Homeostatic regulation of glutamatergic transmission by dopamine D_4 receptors

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Alterations of synaptic transmission have been considered a core feature of mental disorders; thus, we examined the role of dopamine D_4 receptors, which is highly implicated in attention-deficit hyperactivity disorder and schizophrenia, in regulating synaptic functions of prefrontal cortex, a brain region critical for cognitive and emotional processes. We found that D_4 stimulation caused a profound depression or potentiation of AMPA receptor-mediated excitatory synaptic transmission in prefrontal cortex pyramidal neurons when their activity was elevated or dampened, respectively, which was accompanied by a D_4-induced decrease or increase of AMPARs at synapses. The dual effects of D_4 on AMPAR trafficking and function was dependent on the D_4-mediated bidirectional regulation of CaMKII activity via coupling to distinct signaling pathways, which provides a unique mechanism for D_4 receptors to serve as a homeostatic synaptic factor to stabilize cortical excitability.

Prefrontal cortex (PFC) is a brain region critically involved in the high-level "executive" processes, including working memory, novelty seeking, and emotional control (1, 2). PFC neurons are highly innervated by dopaminergic projections (3). Aberrant dopaminergic actions have been implicated in PFC abnormalities associated with mental disorders, such as attention deficit-hyperactivity disorder (ADHD) and schizophrenia (4, 5).

The expression of D_4 receptors is largely restricted to PFC neurons (6). Multiple studies have indicated that ADHD is associated with a D_4 gene polymorphism that weakens D_4 receptor function (7–10). Preclinical studies suggest that D_4 plays a pivotal role in the expression of juvenile hyperactivity and impulsive behaviors associated with ADHD (11). Moreover, elevated D_4 ligand-binding sites and increased D_4 mRNA levels are found in forebrain tissue of schizophrenics (12). The uniquely effective antipsychotic drug used in schizophrenia treatment, clozapine, has a high affinity to D_4 receptors (13, 14). In addition, D_4 receptor antagonists alleviate stress-induced working-memory problems in monkeys (15) and ameliorate cognitive deficits caused by the psychotomimetic drug phencyclidine (16). Mice lacking D_4 receptors exhibit supersensitivity to psychomotor stimulants, reduced exploration of novel stimuli, and cortical hyperexcitability (17–19). These lines of evidence strongly support that D_4 receptors are critically involved in PFC functions both under the normal condition and in many neuropsychiatric disorders (20, 21).

Behavioral studies have found that D_4 receptors exert bidirectional effects on PFC working memory according to the baseline performance (22). To advance our understanding of how this enigmatic dopamine receptor regulates PFC functions, we need to identify its molecular target in PFC neurons. Our previous studies (23, 24) have shown that D_4 receptors exert a complex and dynamic regulation of CaMKII, a multifunctional enzyme that plays a central role in regulating several key postsynaptic targets required for learning and memory (25). One prominent target of CaMKII is the AMPA receptor (26, 27), which directly controls the excitatory output of PFC.

In this study, we have examined the impact of D_4 receptors on AMPAR-mediated synaptic transmission in PFC pyramidal neurons. We found that D_4 receptors exerted an activity-dependent bidirectional regulation of AMPA responses, which involved CaMKII-regulated AMPAR trafficking at synapses. This novel regulation endows D_4 receptors with the ability to dynamically alter glutamatergic transmission according to the activity levels of PFC pyramidal neurons. The homeostatic control of synaptic strength by D_4 receptors could act to stabilize the activity of PFC circuits.

Results

Activity of PFC Pyramidal Neurons Is Dynamically Regulated. To test whether cortical neuronal activity can be manipulated rapidly, we pretreated rat (3–4 wk) PFC slices with bicuculline (Bic, 10 μM, 2 h) to block inhibitory transmission or with TTX (0.5 μM, 2 h) to suppress neuronal firing, similar to the paradigms used by others (28, 29). Bic or TTX was washed off for 0.5 to 2 h before recording. As shown in Fig. 1 *A*–*C*, following Bic or TTX pretreatment, neuronal excitability was significantly elevated or dampened, respectively, as indicated by the significantly increased or decreased spike frequency. We also incubated PFC slices with a low (1 mM) Mg^{2+} artificial cerebrospinal fluid (ACSF) (2 h) (30) or the NMDAR blocker 2-amino-5-phosphonovaleric acid (APV, 25 μM, 2 h), as another paradigm to elevate or dampen neuronal activity, respectively. Consistently, we found that the low Mg^{2+} ACSF increased, and APV pretreatment decreased, neuronal excitability significantly (Fig. 1 *A*, *C*, and *D*). The basic properties, including resting potential, input resistance, and action potential half-width, were not significantly altered by these drug treatments, but the rheobase (the smallest current injection that elicited a single spike) was significantly decreased by Bic or low Mg^{2+} ACSF, and increased by TTX or APV (Table S1).

Next, we investigated whether the altered neuronal activity by these manipulations may be caused by the changes in synaptic strength. As shown in Fig. 1 *D* and *E*, the evoked excitatory postsynaptic currents (eEPSC) was significantly increased by Bic pretreatment, and decreased by TTX pretreatment (nontreated: 103.3 ± 6.5 pA, $n = 16$; +Bic: 151.0 ± 7.9 pA, $n = 14$; +TTX: 49.4 ± 5.4 pA, $n = 13$; $P < 0.01$, ANOVA), but the evoked inhibitory postsynaptic currents (eIPSC) was unchanged (nontreated: 218.8 ± 19.1 pA, $n = 11$; +Bic: 248.3 ± 38.6 pA, $n = 8$; +TTX: 263.1 ± 32.3 pA, $n = 9$; $P > 0.05$, ANOVA). Low Mg^{2+} ACSF also increased, but APV pretreatment decreased, eEPSC (Fig. 1*D*). Moreover, in cultured PFC pyramidal neurons (days in vitro 24–28), the miniature EPSC (mEPSC) amplitude and frequency were significantly increased by Bic-pretreatment, and significantly decreased by TTX-pretreatment (Fig. 1 *F* and *G*) (nontreated: 32.5 ± 1.5 pA, 5.7 ± 0.5 Hz, $n = 10$; +Bic: 39.2 ± 1.7 pA, 7.9 ± 0.5 Hz, $n = 13$; +TTX: 20.6 ± 1.1 pA, 3.5 ± 0.2 Hz, $n = 10$; $P < 0.01$, ANOVA). These data suggest that PFC neuronal excitability and synaptic activity is up-regulated by a short treatment with Bic and down-regulated by a short treatment with TTX. Moreover, these changes were maintained in a prolonged manner (Fig. S1).

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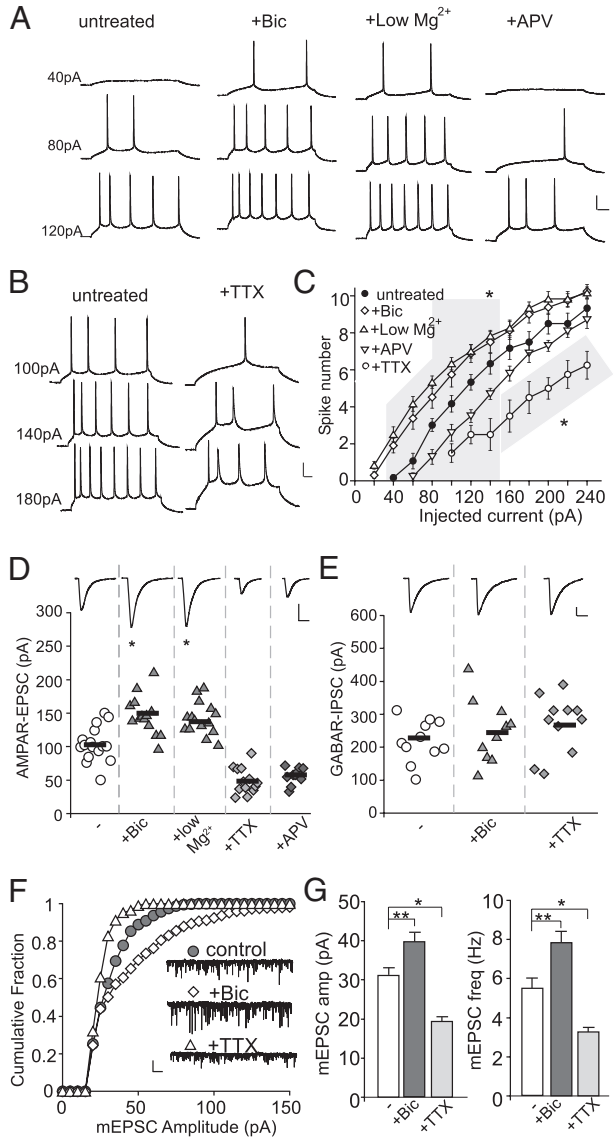

Fig. 1. Excitability and baseline glutamatergic transmission of PFC pyramidal neurons are bidirectionally changed by different treatments. (*A* and *B*) Traces of action potential firing evoked by depolarizing current pulses in representative cells from PFC slices either untreated or pretreated with Bic, a low Mg^{2+} ACSF, APV, or TTX for 2 h. (Scale bars, 20 mV, 50 ms.) Recordings were performed after washing off these agents. (*C*) Summary plot of spike numbers (mean ± SEM) in response to different current injections ($n = 6$ for each of the five groups). $*P < 0.05$. (*D* and *E*) Dot plots showing the amplitude of pharmacologically isolated AMPAR-EPSC (*D*) and $GABA_A$R-IPSC (*E*) in individual neurons from PFC slices with different treatments. The same stimulus intensity and electrode placement for each cell in each group were used to evoke these synaptic events. (*Inset*) Representative synaptic current traces. (Scale bars, 50 pA, 20 ms.) $*P < 0.01$. (*F*) Cumulative plot of mEPSC amplitudes in representative cells from PFC cultures with or without Bic (10 μM, 2 h) or TTX (0.5 μM, 2 h) pretreatment. (*Inset*) Representative mEPSC traces. (Scale bars, 50 pA, 1 s.) (*G*) Bar graphs (mean ± SEM) showing the mEPSC amplitude and frequency in PFC cultures with different treatments. $**P < 0.01$; $*P < 0.001$.

D_4 Induces Activity-Dependent, Bidirectional Regulation of Glutamatergic Transmission in PFC Pyramidal Neurons. To study the potential impact of D_4 receptors on glutamatergic transmission, we examined the effect of D_4R agonist PD168077 on AMPAR-EPSC in PFC pyramidal neurons. Neurons were held at −70 mV, and EPSC was evoked by stimulating neighboring pyramidal neurons. The recorded eEPSC is mediated by AMPA receptors, as it was almost completely blocked by the selective AMPAR antagonist NBQX (10 μM), but was not altered by Bic (10 μM) or APV (100 μM) (Fig. S2). Application of PD168077 (40 μM) caused a significant reduction of AMPAR-EPSC (Fig. 2 *A* and *C*) ($22 \pm 2.2\%, n = 8$). In contrast, PD168077 had almost no effect in striatal medium spiny neurons ($5.0 \pm 3.2\%, n = 5$), consistent with the abundant expression of D_4 receptors in PFC but not in striatum. Furthermore, the D_4R antagonist L-741742 (20 μM) blocked the effect of PD168077 (Fig. S3*A*) ($4.4 \pm 1.5\%, n = 5$), suggesting the mediation by D_4 receptors. The "antagonist chasing" experiment, in which L-741742 was applied after the D_4 effect had been established, indicated that the PD168077 (40 μM)-induced decrease of EPSC in PFC slices failed to return to the baseline level after 20 min of "antagonist chasing" (Fig. S3*B*) ($27.1 \pm 1.3\%, n = 5$), suggesting that D_4R downstream signaling molecules, but not D_4R itself, are mediating the long-lasting effects.

We next examined whether the D_4 regulation of AMPAR-EPSC in PFC pyramidal neurons is influenced by neuronal activity. In Bic-pretreated slices, the reducing effect of PD168077 (40 μM) on AMPAR-EPSC was significantly augmented (Fig. 2 *B* and *C*) ($46.5 \pm 2.4\%, n = 22$), but in TTX-pretreated slices

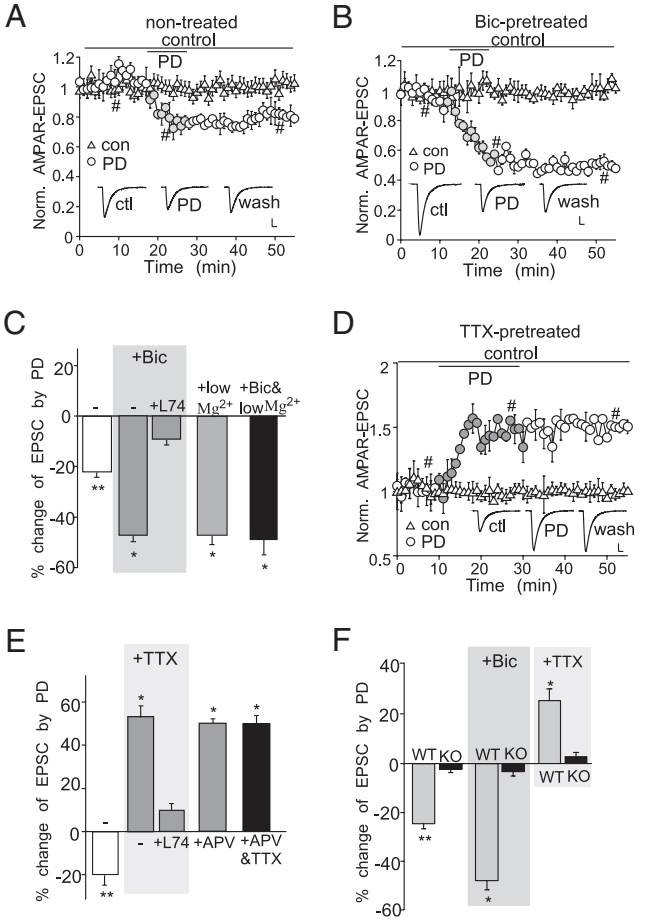

Fig. 2. Activation of D_4 receptors induces a profound depression or potentiation of AMPAR-EPSC in PFC pyramidal neurons at the high-activity or low-activity state, respectively. (*A*, *B*, and *D*) Plot of AMPAR-EPSC showing the effect of PD168077 (40 μM) in representative cells from PFC slices pretreated without (*A*) or with (*B*) Bic (10 μM, 2 h) or (*D*) TTX (0.5 μM, 2 h). (*Inset*) Representative current traces at time points denoted by #. (Scale bars, 25 pA, 10 ms.) (*C*, *E*, and *F*) Bar graphs showing the percentage change of AMPAR-EPSC amplitudes by PD168077 in rat PFC pyramidal neurons with different treatments (*C* and *E*) or in PFC slices (Bic- or TTX-pretreated) from wild-type vs. D_4 knockout mice (*F*). $**P < 0.05$; $*P < 0.001$.

PD168077 caused a profound enhancement of AMPAR-EPSC (Fig. 2 *D* and *E*) (55.6 ± 3.3%, $n = 24$). Both effects were persistent and sustained for 30 min after removal of PD168077. The dual effects of PD168077 were not because of washing off Bic or TTX, as a stable current was recorded in control cells when no PD168077 was applied (Fig. 2 *B* and *D*). Briefer Bic pretreatment failed to significantly augment the reducing effect of PD168077 on AMPAR-EPSC (10 min: 19.5 ± 2.8%, $n = 8$; 1 h: 21.4 ± 6.7%, $n = 5$), but briefer TTX pretreatment led PD168077 to cause no or small enhancement of AMPAR-EPSC (10 min: 6.7 ± 1.1%, $n = 6$; 1 h: 23.0 ± 3.3%, $n = 6$). This finding suggests that a prolonged (2 h) Bic or TTX pretreatment of PFC slices is necessary to enable D_4 to cause a strong depression or potentiation of AMPAR synaptic responses. PD168077 did not alter the paired-pulse ratio of AMPAR-EPSC, a readout that is affected by presynaptic transmitter release (Fig. S3*C*) (nontreated: 3.1 ± 1.2% change, $n = 8$; Bic-pretreated: 2.5 ± 0.6% change, $n = 8$), suggesting that the D_4 regulation of AMPAR-EPSC is through modifying postsynaptic AMPA receptors.

Dose–response data indicate that the reducing or enhancing effect of PD168077 (1–100 μM) in Bic- or TTX-pretreated neurons reached the saturating level at 40 μM; thus, 40 μM of PD168077 was used in most of our experiments to ensure D_4 receptors are fully activated in PFC pyramidal neurons. The dual effects of PD168077 (40 μM) were blocked by the D_4R antagonist L-741,742 (20 μM) [Bic-pretreated: 9.4 ± 2.5% reduction, $n = 5$ (Fig. 2*C*); TTX-pretreated: 8.0 ± 2.8% enhancement, $n = 5$ (Fig. 2*E*)].

When neuronal activity was elevated by the low Mg^{2+} ACSF, PD168077 induced a much bigger reduction of AMPAR-EPSC (46 ± 3.3%, $n = 8$) (Fig. 2*C*), and failed to cause more reduction in PFC slices with combined pretreatment of Bic plus low Mg^{2+} ACSF (48.1 ± 4.6%, $n = 5$), suggesting that the Bic and low Mg^{2+} effects occlude one another. In APV-pretreated slices, PD168077 caused an enhancement of AMPAR-EPSC (52 ± 2.8%, $n = 9$) (Fig. 2*E*), and failed to cause more enhancement in PFC slices with combined pretreatment of TTX plus APV (50.1 ± 3.9%, $n = 9$) (Fig. 2*E*), suggesting that the TTX and APV effects occlude one another.

To further prove that the dual effects of PD168077 are specifically mediated by D_4 receptors, we examined D_4 knockout mice (17). PD168077 lost the capability to decrease AMPAR-EPSC in PFC pyramidal neurons (Bic-pretreated) from D_4 knockout mice (WT: 47.9 ± 3.8%, $n = 5$; KO: 3.2 ± 1.9%, $n = 7$) (Fig. 2*F* and Fig. S4*B*), as well as in nontreated $D_4^{-/-}$ neurons (WT: 24.6 ± 2.1%, $n = 5$; KO: 2.2 ± 1.4%, $n = 6$) (Fig. 2*F* and Fig. S4*A*). PD168077 also lost the capability to increase AMPAR-EPSC in PFC pyramidal neurons (TTX-pretreated) from D_4 knockout mice (WT: 25.1 ± 4.6%, $n = 5$; KO: 2.7 ± 1.6%, $n = 5$) (Fig. 2*F* and Fig. S4*C*). Taken together, these findings show that D_4 activation induces a profound depression of AMPAR transmission in PFC at the high-activity state, and a profound potentiation of AMPAR transmission in PFC at the low-activity state.

D_4-Induced Bidirectional Regulation of AMPAR-Mediated Synaptic Transmission Is Receptor-, Cell Type-, and Target-Specific. To determine whether endogenous activation of D_4 receptors also dynamically regulates AMPARs, we applied the specific dopamine transporter inhibitor GBR-12909 (10 μM) to block the reuptake of dopamine. The $D_{2/3}$ antagonist sulpiride (5 μM) and $D_{1/5}$ antagonist SCH23390 (10 μM) were coapplied to block the activation of non-D_4 dopamine receptors. As shown in Fig. S5, GBR-12909 induced a small reduction of AMPAR-EPSC in nontreated neurons (25.5 ± 3.3%, $n = 8$), a significantly bigger reduction in Bic-pretreated neurons (42.1 ± 3.7%, $n = 9$), and a robust increase in TTX-pretreated neurons (47.6 ± 4.2%, $n = 10$). All these effects were largely blocked by the D_4R antagonist L-741,742. Application of sulpiride plus SCH23390 did not affect basal AMPAR-EPSC, which suggests that activation of D_4 receptors by endogenous dopamine induces an activity-dependent bidirectional regulation of synaptic AMPA responses in PFC pyramidal neurons, similar to the effect of exogenous D_4 agonist.

The dopamine receptor most close to D_4 is D_2. Application of the D_2R agonist quinpirole (20 μM) reversibly reduced AMPAR-EPSC (32.3 ± 2.9%, $n = 4$) (Fig. S6 *A* and *B*) in PFC pyramidal neurons. However, this effect was not altered by pretreatment with Bic (34.0 ± 2.3%, $n = 4$) or TTX (33.3 ± 1.7%, $n = 4$). This finding suggests that, unlike D_4, the D_2 regulation of AMPAR-EPSC is independent of PFC neuronal activity.

D_4 receptors also regulate AMPAR-EPSC in PFC GABAergic interneurons (31). However, PD168077 induced a similar reduction of AMPAR-EPSC in this neuronal population, regardless of activity states (Fig. S6 *C–F*) (53.1 ± 3.0%, $n = 6$; Bic-pretreated: 51.3 ± 2.4%, $n = 4$; TTX-pretreated: 51.2 ± 2.0%, $n = 5$).

Application of PD168077 also decreased the amplitude of NMDAR-EPSC (41.4 ± 3.0%, $n = 5$) and $GABA_A$R-IPSC (34.6 ± 2.6%, $n = 7$) in PFC pyramidal neurons, consistent with our previous reports (32, 33). However, the effects of PD168077 on NMDAR-EPSC and $GABA_A$R-IPSC were not sensitive to Bic pretreatment (41.0 ± 5.5%, $n = 5$; 33.0 ± 2.8%, $n = 6$, respectively), suggesting that, unlike AMPARs, NMDARs and $GABA_A$Rs are down-regulated by D_4 signaling independent of neuronal activity.

To test whether the dual regulation of AMPAR-EPSC by D_4 receptors is a result of the indirect impact of D_4 on $GABA_A$Rs or NMDARs, we also examined the effect of PD168077 on AMPAR-EPSC in PFC slices when Bic and APV were bath applied throughout the recording. As shown in Fig. S7, D_4 still caused a prominent reduction of the pharmacologically isolated AMPAR-EPSC in Bic-pretreated neurons (48 ± 2.7%, $n = 8$), and a marked increase of AMPAR-EPSC in TTX-pretreated neurons (55.6 ± 7.5%, $n = 8$). This finding suggests that D_4 regulates AMPAR responses via a mechanism independent of $GABA_A$Rs or NMDARs.

D_4 Bidirectionally Regulates AMPAR Synaptic Clusters in an Activity-Dependent Manner. To test whether the change in AMPAR trafficking is involved in D_4 regulation of synaptic transmission at different activity states, we carried out a quantitative immunostaining assay. Synaptic GluA1 or GluA2/3 clusters were indicated by those colocalized with the synaptic marker PSD-95 or Synaptophysin. As shown in Fig. 3 *A* and *B*, PD168077 (40 μM, 10 min) caused a small reduction of synaptic GluA1 (colocalized with PSD-95) and synaptic GluA2/3 (colocalized with Synaptophysin) cluster density in untreated PFC pyramidal neurons. In Bic (10 μM, 2 h)-pretreated neurons, synaptic GluA1 and synaptic GluA2/3 clusters were significantly increased, and the reducing effect of PD168077 on both became more prominent. On the other hand, in TTX (0.5 μM, 2 h)-pretreated PFC cultures, synaptic GluA1 and synaptic GluA2/3 clusters were significantly decreased, and an enhancing effect was found with D_4 activation on synaptic GluA1, but not on synaptic GluA2/3. The cluster intensity of total GluA1, total GluA2/3, PSD-95 or Synaptophysin was not changed by Bic- or TTX-pretreatment or PD168077 application. Surface biotinylation assay (34) also demonstrated that PD168077 caused a marked reduction of surface GluA1 and GluA2 expression in PFC slices pretreated with Bic (Fig. 3*C*). On the other hand, in PFC slices pretreated with TTX, PD168077 significantly increased the level of surface GluA1, but not surface GluA2 (Fig. 3*D*).

Next, we performed electrophysiological studies to confirm that D_4 receptors differentially affect AMPAR subunits at high or low activity states. It has been found that GluA2-lacking AMPARs have prominent rectification (35). As shown in Fig. 4*A*, the rectification index of AMPAR-EPSC was not significantly altered by PD168077 in Bic-pretreated PFC pyramidal neurons (control: 2.3 ± 0.31, PD: 2.0 ± 0.18, $n = 7$), but was significantly increased by PD168077 in TTX-pretreated neurons (control: 2.6 ± 0.16, PD: 4.5 ± 0.35, $n = 8$, $P < 0.001$, ANOVA). Moreover, Naspm (100 μM, a selective blocker of GluA2-lacking AMPARs) produced little effect on basal AMPAR-EPSC (10.9 ± 4.8%,

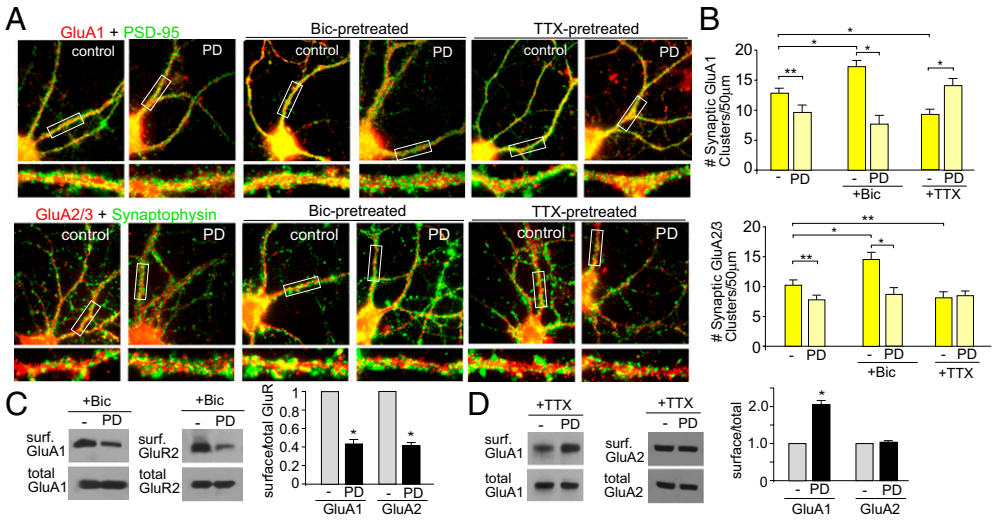

Fig. 3. D_4 bidirectionally regulates synaptic AMPARs in PFC pyramidal neurons at different activity states. (*A*) Immunocytochemical images of costained GluA1 (red) and PSD-95 (green) or GluA2/3 (red) and Synaptophysin (green) in control vs. PD168077-treated PFC pyramidal neurons from cultures pretreated without or with Bic or TTX. Enlarged versions of the boxed regions of dendrites are also shown. (*B*) Quantitative analysis of synaptic GluA1 (PSD-95 colocalized, yellow puncta) or synaptic GluA2/3 (synaptophysin costained, yellow puncta) clusters along dendrites in different conditions. $^{**}P < 0.05$; $^{*}P < 0.01$. (*C* and *D*) Immunoblots of surface and total GluA1 and GluA2 subunits and quantification of the surface/total ratio of GluA1 and GluA2 in PFC slices (Bic- or TTX-pretreated) without or with PD168077 treatment. $^{*}P < 0.01$.

$n = 8$). However, following PD168077 application, Naspm brought the enhanced AMPAR-EPSC to the baseline in TTX-pretreated neurons (−Naspm: 45.3 ± 4.5% enhancement, $n = 9$; +Naspm: 10.2 ± 3.4% enhancement, $n = 11$) (Fig. 4*B*). These results suggest that D_4 suppresses synaptic GluA2-containing AMPAR (GluA1/GluA2) at the high-activity state, but facilitates GluA2-lacking AMPAR (GluA1/GluA1) trafficking to the synaptic membrane at the low-activity state.

CaMKII Is Involved in D_4 Regulation of AMPAR Transmission at Different Activity States. We then examined the possible mechanism by which D_4 bidirectionally regulates AMPARs. It has been found that CaMKII can regulate AMPARs by changing their biophysical properties (36) and their synaptic delivery (27). Our previous studies show that D_4 decreases CaMKII enzymatic activity in PFC at the high-activity state (23), and promotes the activation and synaptic translocation of CaMKII in PFC at the low-activity state (23, 24). Thus, we tested the role of CaMKII in D_4 regulation of AMPAR transmission.

First, we tested the level of active CaMKII (Thr^{286}-phosphorylated) in slices pretreated with Bic or TTX. As shown in Fig. S8*A*, Bic or TTX pretreatment significantly increased or decreased p-CaMKII levels, respectively. After 1 h of washing, p-CaMKII returned to the basal level in slices with a short (10 min or 1 h) Bic or TTX pretreatment, but stayed at the elevated or reduced level in slices with 2 h Bic or TTX pretreatment. This finding suggests that prolonged elevation or suppression of neuronal activity is required for maintaining high or low CaMKII activity in a sustained manner.

Next, we tested the effect of D_4 receptors on CaMKII activity in slices with 2 h Bic or TTX pretreatment followed by 1 h washing. As shown in Fig. S8*B*, PD168077 (40 μM, 10 min) significantly reduced p-CaMKII in Bic-pretreated PFC slices, but significantly increased p-CaMKII in TTX-pretreated slices, confirming that D_4 exerts an activity-dependent, bidirectional regulation of CaMKII enzymatic activity (23).

To examine whether the D_4 reduction of AMPAR-EPSC at the high-activity state is through suppression of CaMKII, we dialyzed neurons with an EGTA-free internal solution containing purified CaMKII (0.6 μg/mL), calmodulin (30 μg/mL), and $CaCl_2$ (0.3 mM). The active CaMKII protein itself did not significantly alter the basal EPSC in Bic-pretreated neurons (5.7 ± 2.9%, $n = 7$), which is likely because of the high level of CaMKII in these cells. The PD168077-induced depression of AMPAR-EPSC in Bic-pretreated neurons was largely blocked by intracellular infusion of the active CaMKII protein (Fig. 5 *A* and *C*) (14.6 ± 3.2%, $n = 7$), but not the heat-inactivated CaMKII protein (48.6 ± 4.0%, $n = 6$) (Fig. 5*C*). On the other hand, dialysis with the CaMKII inhibitor, AIP peptide (5 μM), caused a significant decrease of basal EPSC in Bic-pretreated neurons (Fig. 5*B*) (36.2 ± 3.6%, $n = 9$) and occluded the reducing effect of subsequently added PD168077 (Fig. 5 *B* and *C*) (10.7 ± 2.3%, $n = 8$), although the scrambled peptide was ineffective.

To examine whether the D_4 enhancement of AMPAR-EPSC at the low-activity state is through stimulation of CaMKII, we dialyzed the AIP peptide to specifically inhibit active CaMKII. AIP (5 μM) itself did not alter the basal EPSC in TTX-pretreated neurons (2.7 ± 2.6%, $n = 7$), which is likely because of the low level of CaMKII in these cells. The increase of AMPAR-EPSC by PD168077 in TTX-pretreated neurons was blocked in AIP-dialyzed neurons (Fig. 5 *D* and *F*) (5.4 ± 1.6%, $n = 5$), but not in neurons loaded with a scrambled peptide (51.8 ± 7.9%, $n = 5$) (Fig. 5*F*). On the other hand, dialysis with the active CaMKII caused a significant increase of basal EPSC in TTX-pretreated neurons (Fig. 5*E*) (45.3 ± 5.4%, $n = 8$) and occluded the enhancing effect of subsequently added PD168077 (Fig. 5 *E* and *F*) (5.8 ± 3.5%, $n = 6$), although the heat-inactivated CaMKII was ineffective. Injecting a high concentration (10 mM) of Ca^{2+} chelator BAPTA also blocked the enhancing effect of PD168077 on AMPAR-EPSC (low BAPTA: 53.2 ± 2.0%, $n = 5$; high BAPTA: 9.4 ± 3.2%, $n = 5$) (Fig. 5*F*). Taken together, these data suggest that D_4 reduces AMPAR-EPSC through suppression of CaMKII at the high-activity state, and D_4 enhances AMPAR-EPSC through stimulation of CaMKII at the low-activity state.

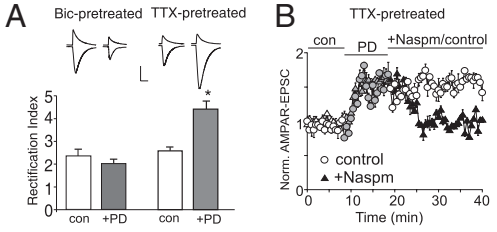

Fig. 4. D_4 activation differentially affects the subunit composition of synaptic AMPARs at different activity states. (*A*) Bar graphs showing the AMPAR-EPSC rectification index in PFC pyramidal neurons before and after PD168077 (40 μM) from slices pretreated with Bic (10 μM, 2 h) or TTX (0.5 μM, 2 h). Rectification of AMPA responses (ratio of the current amplitude at −60 mV to that at +40 mV) was measured with a spermine (100 μM)-containing intracellular solution. (*Inset*) Representative AMPAR-EPSC traces at −60 mV and +40 mV. $^{*}P < 0.001$. (Scale bars, 50 pA, 10 ms) (*B*) Plot of AMPAR-EPSC showing the effect of PD168077 in the absence or presence of the subsequently applied Naspm (100 μM), in representative cells from TTX-pretreated slices.

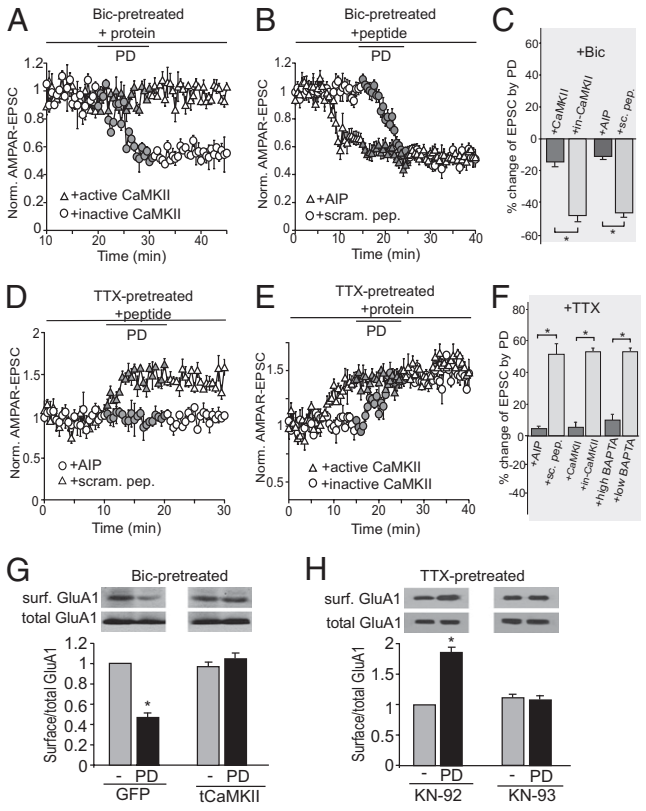

Fig. 5. CaMKII is involved in the activity-dependent bidirectional regulation of AMPAR-EPSC by D_4. (*A*, *B*, *D*, and *E*) Plot of AMPAR-EPSC showing the effect of PD168077 (40 μM) in representative cells infused with the active CaMKII protein (0.6 μg/mL) (*A* and *E*) or the CaMKII inhibitory peptide AIP (5 μM) (*B* and *D*) from PFC slices pretreated with Bic (*A* and *B*) or TTX (*D* and *E*). (*C* and *F*) Bar graphs showing the effect of PD168077 on EPSC in the presence of various agents in Bic- (*C*) or TTX- (*F*) pretreated PFC pyramidal neurons. $*P < 0.001$. (*G* and *H*) Immunoblots and quantification of GluA1 (surface and total) in PFC slices infected with GFP vs. GFP-tCaMKII Sindbus virus (*G*) or treated with KN-92 vs. KN-93 (*H*). Twenty-four hours after viral infection, slices were treated with Bic (10 μM, 2 h) or TTX (0.5 μM, 2 h) before being exposed to PD168077 (40 μM, 10 min). KN-93 or KN-92 was added at 30 min before PD168077. $*P < 0.01$.

Consistent with the role of CaMKII in D_4 regulation of AMPAR-EPSC, the enhancing effect of Bic pretreatment (2 h) on mEPSC was prevented by CaMKII inhibitor KN-93 (control: 30.2 ± 1.1 pA, 5.4 ± 0.6 Hz, $n = 5$; +Bic: 28.0 ± 2.6 pA, 6.0 ± 1.0 Hz, $n = 6$) (Fig. S8 *C* and *E*). On the other hand, the reducing effect of TTX pretreatment (2 h) on mEPSC was diminished in neurons infected with the truncated (constitutively active) CaMKII (tCaMKII) Sindbis virus (27) (control: 35.2 ± 1.1 pA, 7.4 ± 0.8 Hz, $n = 5$; +TTX: 34.6 ± 1.0 pA, 7.6 ± 0.9 Hz, $n = 5$) (Fig. S8 *D* and *E*), but was not prevented by application of the transcription inhibitor Actinmyocin D (control: 29.0 ± 0.6 pA, 5.8 ± 0.5 Hz, $n = 8$; +TTX: 20.7 ± 1.2 pA, 2.7 ± 0.6 Hz, $n = 10$; $P < 0.001$) (Fig. S8 *D* and *E*). This finding suggests that Bic or TTX pretreatment also converges on CaMKII stimulation or CaMKII suppression to up- or down-regulate synaptic AMPAR responses, respectively.

To complement electrophysiological studies, we also examined the role of CaMKII in D_4 regulation of surface GluA1 expression at different states. To elevate CaMKII activity, PFC slice cultures were infected with tCaMKII Sindbis virus. As shown in Fig. 5 *G* and *H*, the reducing effect of PD168077 on surface GluA1 expression was abolished in tCaMKII-infected PFC slices pretreated with Bic (GFP: 0.47 ± 0.1-fold of control, $n = 4$, $P < 0.01$, ANOVA; tCaMKII: 1.05 ± 0.06-fold of control, $n = 4$). On the other hand, in PFC slices pretreated with TTX, the enhancing effect of PD168077 on surface GluA1 expression was blocked by the CaMKII inhibitor KN-93 (20 μM, 1.08 ± 0.07-fold of control, $n = 5$), but not the inactive analog KN-92 (20 μM, 1.85 ± 0.09-fold of control, $n = 5$, $P < 0.01$, ANOVA). This finding further suggests that D_4 regulates AMPAR membrane trafficking dynamically via a CaMKII-dependent mechanism.

Discussion

Intensive research in the activity-dependent bidirectional changes of AMPAR-mediated synaptic transmission, such as long-term potentiation (LTP) induced by high-frequency stimulation and long-term depression (LTD) induced by low-frequency stimulation, has shown that these forms of synaptic plasticity are important for learning and memory (37). In this study, we demonstrate that a neuromodulator, the D_4 receptor, can uniquely regulate glutamatergic transmission in an activity-dependent bidirectional manner in PFC pyramidal neurons. At the high-activity state, D_4 receptors induce a persistent depression of AMPAR-EPSC, but at the low-activity state, D_4 receptors cause a sustained potentiation of AMPAR-EPSC. Thus, D_4 receptors serve as a homeostatic synaptic factor to stabilize the excitability of PFC pyramidal neurons, which provides a potential mechanism for the cortical hyperexcitability exhibited in D_4-deficient mice (18). Given the key role of PFC glutamatergic transmission in working memory (1), our results suggest that D_4 receptors may regulate cognitive processes in a dynamic manner depending on the basal output signal of PFC.

The activity states were set by pretreating PFC slices or cultures with Bic or TTX for 2 h, a paradigm often used to acutely elevate or dampen neuronal activity in vitro, respectively (28, 29). It has been found that chronic (over a period of days) perturbation of network activity can trigger compensatory changes in synaptic function that contribute to the homeostatic restoration of neuronal activity (38). However, we found that the neuronal activity level (as indicated by spiking frequency and excitatory synaptic strength) of PFC pyramidal neurons was elevated by Bic pretreatment (2 h), and was reduced by TTX pretreatment (2 h), and these changes could sustain for hours after washout of Bic or TTX, suggesting that homeostatic synaptic scaling has not occurred in mature cortical neurons after the short-term Bic or TTX treatment.

Mounting evidence has demonstrated that the primary basis for synaptic plasticity like LTP or LTD is the trafficking of AMPAR subunits to and from the synapse (39, 40). Consistent with this notion, the D_4-induced depression or potentiation of AMPAR-EPSC at different activity states is accompanied by reduced or increased expression of synaptic AMPARs in PFC pyramidal neurons, respectively. In agreement with our results, D_4 activation reduces GluA1 surface expression in hippocampal cultures after chemical LTP induction, and D_4 reduces AMPAR-EPSC amplitudes at potentiated hippocampal synapses (41). Interestingly, GluA1 and GluA2 subunits are differentially regulated by D_4 receptors. At the high-activity state, D_4 activation removes GluA1/2 heteromeric AMPAR channels from synapses, but at the low-activity state, D_4 activation delivers more GluA1/1 homomeric AMPAR channels to synapses, similar to the trafficking of AMPAR subunits in LTD/LTP induced by electrical stimulation (39).

CaMKII, a molecular switch that is capable of long-term memory storage (42), plays a central role in the dual regulation of AMPAR trafficking and function by D_4 receptors. Previously, we have found that D_4 receptors exert a dynamic regulation of CaMKII enzymatic activity and synaptic distribution (23, 24). At the high-activity state, D_4 couples to the canonical G_i pathway to inhibit PKA and cause a decrease in the phosphorylation of inhibitor 1, which leads to disinhibition of protein phosphatase I and reduced activity of CaMKII (23, 32). At the low-activity state, D_4 utilizes the G_q pathway, which leads to activation of phospholipase C, elevation of intracellular Ca^{2+}, and ensuing CaMKII activation (23). The dynamic regulation of CaMKII enables D_4 to regulate CaMKII synaptic substrates in a flexible manner. In this study, we have found that, the D_4 depression of

AMPARs at the high-activity state is blocked by activated CaMKII and occluded by CaMKII inhibitors, suggesting its dependence on CaMKII inhibition; however, the D_4 potentiation of AMPARs at the low-activity state is blocked by CaMKII inhibitors and occluded by activated CaMKII, suggesting its dependence on CaMKII activation. This dual regulation of CaMKII activity is unique to D_4 receptors. Activation of D_2 receptors always decreases CaMKII activity, regardless of neuronal activity (23), which explains the unidirectional regulation of AMPAR-EPSC by D_2 receptors.

In summary, we have revealed a unique action of D_4 receptors in PFC pyramidal neurons: regulation of AMPAR trafficking and function in an activity-dependent manner, which enables D_4 receptors to serve as a homeostatic synaptic factor to stabilize the excitability of cortical circuits. Because dysfunction of glutamatergic transmission is the core feature and fundamental pathology of mental disorders (43), our results provide a potential framework for understanding the link of aberrant D_4 functions to ADHD (7–11) and schizophrenia (14).

Materials and Methods

AMPAR-EPSC was recorded in PFC neurons from young SD rats as previously described (34). Standard immunocytochemical and biochemical approaches were used to measure synaptic and surface AMPARs (24, 31, 34). For details, see *SI Materials and Methods*.

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