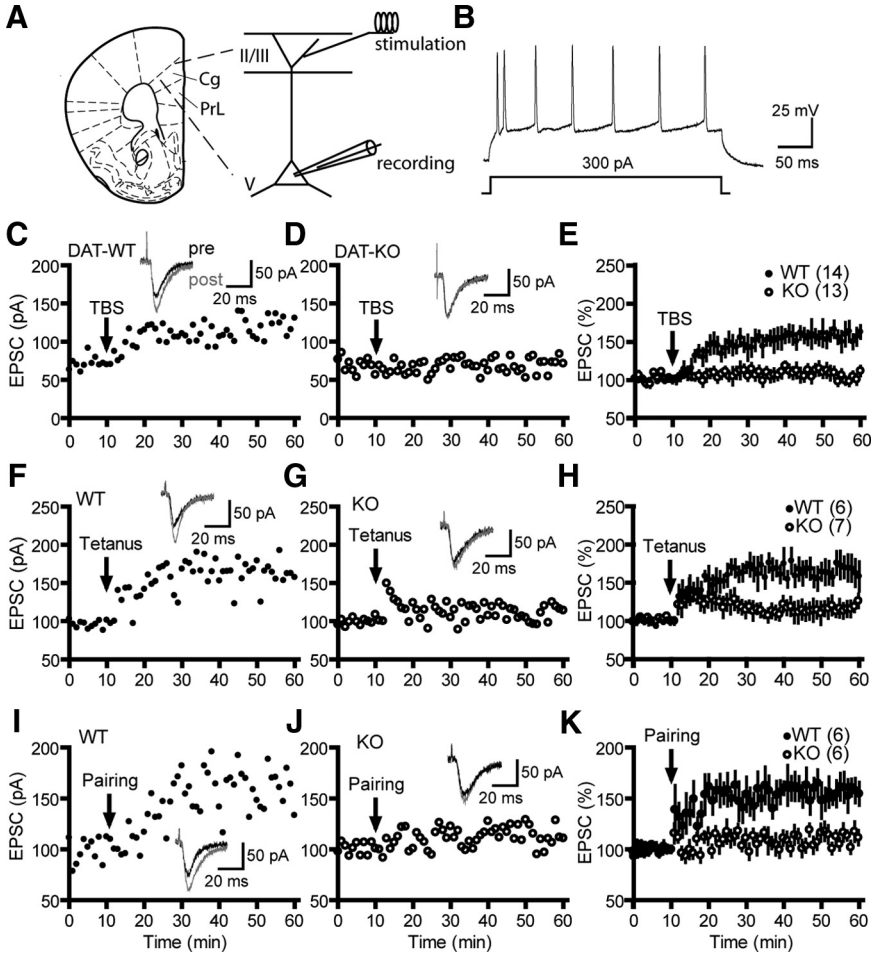

Figure 1. LTP is abolished in mPFC neurons in DAT KO mice. ***A***, Schematic of stimulation and recording configuration from a coronal PFC slice. Cg, Cingulated cortex. ***B***, Characteristic adaptive firing patterns recorded under the current-clamp configuration from a layer V pyramidal neuron. ***C–E***, LTP was induced in a WT (***C***), but not a KO (***D***), neuron by the TBS protocol (indicated by arrows) and the summary of normalized LTP (***E***). ***F–H***, LTP induced in a WT (***F***), but not a KO (***G***), neuron by the tetanus protocol (arrows) and the summary data for the normalized LTP (***H***). ***I–K***, LTP induced in a WT (***I***), but not a KO (***J***), neuron by the pairing protocol (arrows) and the summary data for the normalized LTP (***K***). Traces (insets) are averages of five EPSCs recorded 5 min before and 30 min after the respective induction procedures. For this and following figures, values in parentheses indicate numbers of cells examined except noted otherwise.

depolarization mechanisms for LTP induction by high-frequency stimulation protocols. Thus, our data suggest that mechanisms intrinsic to LTP induction in postsynaptic neurons are impaired in the mutant synapses. Together, the capability to induce LTP in the PFC was lost in DAT KO mice, regardless of the induction protocols.

Elevated DA levels in PFC impair LTP in mutant and normal mice

To measure the impact of DAT deletion on the "true" basal extracellular concentrations of DA in the PFC, we applied a quantitative low perfusion rate microdialysis approach (Smith et al., 1992; Cyr et al., 2006) (Fig. 2*A*,*B*). As shown in Figure 2*B*, basal DA level in the PFC was markedly (~3.6-fold) higher in DAT KO mice compared with their WT littermates. The enhanced DA tone in the PFC of mutant mice is consistent with a deficient DA clearance mechanism that causes accumulation of DA in the extracellular space in these mice.

To test whether pharmacological elevation of dopaminergic tone also impairs prefrontal LTP in normal animals, we acutely injected WT mice with various DA agonists and killed the animals 30 min later for LTP assessments. Amphetamine is a potent DA agonist that raises extracellular DA levels in several brain areas, including the mouse PFC (Ventura et al., 2004), by blocking and reversing the action of DAT (Amara and Kuhar, 1993). At a psychotomimetic dose (10 mg/kg, i.p.) (Ralph et al., 1999), amphetamine completely abolished prefrontal LTP (Fig. 2*D*,*F*), whereas the saline injection was without effect (Fig. 2*C*,*F*). Transient elevation of extracellular DA by the selective DAT blocker GBR12909 (10 mg/kg, i.p.) (Carboni et al., 2006) also severely impaired LTP induction (Fig. 2*E*,*F*). These results suggest that pharmacological treatments that acutely increase prefrontal DA levels *in vivo* can block induction of PFC LTP. It is thus likely that the absence of LTP in DAT KO mice is attributable to the hyperdopaminergic tone and less to compensatory mechanisms in the synapse that may be associated with the lack of DAT during development.

Stimulation of D_2-class receptors mediates hyperdopaminergic impairment of prefrontal LTP

We next investigated which receptor class might mediate the DA effect in attenuating prefrontal LTP. LTP was unaffected by intraperitoneal injections of SKF81297, a full D_1 agonist at either moderate (3 mg/kg) (Fig. 3*A*,*C*) or high (10 mg/kg) (Fig. 3*B*,*C*) doses. In contrast, stimulation of D_2-class receptors by the agonist quinpirole (intraperitoneally) inhibited LTP in a dose-dependent manner (Fig. 3*D–F*). To further investigate the inhibition of D_2 overactivation on LTP, we examined LTP in WT slices by including quinpirole in the extracellular recording solution *in vitro*. Bath-applied quinpirole (10 μM) completely abolished prefrontal LTP (95.3 ± 4.2%; $p < 0.001$ vs WT) (Fig. 3*G*). Collectively, these data demonstrate that excessive activation of D_2-class receptors is detrimental to LTP induction in the PFC.

In DAT KO mice, a sustained hyperdopaminergic tone alters sensitivity of both postsynaptic D_1- and D_2-class receptors (Gainetdinov et al., 1999). Because D_1-class receptor-mediated intracellular signaling classically opposes that mediated by the D_2 class, our data suggests that, in response to the same elevated DA tone, D_2-class dominates D_1-class receptors in shutting off prefrontal LTP. The blockade of prefrontal LTP by acute amphetamine or GBR12909 administration suggests that this dominance also occurs in normal animals. To further investigate this hypothesis, we injected WT mice with a mixture of SKF81297 and quinpirole to simultaneously stimulate both D_1- and D_2-class receptors. LTP recorded from slices prepared from these animals was markedly impaired (119.5 ± 3.0%; $p < 0.01$ vs WT), mimicking the effect of quinpirole alone (Fig. 3*H*). Thus, despite their opposite effects in many signaling pathways, D_2-class receptors appear to dominate D_1-class receptors in the regulation of prefrontal LTP.

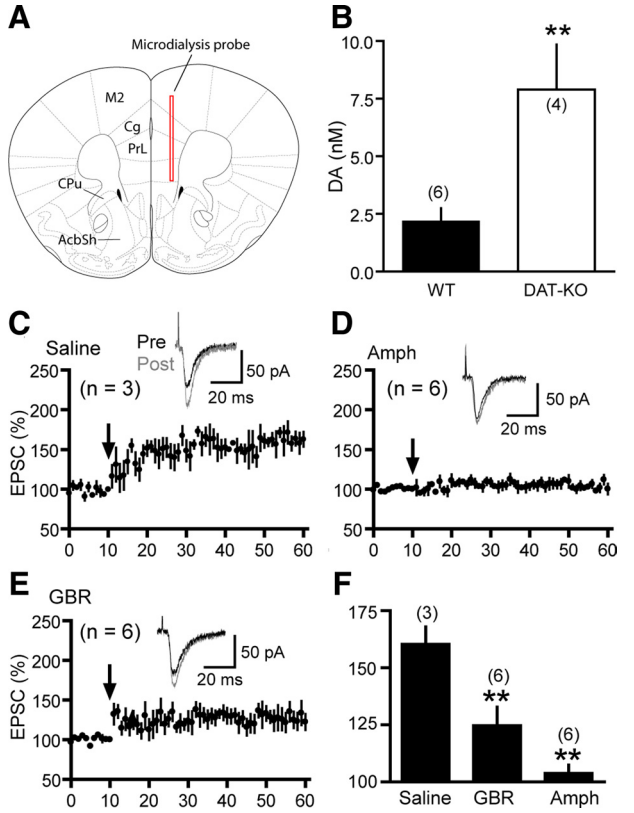

Figure 2. Elevation of extracellular DA levels impairs LTP in the PFC. ***A***, Schematic showing localization of the *in vivo* microdialysis probe (red rectangular) in the PFC. CPu, Caudate–putamen; AcbSh, accumbens nucleus shell; M2, secondary motor cortex. ***B***, Extracellular DA levels in the PFC of freely moving mice measured using quantitative low perfusion rate microdialysis. ***C–E***, LTP induced on slices prepared from WT mice that received single injections of saline (***C***), amphetamine (10 mg/kg, i.p.; Amph; ***D***), or GBR12909 (10 mg/kg, i.p.; GBR; ***E***). ***F***, Summary of effects of *in vivo* dopaminergic manipulations on prefrontal LTP. LTP was induced by the TBS protocol (arrows). Insets show representative EPSCs recorded before and after LTP induction. In this and following figures, mice were killed 30 min after drug injection. $^{**}p < 0.01$ vs WT (***B***) or saline (***F***), two-tailed Student's *t* tests.

Inhibition of D_2 receptors rescues prefrontal LTP in DAT KO mice

Given the dominant role of D_2 receptors in negatively gating LTP in the PFC, we examined whether antagonizing D_2-class receptors could restore LTP in DAT KO mice (Fig. 4). Bath application of haloperidol (10 μM), a typical antipsychotic drug and a potent inhibitor of D_2 receptors with relatively high specificity (Seeman and Van Tol, 1994), enabled LTP (Fig. 4*A*), indicating that D_2 inhibition rescued LTP *in vitro*. More importantly, acute blockade of the D_2-class receptors after a single intraperitoneal injection of haloperidol (1 mg/kg) similarly rescued LTP in KO mice (Fig. 4*B*,*F*), demonstrating that the LTP deficit in the mutant mice can be rescued *in vivo*. Haloperidol at the same dose had no effect on prefrontal LTP in WT mice, nor did saline injection on LTP in KO mice (Fig. 4*F*). We also observed similar *in vivo* rescuing effects of LTP by another selective D_2-class antagonist, raclopride (3 mg/kg) (Seeman and Van Tol, 1994), and by the atypical antipsychotic drug clozapine (6 mg/kg) (Fig. 4*F*) (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). It should be noted that the pharmacological profile of clozapine also includes antagonism at D_1-class receptors, 5-HT_2 receptors, and α1-adrenergic receptors (Ereshefsky et al., 1989; Meltzer 1994). The *in vivo* rescue of LTP by acute D_2 blockade in the KO mice (haloperidol, 137.7 ± 6.8%; raclopride, 140.0 ± 10.8%; clozapine, 133.5 ± 9.7%) appeared submaximal when compared with WT (157.1 ± 7.2%) or saline-treated WT (159.6 ± 8.3%) mice. However, chronic exposure to haloperidol ($0.5\ mg \cdot kg^{-1} \cdot d^{-1}$, 14 d) (Fig. 4*E*) or clozapine ($4\ mg \cdot kg^{-1} \cdot d^{-1}$, 14 d) (supplemental Fig. S3, available at www.jneurosci.org as supplemental material) completely restored LTP in the mutant mice (Fig. 4*F*).

Postsynaptic D_1-class DA receptors are also dysregulated by the sustained hyperdopaminergic tone in DAT KO mice (Gainetdinov et al., 1999). We found that acute administration of SCH23390 (0.01 mg/kg, s.c.), a D_1-class receptor antagonist, to the mutant mice did not rescue LTP (Fig. 4*C*,*F*). Furthermore, administration of SKF81297 (3 mg/kg, i.p.) failed to enable LTP (Fig. 4*D*,*F*). Thus, despite antagonism of certain aspects of D_2-mediated signaling, activation of D_1 was unable to mimic the effect of D_2 blockade in rescuing LTP in the PFC. These data further support the view that D_2 signaling may override D_1 signaling under the same hyperdopaminergic condition, blocking LTP.

Altered synaptic transmission in PFC synapses in DAT KO mice

Deficits in basal synaptic properties could prevent LTP induction. For instance, impairment to NMDAR function could inhibit induction of LTP (Kiyama et al., 1998), and saturation of AMPARs, in some cases (Ungless et al., 2001), could occlude LTP induction. Thus, we performed a detailed characterization of basal synaptic properties in the PFC of DAT KO mice. To evaluate synaptic NMDAR and AMPAR functions, we measured the ratio of NMDAR- to AMPAR-mediated EPSCs (NMDA/AMPA ratio) using two methods. In the kinetics based method, the AMPAR- and NMDAR-mediated components were distinguished by their differential activation and inactivation kinetics (Fig. 5*A*). In the pharmacology-based method, the two components were isolated by recording EPSCs at +40 mV in the absence followed by the presence of AP-5 (50 μM) (Fig. 5*B*). In both cases, the NMDA/AMPA ratio was significantly decreased in DAT KO mice.

The decreased NMDA/AMPA ratio in the mutant synapse could be attributable to an enhancement in AMPAR number and/or function, a suppression in NMDAR number and/or function, or both. To investigate these possibilities, we analyzed the AMPAR-mediated mEPSCs (Fig. 5*C–E*). We found no differences in the amplitude of mEPSCs between WT and KO mice (Fig. 5*D*), suggesting that postsynaptic AMPARs are normal in the mutant mice. In contrast, the frequency of mEPSCs was significantly reduced in KO mice (Fig. 5*E*). We also found that the paired-pulse ratio (PPR), a reliable measure of transmitter release probability (Zucker, 1989), was significantly increased in the mutant mice at all intervals examined (Fig. 6), suggesting that the glutamatergic terminals in DAT KO mice are impaired. Thus, the reduction in mEPSC frequency likely reflects a decrease in the probability of neurotransmitter release.

The unaltered AMPAR system suggests that the decreased NMDA/AMPA ratio in DAT KO mice was attributable to a decrease in postsynaptic NMDAR number and/or function. To test this possibility more directly, we analyzed the NMDAR component of mEPSCs. mEPSCs mediated by both AMPARs ($mEPSC_{AMPA}$) and NMDARs ($mEPSC_{NMDA}$) were collected in Mg^{2+}-free extracellular solution, and $mEPSC_{AMPA}$ was recorded in the presence of Mg^{2+} (Fig. 5*F*). $mEPSC_{NMDA}$ was calculated by subtracting the average $mEPSC_{AMPA}$ from the average total mEPSC and measured as the charge transfer. This analysis showed

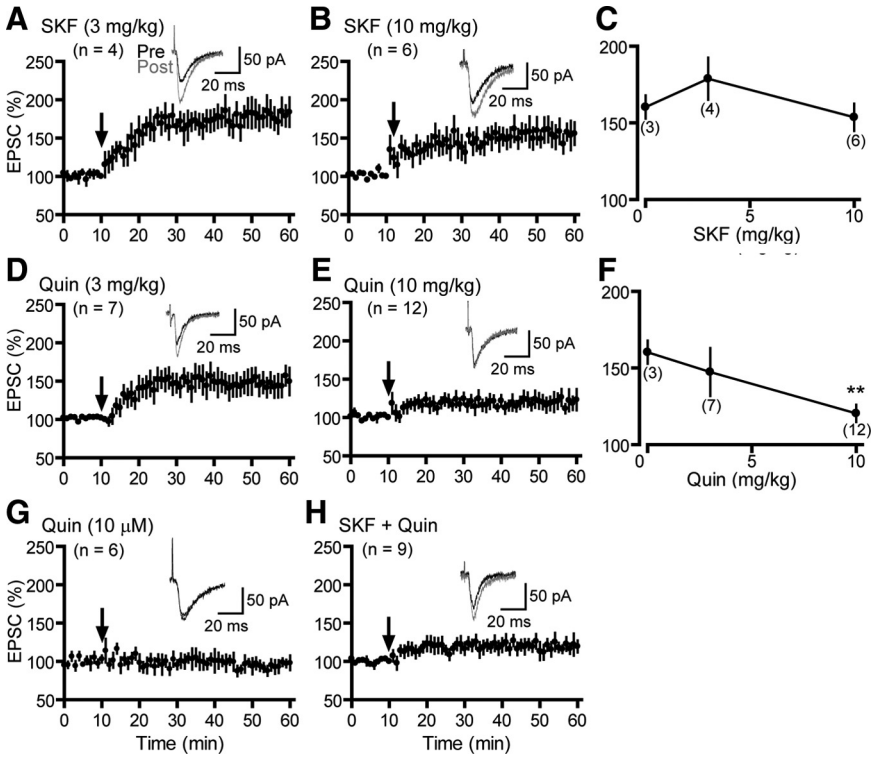

Figure 3. D_2 stimulation inhibits PFC LTP in normal mice. ***A–C***, Lack of effect of SKF81297 (SKF; ***A***, 3 mg/kg; ***B***, 10 mg/kg; ***C***, summary, i.p.) on LTP. Despite the trend, the LTP differences between 0, 3, and 10 mg/kg SKF81297 were not statistically significant (Student's *t* tests). ***D–F***, Dose-dependent blockade of LTP by quinpirole (Quin; ***D***, 3 mg/kg; ***E***, 10 mg/kg; ***F***, summary, i.p.). $^{**}p < 0.01$ vs saline, two-tailed Student's *t* tests. ***G***, Effect of quinpirole (10 μM) on LTP *in vitro*. ***H***, Effect of coinjections of SKF81297 (3 mg/kg) and quinpirole (10 mg/kg) on LTP. Arrows indicate LTP induction by TBS. Insets show representative EPSCs recorded before and after LTP induction.

a significantly reduced $mEPSC_{NMDA}$ in the mutant mice (Fig. 5*G*), further supporting our conclusion that synaptic NMDAR function is diminished in DAT KO mice.

Redistribution of NMDARs from synaptic to extrasynaptic sites in DAT KO mice

To gain insights into the diminished synaptic NMDAR activity in DAT mutant mice at the molecular level, we performed Western blot analysis of glutamate receptors on biochemically fractionated subcellular compartments (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). The TIF is enriched with PSDs and is often used to measure glutamate receptor abundance in the postsynaptic compartment (Gardoni et al., 2001, 2006; Picconi et al., 2004). Protein levels of NMDAR and AMPAR subunits in total cortical homogenates were not different between WT and DAT KO mice (supplemental Fig. S4*C*,*D*, available at www.jneurosci.org as supplemental material), suggesting that expressions of glutamate receptors are essentially unaffected in the mutant cortex. However, we observed a significantly reduced NR1 and slight but not significant reduction of NR2A or NR2B levels in the TIF fraction prepared from DAT KO cortices (supplemental Fig. S4*E*,*F*, available at www.jneurosci.org as supplemental material), indicating a redistribution of NR1 subunit from insoluble to soluble membrane fractions in DAT KO mice. Because TIF may contain both surface and intracellular proteins, these results do not directly imply trafficking of NMDARs from synaptic to extrasynaptic sites but do suggest that NMDARs, particularly the obligatory NR1 subunit, is redistributed away from the postsynaptic compartment of the synapse.

To further explore the potential trafficking of NMDARs between synaptic and extrasynaptic compartments, we performed cell surface biotinylation experiments on PFC slices (Fig. 7). NHS-SS-biotin binds to free amino groups of proteins and, because it is membrane impermeable, can be used to distinguish between cell surface and intracellular proteins. We found that the surface, but not the total, levels of NR1 and NR2A subunits were significantly reduced in DAT KO mice, whereas NR2B subunits displayed a slight but not statistically significant reduction. These results suggest a redistribution of NR1 and NR2A subunits from surface to intracellular compartments. Together with the TIF experiments, our data support the idea that postsynaptic NMDARs, particularly those containing the NR2A subunit, may have been removed from the synapse under hyperdopaminergic conditions.

Amphetamine acutely mimics synaptic deficits in DAT KO mice

To exclude the possibility that the synaptic deficits observed in DAT KO mice might be related to nonspecific adaptive or compensatory changes associated with the constitutive DAT deletion during development, we repeated the electrophysiological analyses in amphetamine-treated WT mice. Acute single injection of amphetamine (10 mg/kg, i.p.) reduced the NMDA/AMPA ratio (Fig. 5*A*), diminished the mEPSC frequency (Fig. 5*C*,*E*), increased PPR (Fig. 6), and suppressed $mEPSC_{NMDA}$ (Fig. 5*G*). These data recapitulated the synaptic deficiencies observed in DAT KO mice, further supporting the role of elevated DA tone per se in the synaptic transmission abnormalities in these hyperdopaminergic models.

Normalizing NMDAR function does not fully restore prefrontal LTP

We next investigated the synaptic mechanism that underlies the loss of LTP in the PFC synapse of DAT KO mice. Because prefrontal LTP depends on the activation of NMDARs (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), we first tested whether the LTP deficit might result from hypofunctional NMDARs. For this purpose, prefrontal LTP was evaluated in DAT KO slices under a condition in which the compromised NMDAR function was pharmacologically normalized. ALX5407, a selective inhibitor of the glycine transporter, functions as an indirect glycine site NMDAR agonist (Atkinson et al., 2001). ALX5407 (1 μM) (Konradsson et al., 2006) did not have significant effect on NMDA/AMPA ratio in WT but completely restored the ratio in KO mice (Fig. 8*A*,*B*). This restoration occurred in the absence of any modulation of $EPSC_{AMPA}$ (Fig. 8*C*), suggesting that ALX5407 normalized NMDAR function in the KO mice. We then examined whether ALX5407 could restore LTP in the mutant synapse. We found that bath application of ALX5407 only partially rescued prefrontal LTP in the mutant mice ($135.4 \pm 7.6\%$, $p < 0.05$ vs WT, or ~62% of WT control) (Figs. 8*D*, 9*E*) and had no effect on LTP in WT synapses (Fig. 9*E*).

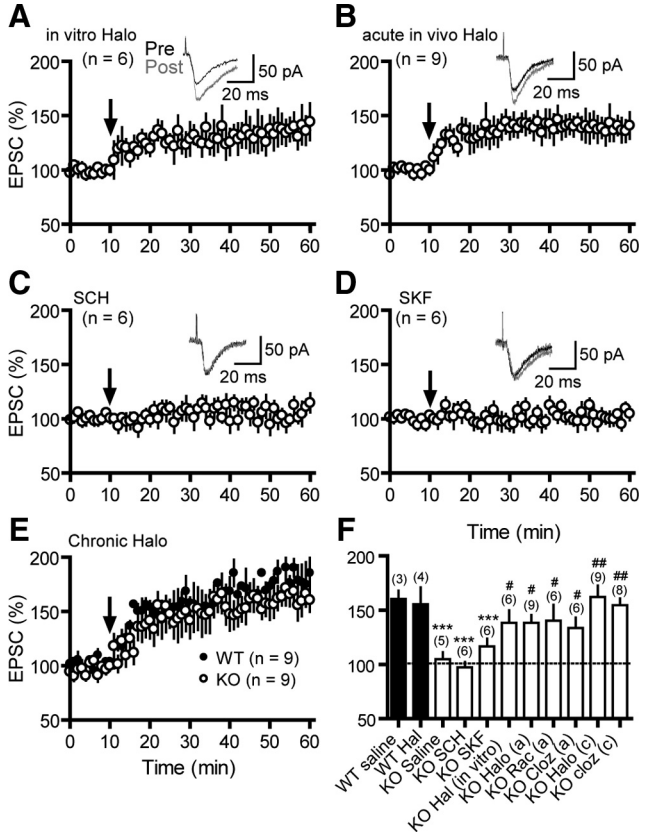

Figure 4. Rescue of prefrontal LTP by blockade of D_2 receptors in DAT KO mice *in vitro* and *in vivo*. ***A***, Bath application of haloperidol (Halo; 10 μM) enabled LTP in KO slices. ***B***, LTP induced on slices prepared from KO mice that received single acute injection of haloperidol (1 mg/kg, i.p.). ***C***, ***D***, Lack of effect of SCH23390 (0.01 mg/kg, s.c.; SCH; ***C***) or SKF81297 (3 mg/kg, i.p.; SKF; ***D***) on LTP in KO mice. ***E***, LTP induced on slices obtained from WT and KO mice treated daily with haloperidol (0.5 mg/kg, i.p.) for 14 d. Mice were killed 30 min after the last injection. ***F***, Summary of LTP under different conditions. Arrows indicate LTP induction by TBS. Insets are representative EPSCs recorded before and after LTP induction. The dashed line in ***F*** indicates the baseline synaptic response. $^{***}p < 0.001$ vs WT saline; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs KO saline, one-way ANOVA followed by Tukey–Kramer tests. Rac, Raclopride; Cloz, clozapine.

Thus, although hypofunctional NMDARs may contribute to the LTP deficiency in the KO mice, additional mechanisms must be involved.

Inhibition of postsynaptic PP1 rescues prefrontal LTP independent of NMDAR modulation

We explored the hypothesis that PP1, a protein phosphatase enriched in the PSD (Shields et al., 1985), may be involved in PFC LTP. PP1 can gate hippocampal LTP by regulating the activity of its substrate, CaMKII in postsynaptic neurons (Blitzer et al., 1995). Overactivation of D_2-class receptors in response to hyperdopaminergic tone may elevate PP1 activity by antagonizing this cAMP/PKA cascade or by triggering other Ca^{2+}-dependent signaling modules, e.g., the calcineurin (PP2B) (Greengard et al., 1999), preventing induction of LTP. Consistent with this idea, we found that the total level of α-CaMKII was normal, but the phosphorylation of α-CaMKII at Thr286 was markedly reduced in the cortex of DAT KO mice compared with WT littermates. In addition, NMDA-stimulated α-CaMKII phosphorylation was lost in the mutant slices (supplemental Fig. S5, available at www.jneurosci.org as supplemental material). These results suggest that cortical PP1 activity is constitutively elevated in DAT KO mice.

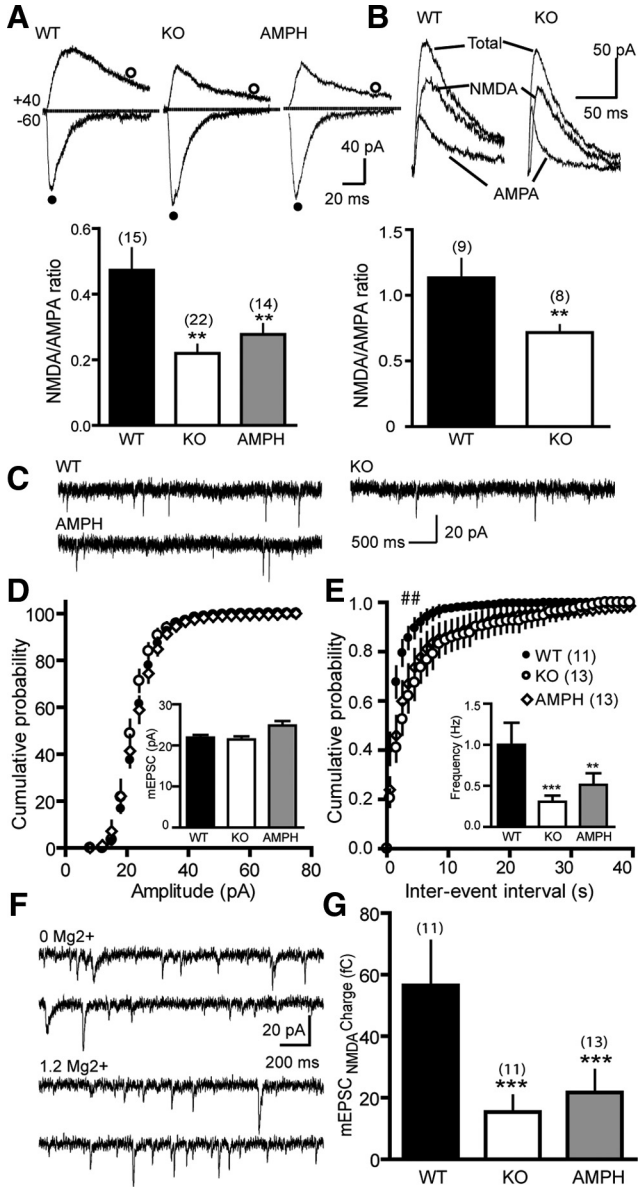

Figure 5. Altered synaptic transmission in the PFC of DAT KO and amphetamine-treated mice. ***A***, NMDA/AMPA ratio determined based on the differential kinetics of $EPSC_{NMDA}$ and $EPSC_{AMPA}$. Top, Sample EPSCs recorded at holding potentials of -60 (to record $EPSC_{AMPA}$) and $+40$ mV (to record both $EPSC_{AMPA}$ and $EPSC_{NMDA}$) from slices prepared from WT, KO, and amphetamine (AMPH; 10 mg/kg, i.p.)-treated WT mice. Bottom, summary of mean NMDA/AMPA ratios. NMDA/AMPA ratio is defined as the amplitude of the NMDAR component 80 ms after stimulation at $+40$ mV (○) divided by the peak AMPAR component at -60 mV (●). ***B***, NMDA/AMPA ratio determined based on pharmacologically isolated $EPSC_{NMDA}$ and $EPSC_{AMPA}$. Top, Examples of total EPSC, $EPSC_{AMPA}$, and $EPSC_{NMDA}$ recorded from a WT and a KO neuron. Bottom, Summary of mean NMDA/AMPA ratios. ***C***, Sample mEPSCs recordings. ***D***, ***E***, Cumulative probabilities of amplitude (***D***) and interevent interval (***E***) distributions of mEPSCs. Insets, Mean amplitudes and frequencies. ***F***, Sample mEPSCs recorded at -60 mV in the absence and presence of Mg^{2+}. ***G***, Summary of charge transfer mediated through $mEPSC_{NMDA}$. $mEPSC_{NMDA}$ was derived by subtracting the average $mEPSC_{AMPA}$ from the average mEPSC, and the area under the resultant average $mEPSC_{NMDA}$ was measured as the charge transfer. $^{\#\#}p < 0.01$, Kolmogorov–Smirnov tests vs WT. $^{**}p < 0.01$; $^{***}p < 0.001$ vs WT, two-tailed Student's *t* tests.

To investigate whether the overactivation of postsynaptic PP1 contributes to the LTP failure in DAT KO mice, we tested the effect of PP1 inhibition on LTP induction. Loading postsynaptic cells with microcystin LR (10 μM) (Launey et al., 2004; Belmeguenai and Hansel, 2005), a potent inhibitor of PP1/PP2A,

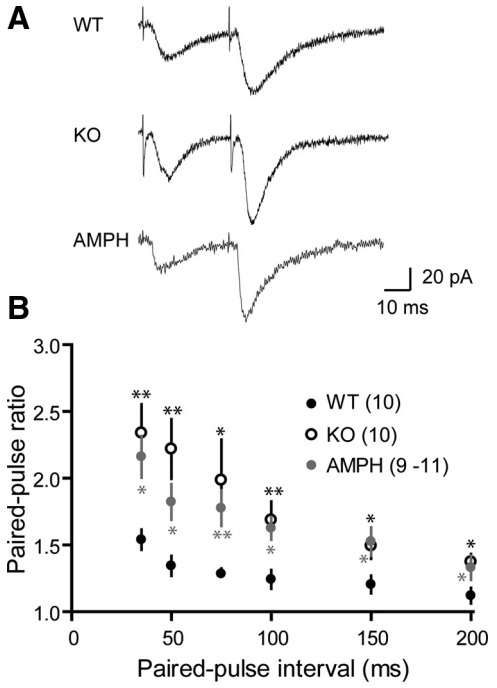

Figure 6. Increased paired-pulse facilitation in DAT KO and amphetamine-treated WT mice. ***A***, Representative recordings of PPR at the interpulse interval of 35 ms from slices prepared from WT, KO, and amphetamine (AMPH; 10 mg/kg, i.p.)-treated WT mice. ***B***, Summary of mean PPR at various interpulse intervals. $*p < 0.05$; $**p < 0.01$ vs corresponding WT values (*t* tests).

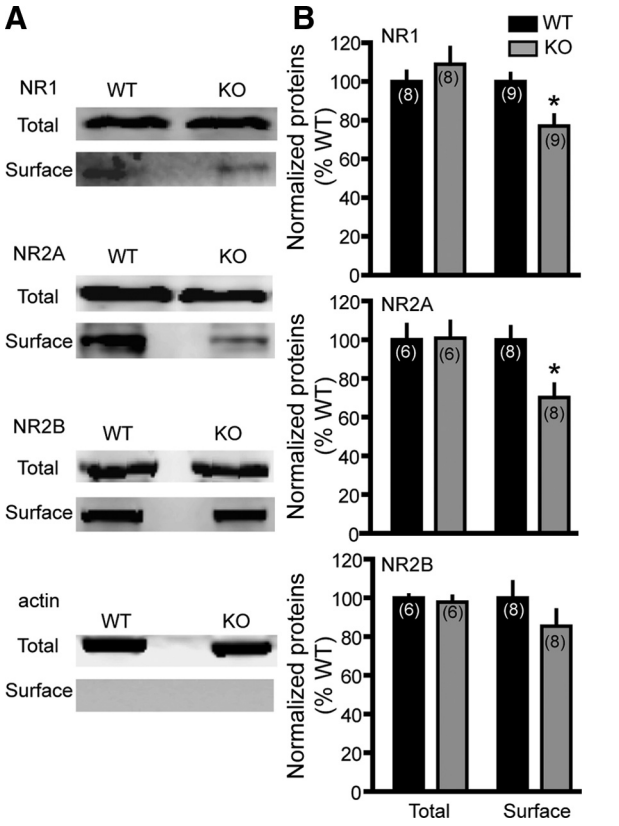

Figure 7. Reduced surface NMDAR receptors in DAT KO mice as analyzed by surface biotinylation. Sample blots (***A***) and densitometric summary (***B***) show significantly reduced surface NR1 and NR2A subunit levels in DAT KO mice. Surface NR2B level was also lower in KO mice, but the decrease did not reach a significance level. Total levels of NR1, NR2A, and NR2B were similar between WT and mutant mice, consistent with the results obtained from the total homogenates in the absence of biotinylation (supplemental Fig. S4 *A*, *B*, available at www.jneurosci.org as supplemental material). Note the absence of actin bands from the surface samples (***A***, bottom), confirming the validity of the approach. The same amount of protein was loaded per lane. $*p < 0.05$, Student's *t* test. Numbers of mice analyzed were indicated in parentheses. Results are presented in arbitrary units normalized to corresponding protein levels observed in WT mice.

restored LTP in DAT KO slices (Fig. 8*E*). In contrast, loading cells with the PP2A-specific inhibitor fostriecin (100 nM) (Launey et al., 2004; Belmeguenai and Hansel, 2005) was without effect (Figs. 8*H*, 9*E*). Thus, the rescue of LTP by microcystin LR in DAT KO cells was mediated principally by inhibition of PP1. Microcystin LR had little effect on LTP in WT neurons (Fig. 9*E*), indicating that the endogenous PP1 activity in the synapse under normal conditions is sufficiently low so that its additional inhibition would not further facilitate LTP. Microcystin LR loading affected neither $EPSC_{AMPA}$ nor $EPSC_{NMDA}$ (Fig. 8*F*, *G*), consistent with a previous report that PP1 does not affect basal synaptic strength (Morishita et al., 2001). Our data exclude the possibility that the LTP enabling by PP1 inhibition was mediated by PP1 modification of NMDA and/or AMPA receptors and support the notion that, during LTP induction, PP1 gains access to synaptic substrates whose activity, or lack thereof, is critical for LTP.

If the PP1-dependent rescue of prefrontal LTP in mutant synapses was independent of NMDAR modifications as a consequence of PP1 inhibition, NMDAR normalization and PP1 inhibition should result in LTP that is greater than either manipulation alone. Indeed, bath-applied ALX5407 elicited additional LTP in cells postsynaptically loaded with microcystin (Figs. 8*I*, 9*E*), supporting the idea that the NMDAR- and PP1-dependent rescues of PFC LTP in mutant synapses are additive. Thus, the loss of PFC LTP in DAT KO mice is likely mediated by two separable mechanisms, hypofunctional NMDARs and hyperactive PP1 signaling.

LTP rescue by D_2 receptor blockade is mediated by postsynaptic PP1

We finally determined which of the mechanisms, the NMDAR hypofunction or the PP1 hyperactivation, mediates the D_2-dependent rescue of prefrontal LTP. We found that a single injection of haloperidol did not significantly affect the NMDA/AMPA ratio in both WT and KO mice (Fig. 8*A*,*B*), suggesting that haloperidol-restored LTP was independent of NMDAR modification. In contrast, loading cells with PKI(6–22) (20 μM) (Yasuda et al., 2003), a membrane-impermeable inhibitory peptide of PKA, prevented LTP in slices prepared from haloperidol-treated DAT KO mice (Fig. 9*A*). Because the D_2-mediated PP1 signaling can presumably be blocked by inhibition of the downstream PKA in postsynaptic cells, these data suggest that blocking D_2 receptors rescued LTP via suppression of the postsynaptic PP1 signaling. In support of this interpretation, we found that loading cells with microcystin LR (10 μM) in slices from haloperidol-treated mice resulted in LTP (135.2 ± 11.7%) that was comparable with LTP rescued by either PP1 inhibition (141.5 ± 8.3%) or haloperidol (137.7 ± 6.8%) alone (Fig. 9*E*). In contrast, incubation of slices prepared from haloperidol-treated DAT KO mice in the presence of ALX5407 (1 μM) resulted in LTP (157.3 ± 5.9%) that was significantly greater than LTP enabled by either ALX5407 (135.4 ± 7.6%) or haloperidol (Fig. 9*C*,*E*). Finally, if D_2 receptors "gate" LTP via regulating the activity of PP1, constitutive suppression of PP1 should permit LTP even when D_2 is excessively activated. Indeed, we observed that loading cells with microcystin LR (10 μM) prevented the quinpirole-induced inhibition of LTP (Fig. 9*D*,*E*). Together, our findings demonstrate

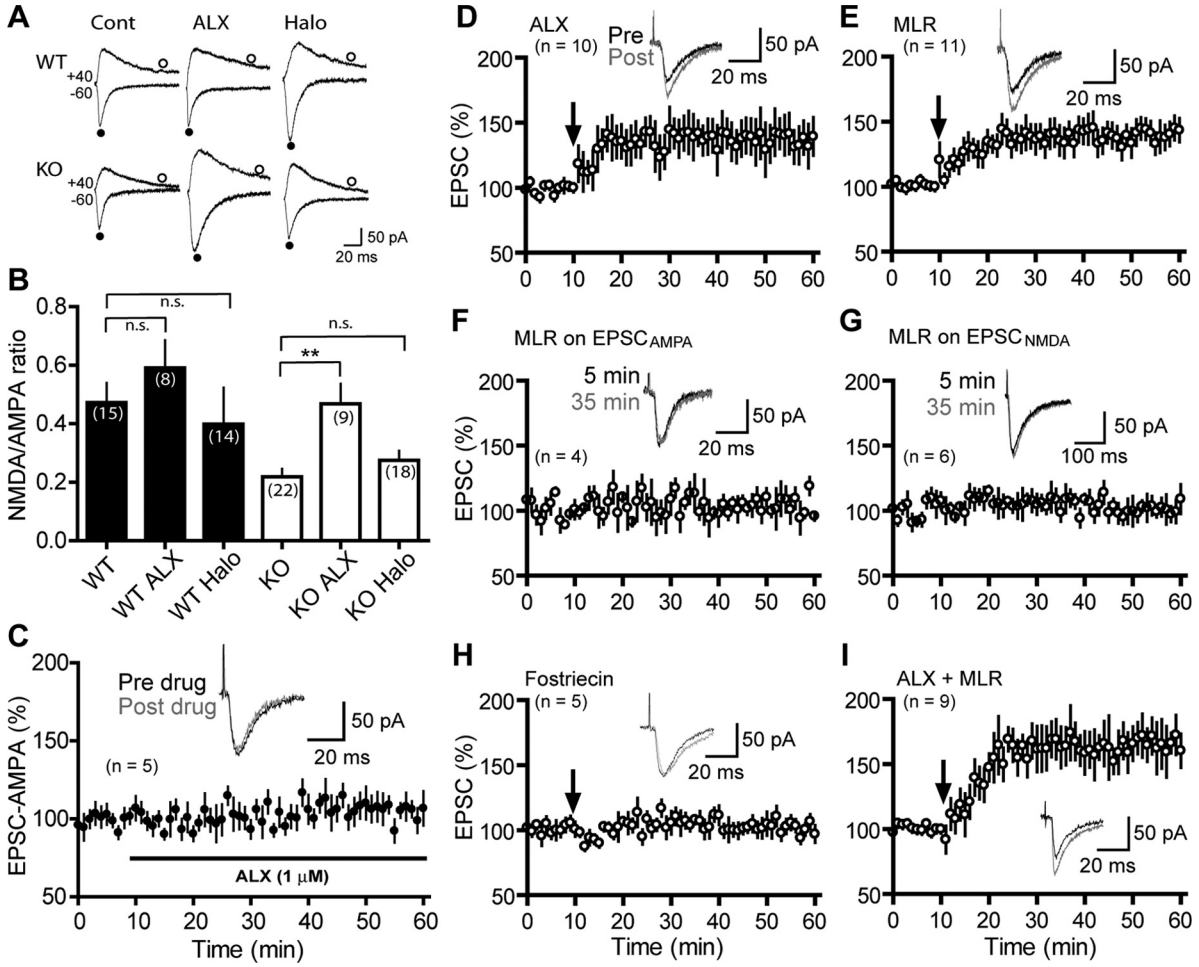

Figure 8. Normalizing NMDARs or inhibiting postsynaptic PP1 independently rescues prefrontal LTP in DAT KO mice. ***A***, Effects, or lack thereof, bath-applied ALX5407 (ALX; 1 μM) or *in vivo* injected haloperidol (Halo; 1 mg/kg, i.p.) on representative EPSCs recorded at −60 or +40 mV. Cont, Control. ***B***, Mean NMDA/AMPA ratios determined using the kinetics-based method. $^{**}p < 0.01$, two-tailed Student's *t* tests; n.s., not significant. ***C***, Lack of effect of ALX5407 (1 μM) on EPSC$_{AMPA}$ in WT slices. ***D***, Bath application of ALX5407 (1 μM) rescued LTP in KO slices. ***E***, Loading cells with microcystin LR (MLR; 10 μM) rescued LTP in KO neurons. ***F***, ***G***, Loading MLR postsynaptically had no effect on basal EPSC$_{AMPA}$ (***F***) or EPSC$_{NMDA}$ (***G***) in KO neurons. ***H***, Loading fostriecin (100 nM) in postsynaptic cells failed to rescue LTP in KO neurons. ***I***, Bath application of ALX5407 (1 μM) further enhanced MLR-rescued LTP in KO neurons. Arrows indicate LTP induction by TBS. Insets show example EPSCs recorded before and after LTP induction (***D***, ***E***, ***H***, ***I***) or drug application (***C***) or 5 and 35 min after the establishment of whole-cell configuration (***F***, ***G***). Data presented in ***D***, ***E***, ***H***, and ***I*** are also summarized in Figure 9*E*.

that postsynaptic D_2 receptors tightly couple to the PP1 signaling, rather than NMDAR activity, in postsynaptic cells to control LTP induction in the PFC.

Discussion

In this study, we show that excessive DA tone impairs induction of LTP in the PFC. Although presynaptic contributions cannot be excluded, the LTP blockade can be mediated by overstimulation of postsynaptic D_2-class receptors that results in elevated postsynaptic PP1 activity via a heterosynaptic gating mechanism (Fig. 10). Excessive PP1 activity may keep an abnormally large portion of synaptic CaMKII unphosphorylated, locking the bistable CaMKII/PP1 switch in the "off" state refractory to activation by NMDARs during LTP induction, thus preventing LTP (Lisman and Zhabotinsky, 2001).

Dopaminergic modulation of prefrontal transmission and plasticity

It is believed that DA transmission plays a permissive or facilitating role in LTP in the PFC. In slices, activation of D_1-class receptors enhances, and blockade of them attenuates the late, protein synthesis-dependent maintenance phase of LTP in layer V prefrontal synapses; blockade of D_2-class receptors is without effect (Huang et al., 2004). Background or phased DA has also been shown to facilitate LTP induction (Blond et al., 2002; Matsuda et al., 2006). Similar facilitating effects of DA on LTP have been observed in anesthetized (Gurden et al., 1999, 2000) and behaving animals performing learning tasks (Li et al., 2003; Lemon and Manahan-Vaughan, 2006). The dopaminergic facilitation of LTP is thought to be achieved by DA released during LTP induction and is, in most cases, mediated through the cAMP/PKA signaling (Jay et al., 1998; Gurden et al., 2000; Otani et al., 2003; Huang et al., 2004). These studies highlight the role of phasic DA and postsynaptic D_1-, but not D_2-, class receptors in promoting synaptic plasticity.

Overactive dopaminergic signaling is implicated in prefrontal dysfunctions associated with schizophrenia, ADHD, and stress (Seeman, 1987; Goldman-Rakic, 1995; Castellanos and Tannock, 2002; Arnsten, 2009). How elevated DA tone affects LTP, and synaptic transmission in the PFC in general, has not been investigated. Using a genetic model of hyperdopaminergia, we dem-

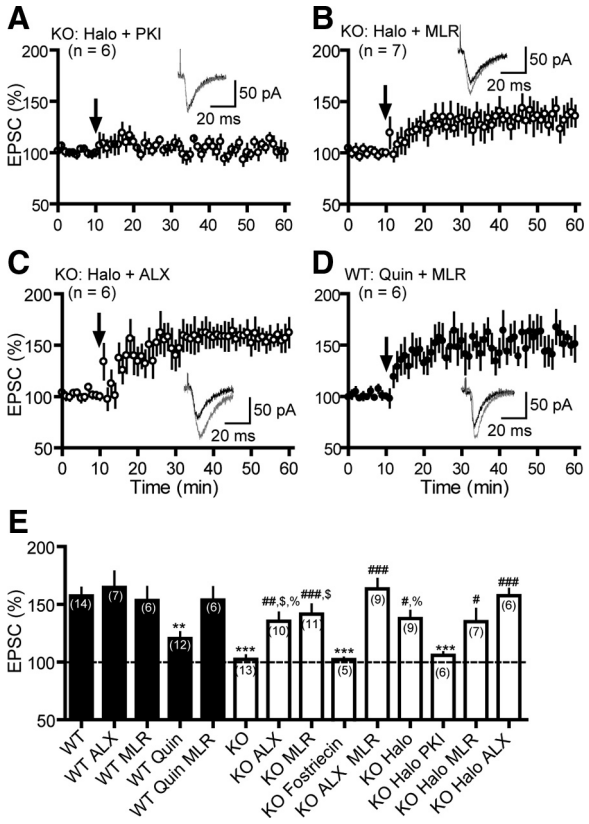

Figure 9. Rescue of prefrontal LTP by D_2 blockage depends on postsynaptic PP1 signaling but not NMDAR modulation. ***A***, Postsynaptic loading of PKI(6–22) amide (PKI; 20 μM) blocked haloperidol (Halo)-rescued LTP. ***B***, Loading postsynaptic neurons with microcystin LR (MLR; 10 μM) did not further enhance haloperidol-rescued LTP in KO neurons. ***C***, LTP induced from slices prepared from haloperidol (1 mg/kg, i.p.)-treated DAT KO mice in the presence of ALX5407 (ALX; 1 μM) in the bath. ***D***, Loading cells with MLR (10 μM) prevented the quinpirole (Quin; 10 mg/kg, i.p.) blockade of LTP. ***E***, Summary of LTP under various conditions. Dashed line indicates the baseline synaptic response. Arrows indicate TBS stimulation. Insets show representative EPSCs recorded before and after LTP induction. $^{**}p < 0.01$, $^{***}p < 0.001$ vs WT; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs KO; $^{\$}p < 0.05$ vs ALX + MLR (KO); $^{\%}p < 0.05$ vs Halo + ALX (KO); one-way ANOVA with *post hoc* Tukey–Kramer tests.

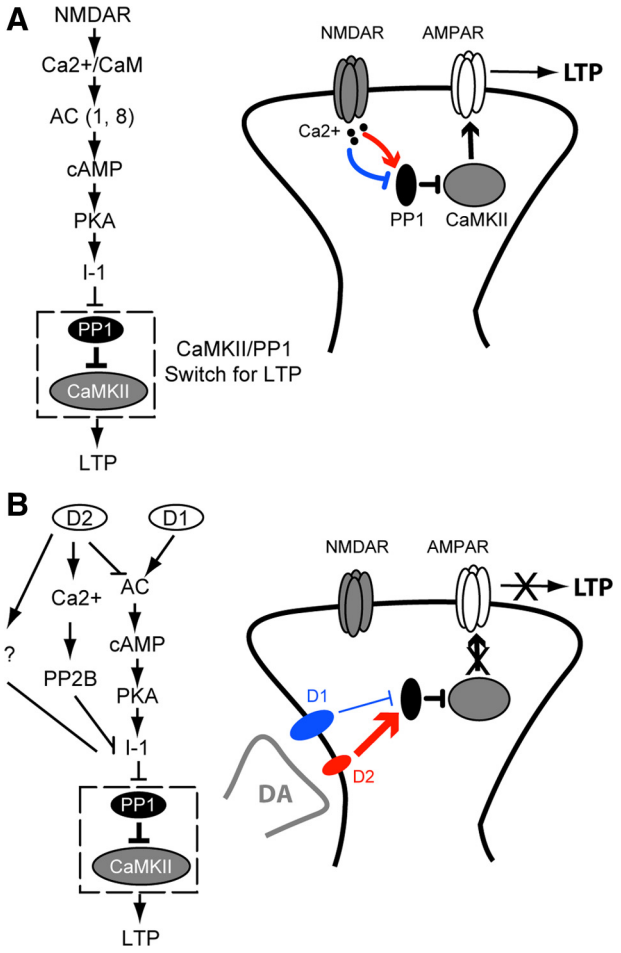

Figure 10. Working models for homosynaptic versus heterosynaptic gating of LTP in postsynaptic neurons. ***A***, NMDAR-operated homosynaptic gating model (Blitzer et al., 1995). Left, Key components of the LTP gating pathway. During LTP induction, Ca^{2+} enters through NMDARs and binds to CaM, resulting in the activation of CaM-stimulated adenylyl cyclases [AC (1 and 8)]. Adenylyl cyclases stimulate the production of cAMP, which in turn activates PKA. PKA then phosphorylates I-1 to reduce the activity of PP1, which regulates the phosphorylation of CaMKII. Right, This gating (blue) occurs transiently during LTP induction within the activated synapse, is most effective to permit LTP induced by certain patterns of synaptic stimulation that activate protein phosphatases (red arrow), and represents an activity-dependent homosynaptic gate for LTP. ***B***, DA receptor-operated heterosynaptic gating model (this study). Left, Schematic of intracellular DA signaling pathways and their coupling to the CaMKII/PP1 switch for LTP induction. DA stimulates D_2 receptors and elevates postsynaptic PP1 activity, presumably through inhibition of the cAMP/PKA-dependent signaling, activation of the Ca^{2+}-dependent PP2B/calcineurin signaling, other unidentified pathways (?), or combinations of the above. Excessive activation of PP1 may lock CaMKII at a stable, dephosphorylated state refractory to activation by NMDARs during LTP induction (Lisman and Zhabotinsky, 2001). Stimulation of D_1-class receptors can, in principle, activate the cAMP/PKA pathway and suppress PP1 activity. This heterosynaptic gating may occur at a dendritic spine harboring D_1 and D_2 DA receptors and receiving dopaminergic input (right). Although significantly less represented in spines, D_2 receptors dominate spine D_1 receptors in the regulation of PP1 activity under hyperdopaminergic conditions. This scheme provides a powerful, constitutive control of LTP by background DA tone and may influence LTP regardless of induction protocols. We note that these models represent simplified schemes, because other intracellular signaling processes in postsynaptic neurons, impairments to NMDARs, and presynaptic mechanisms may also contribute to hyperdopaminergic impairments of LTP.

onstrate that elevated DA tone acts at the D_2-class receptors to impair prefrontal LTP through increasing the postsynaptic PP1 activity. In addition, the hyperdopaminergic tone impairs at least two other synaptic mechanisms. First, glutamate release at glutamatergic terminals (Fig. 6), as well as DA release/clearance at dopaminergic terminals (Jones et al., 1998), are impaired in the KO mice. Second, postsynaptic NMDARs are diminished. This diminishment is mediated, at least in part, by the removal of NR2A-containing NMDARs from mutant synapses, as suggested by the subcellular fractionation and surface biotinylation experiments. Because TIF and PSD contain both surface and intracellular proteins, the decreased NR1 level in TIF simply suggests a reduced availability of this NMDAR obligatory subunit in the postsynaptic compartment and does not necessarily suggest a reduction or subunit dysregulation of assembled NMDARs at the surface. Conversely, the selective reduction of surface, but not total, NR1 and NR2A subunits in mutant mice suggests that NR1/NR2A NMDARs are removed from the surface pool. Assuming that synaptic and nonsynaptic surface receptors (extrasynaptic) are uniformly affected, this result, together with the postsynaptic TIF data, strongly suggests a redistribution of postsynaptic NMDARs, especially those containing NR2A from synaptic to nonsynaptic sites under hyperdopaminergic conditions. Redistribution of NMDAR subunits between synaptic and extrasynaptic compartments has been shown to be highly sensitive to DA signaling and psychostimulant action and can be mediated by a number of mechanisms, including phosphorylation, ubiquitination, and scaffolding (Dunah and Standaert, 2001; Gardoni et al., 2006; Hallett et al., 2006; Schilström et al., 2006; Gao and

Wolf, 2008; Huang et al., 2009; Mao et al., 2009). Additional experiments are needed to elucidate the mechanisms underlying redistribution of NMDAR subunits in response to elevated DA tone.

Our quantitative microdialysis experiments demonstrate that extracellular levels of DA in the PFC of DAT KO are increased by ~3.6-fold from 2.2 nM in WT to 7.9 nM in KO mice. These direct measurements support previous indirect indications of altered DA function in the frontal cortex of these mice, such as blunted tissue levels of DA and its extracellular metabolite homovanillic acid (Pogorelov et al., 2005). Notably, in the striatum of DAT KO mice, extracellular DA is increased by approximately fivefold from 7–10 nM in WT mice to 35–45 nM in KO mice (Jones et al., 1998; Cyr et al., 2006). Given the relatively low level of DAT expression in the cortex (Freed et al., 1995; Sesack et al., 1998), it might be expected that the impact of DAT deletion on DA dynamics in the PFC could be significantly less than what we observed here. A potential explanation for this observation could be that striatal and accumbal DA may spill over (leak) to neighboring regions, thus elevating DA levels in regions that normally have very low levels of extracellular DA. In fact, it has been estimated that DA released to striatal neurons of DAT KO mice can diffuse for millimeters, thus affecting large populations of neurons via "volume transmission" (Jones et al., 1998). It should be noted also that the effect of the trauma layer in the tissue surrounding the microdialysis probe on the amount of DA recovered in dialysates (Bungay et al., 2003; Borland et al., 2005) might differ in WT and DAT KO mice, which may potentially contribute to the difference in DA levels observed between genotypes.

Several lines of evidence presented here support the view that the synaptic deficits in DAT KO mice are likely direct consequences of the high DA tone. First, total levels of cortical NMDAR and AMPAR subunits are unaltered in the KO mice. Second, acute suppression of postsynaptic PP1 mimics and occludes the D_2-mediated rescue of LTP in KO mice, which together with an acute pharmacological normalization of NMDAR activity, fully restores LTP in the mutant mice. Finally, a single injection of amphetamine to WT mice recapitulates the synaptic deficits in the KO mice. These findings suggest that the elevated extracellular DA and dysregulation of downstream DA signaling are primarily responsible for the synaptic transmission and plasticity abnormalities associated with the sustained hyperdopaminergia.

Our findings do not diminish the importance of D_1 receptors in LTP induction but rather reveal a previously unrecognized inhibitory role for D_2-class receptors that becomes dominant under abnormal, hyperdopaminergic conditions. Although both classes of receptors are localized on distal dendrites and spines of layer V PFC pyramidal neurons, D_1 receptors significantly outnumber D_2 receptors (Gaspar et al., 1995). Thus, the overriding role of D_2 receptors may seem surprising. The D_2 dominance in this synapse may reflect a more enriched representation of the signaling component(s) within D_2-coupled signaling cascades. For instance, $G\alpha_i$, a G-protein subunit that inhibits adenylyl cyclase, is highly abundant in cortical postsynaptic densities (Wu et al., 1992). Alternatively, a compartmentalized, D_2-specific signaling system tightly coupled to the LTP induction machinery in the synapse may account for the D_2 dominance. Regardless of the mechanism, our studies identify an important prefrontal process highly sensitive to D_2 dysregulation.

A constitutive heterosynaptic PP1 gate for LTP induction in the PFC

Postsynaptic PP1 plays a gating role in LTP induction in hippocampal CA1 neurons (Blitzer et al., 1995). Certain LTP stimulation patterns activate not only protein kinases necessary for LTP induction but also postsynaptic phosphatases that oppose the induction (O'Dell and Kandel, 1994; Blitzer et al., 1995). Under such conditions, the transient surge of phosphatase activity needs to be suppressed to permit LTP induction. This is presumably achieved through a signaling cascade that involves activation of synaptic NMDARs, influx of Ca^{2+}, stimulation of Ca^{2+}-sensitive adenylyl cyclases, rise of intraspine cAMP, activation of PKA, and stimulation of I-1, leading to inhibition of PP1 that maintains CaMKII at the phosphorylation state necessary for LTP induction (Fig. 10*A*) (Blitzer et al., 1995). This gating occurs during LTP induction within activated synapses, thus can be regarded as a transient, activity-dependent homosynaptic gate for LTP (Fig. 10*A*).

Our studies suggest that a PP1 gate can also be operated by heterosynaptic dopaminergic systems (Fig. 10*B*). The localization of DA receptors in cortical triads that receive both glutamatergic and dopaminergic innervations provides the anatomical basis for a heterosynaptic modulation of excitatory transmission and plasticity at individual cortical synapses by DA. Key components that mediate DA signaling, e.g., PKA and their anchoring proteins, Ca^{2+}-responsive signaling molecules, and major protein phosphatases, are also well represented in dendritic spines (Jordan et al., 2004; Li et al., 2004; Peng et al., 2004). Although the precise signaling intermediates that link D_2 stimulation and PP1 activation in the synapse leading to LTP erosion remains to be determined, activation of spine D_2-class receptors can presumably elevate postsynaptic PP1 activity through inhibition of the cAMP/PKA-dependent signaling, activation of the Ca^{2+}-dependent calcineurin signaling, and/or other unidentified pathways. In principle, stimulation of spine D_1-class receptors, and perhaps other neuromodulatory receptors, can activate the cAMP/PKA pathway and suppress the postsynaptic PP1 activity, opposing the D_2 signaling. Despite the competing D_1 and D_2 signaling, the augmented PP1 activity in mutant PFC synapses suggests that, in response to a same excessive DA tone, the D_2-mediated PP1 activation overrides the D_1-mediated PP1 suppression.

This heterosynaptic gating differs mechanistically from homosynaptic gating. The homosynaptic gate is operated by NMDARs, occurs during the transient induction phase of LTP, and manifests under certain patterns of synaptic stimulation that activate protein phosphatases. Ca^{2+}-stimulated adenylyl cyclases are presumably required to trigger the cAMP/PKA-dependent signaling to maintain an open gate. In contrast, the heterosynaptic gating illustrated here is operated by DA receptors, occurs constitutively because of the continuous presence of background DA tone, does not depend on Ca^{2+}-sensitive adenylyl cyclases, and may influence LTP induction by any protocols. The failure to induce LTP in DAT KO mice by all three protocols tested (TBS, tetanus, and pairing), none of which are thought to activate phosphatases, is consistent with this idea. In addition, the sustained elevation of postsynaptic PP1 activity and a concomitant ~30% reduction of the basal phosphorylation of α-CaMKII, a substrate of synaptic PP1, in the KO mice supports the constitutive nature of the gate.

In summary, our data uncover a mechanism by which excessively elevated DA tone impairs LTP in the mouse mPFC, considered a homolog to the primate dorsolateral PFC (Kolb and

Cioe, 2004) that is important for higher-order cognitive functions, including working memory (Goldman-Rakic, 1995). Recent work has also implicated the mPFC in remote spatial and contextual memory storage (Frankland et al., 2004; Maviel et al., 2004). By abolishing LTP in the mPFC, excessive DA may inhibit encoding and storage of lasting memory traces in this region and impair related behaviors that depend on these memories. Consistent with this view, DAT KO mice display several cognitive deficits, including spatial learning, behavioral flexibility, and the ability to suppress inappropriate responses (Gainetdinov et al., 1999; Morice et al., 2007; Weiss et al., 2007; Dzirasa et al., 2009). In addition, although the beneficial effects of stimulants in improving attention and cognition have been well recognized, it is now clear that high doses of amphetamine impair prefrontal cognitive functions (Arnsten, 2006; Ko and Evenden, 2009; Wood and Anagnostaras, 2009). The detrimental effects are often characterized as inverted-U actions of DA mediated by superanomal stimulation of D_1-class receptors (Zahrt et al., 1997; Seamans and Yang, 2004; Vijayraghavan et al., 2007). Here we show that high-dose amphetamine abolishes PFC LTP via a D_2 receptor-coupled phosphatase gating mechanism. These findings represent a novel mechanism by which excessive DA could impair prefrontal cognitive functions via D_2 receptors, in addition to the inverted-U shaped D_1 receptor-mediated mechanism.

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