Restoration of Glutamatergic Transmission by Dopamine D4 Receptors in Stressed Animals*

Eunice Y. Yuen, Ping Zhong, Xiangning Li, Jing Wei, and Zhen Yan

From the Department of Physiology and Biophysics, State University of New York at Buffalo, School of Medicine and Biomedical Sciences, Buffalo, New York 14214

Background: The dopamine D4 receptors in prefrontal cortex (PFC) play a key role in mental health and disorders.

Results: D4 activation caused a bi-directional, homeostatic regulation of glutamatergic responses in rats exposed to acute or chronic stress.

Conclusion: The altered synaptic excitation in stress conditions is restored by D4 signaling in PFC.

Significance: It provides a potential mechanism for the role of D4 in stress-related neuropsychiatric disorders.

The prefrontal cortex (PFC), a key brain region for cognitive and emotional processes, is highly regulated by dopaminergic inputs. The dopamine D4 receptor, which is enriched in PFC, has been implicated in mental disorders, such as attention deficit-hyperactivity disorder and schizophrenia. Recently we have found homeostatic regulation of AMPA receptor-mediated synaptic transmission in PFC pyramidal neurons by the D4 receptor, providing a potential mechanism for D4 in stabilizing cortical excitability. Because stress is tightly linked to adaptive and maladaptive changes associated with mental health and disorders, we examined the synaptic actions of D4 in stressed rats. We found that neural excitability was elevated by acute stress and dampened by repeated stress. D4 activation produced a potent reduction of excitatory transmission in acutely stressed animals and a marked increase of excitatory transmission in repeatedly stressed animals. These effects of D4 targeted GluA2-lacking AMPA receptors and relied on the bi-directional regulation of calcium/calmodulin kinase II activity. The restoration of PFC glutamatergic transmission in stress conditions may enable D4 receptors to serve as a synaptic stabilizer in normal and pathological conditions.

The dopaminergic system in prefrontal cortex (PFC) plays a key role in regulating high level cognitive functions, such as working memory (1, 2). Dopamine D4 receptors, which are largely restricted to PFC neurons (3, 4), are critically involved in PFC functions both under normal condition and in many neuropsychiatric disorders (5, 6). For example, attention deficit hyperactivity disorder and the responsiveness to its treatments have been associated with a D4 gene polymorphism that weakens D4 receptor function (7–10). The highly effective antipsychotic drug used in schizophrenia treatment, clozapine, has a high affinity to D4 receptors (11, 12). Preclinical studies also indicate that D4 receptor antagonists alleviate stress-induced working memory problems in monkeys (13) and ameliorate cognitive deficits caused by psychotomimetic drugs (14). Mice lacking D4 receptors exhibit supersensitivity to psychomotor stimulants, reduced exploration of novel stimuli, cortical hyperexcitability, and juvenile hyperactivity and impulsive behaviors associated with attention deficit hyperactivity disorder (15–18).

To understand the role of D4 in mental health and disorders, it is important to elucidate the molecular and cellular mechanisms underlying the impact of D4 on cortical excitability and working memory. It has been proposed that glutamate receptor-mediated synaptic transmission that controls PFC neuronal activity is crucial for working memory (19). Dysfunction of glutamatergic transmission is considered as the core feature and fundamental pathology of mental disorders (20–22). Recently we have demonstrated that D4 stimulation causes a profound depression of AMPA receptor (AMPAR) responses in PFC pyramidal neurons when their activity is elevated in vitro and causes a marked potentiation of AMPAR responses when their activity is dampened in vitro (23, 24). It suggests that D4 receptors may use the homeostatic control of glutamatergic transmission to stabilize the activity of PFC circuits.

The PFC is one of the primary targets of stress hormones (25). Stress has profound and divergent effects on the body and the brain. Acute stress is essential for adaptation and maintenance of homeostasis, whereas chronic stress often exacerbates deficiencies in emotional and cognitive processes associated with psychiatric disorders, such as depression, anxiety, and posttraumatic stress disorder (25, 26). In this study, we sought to determine whether animals exposed to acute or repeated behavioral stress have altered neuronal activity in vivo and whether D4 exerts an activity-dependent regulation of glutamatergic transmission in PFC pyramidal neurons from stressed animals. Knowledge gained from this study should help us understand the role of D4 in the adaptive and maladaptive changes linked to stress.
**EXPERIMENTAL PROCEDURES**

*Stress Paradigm*—All experiments were conducted in accordance to the Institutional Animal Care and Use Committee (IACUC) of the State University of New York at Buffalo. Juvenile (3–4 weeks old) Sprague-Dawley male rats were used in this study. For acute stress, rats were restrained in air-accessible cylinders for 2 h (10:00 a.m. to 12:00 p.m.) as described previously (27, 28). For repeated stress, rats were restrained daily (2 h) for 5–7 days.

*Electrophysiological Recording in Slices*—PFC slice preparation procedures were similar to what was described previously (29). In brief, animals were anesthetized by inhaling 2-bromo-2-chloro-1,1,1-trifluoroethane (1 ml/100 g; Sigma) and decapitated. Brains were quickly removed and sliced (300 μm) with a Leica VP1000S Vibrotome while bathed in a HEPES-buffered salt solution. Slices were then incubated for 1–5 h at room temperature (22–24 °C) in artificial cerebrospinal fluid (ACSF) (130 mM NaCl, 26 mM NaHCO3, 1 mM CaCl2, 5 mM MgCl2, 3 mM KCl, 10 mM glucose, 1.25 mM NaH2PO4) bubbled with 95% O2, 5% CO2.

Standard voltage clamp recording techniques were used to measure AMPAR-EPSC in layer V PFC pyramidal neurons as described before (23, 28). Neurons were visualized with a ×40 water-immersion lens and illuminated with near infrared (IR) light, and the image was captured with an IR-sensitive CCD camera. Recordings were obtained using a Multiclamp 700A amplifier (Axon Instruments). Tight seals (2–10 gigohms) were obtained. Brains were quickly removed and sliced (300 μm) with a Leica VP1000S Vibrotome while bathed in a HEPES-buffered salt solution. Slices were then incubated for 1–5 h at room temperature (22–24 °C) in artificial cerebrospinal fluid (ACSF) (130 mM NaCl, 26 mM NaHCO3, 1 mM CaCl2, 5 mM MgCl2, 3 mM KCl, 10 mM glucose, 1.25 mM NaH2PO4) bubbled with 95% O2, 5% CO2.

Western Blotting—After treatment, slices were homogenized in boiling 1% SDS, followed by centrifugation (13,000 × g, 20 min). The supernatant fractions were subjected to 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat dry milk for 1 h at room temperature followed by incubation with various primary antibodies of CaMKII (1:1000; Santa Cruz Biotechnology sc-9035) and Thr(P)286-CaMKII (1:1000; Santa Cruz Biotechnology sc-12886). After incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich), the blots were exposed to the enhanced chemiluminescence substrate (Amersham Biosciences). Quantification was obtained from densitometric measurements of immunoreactive bands on film using ImageJ software.

**RESULTS**

Neuronal Excitability, Which Is Elevated by Acute Stress and Dampened by Repeated Stress, Is Restored by D4 Activation—To compare overall excitability of PFC circuits in control versus stressed animals, we measured the spontaneous action potential firing, which helps to reveal the circuit excitability changes induced by altered synaptic drive onto pyramidal neurons (32). As shown in Fig. 1A, compared with neurons from nonstressed (NS) control rats, the firing rate was significantly increased in neurons from acutely stressed (AS) animals (NS: 1.14 ± 0.25 Hz, n = 9; AS: 2.04 ± 0.31 Hz, n = 10, p < 0.01, t test). On the other hand, a significant reduction in the firing rate was observed in repeatedly stressed (RS) animals (Fig. 1B, NS: 1.25 ± 0.41 Hz, n = 8; RS: 0.37 ± 0.10 Hz, n = 11, p < 0.01, t test). These data indicate that the excitability of PFC pyramidal neurons is elevated by acute stress and dampened by chronic stress. Thus, stress provides a physiological condition that has bi-directional changes in neuronal activity in vivo.

We then examined the impact of D4 receptor activation on the excitability of PFC pyramidal neurons from stressed animals. As shown in Fig. 1C and D, application of the D4 receptor agonist PD168077 (40 μM) produced a significant reducing effect on the firing rate in acutely stressed animals (control: 2.14 ± 0.04 Hz, PD: 1.33 ± 0.03 Hz, n = 7). On the other hand, PD168077 significantly enhanced the firing rate in repeatedly stressed animals (control: 0.39 ± 0.02 Hz, PD: 0.97 ± 0.04 Hz, n = 5). Thus, the PFC neuronal excitability has been restored by D4 in stress conditions.

D4 Restores the Excitatory Synaptic Transmission in Stressed Animals—Because D4 stimulation induces an activity-dependent regulation of glutamatergic transmission in vitro (23), we next examined the effect of D4 in PFC neurons from animals whose neural activity has been perturbed by in vivo stressors. As shown in Fig. 2, A–C, the basal evoked AMPAR-EPSC amplitude was markedly increased by acute stress (NS: 109.8 ± 5.1

**D4 Regulation of AMPA Receptors in Stressed Rats**
**FIGURE 1.** In vivo stress alters the excitability of PFC pyramidal neurons. A and B, representative spontaneous firing recordings and scatter plots of firing rates of PFC pyramidal neurons from nonstressed versus acutely stressed (A) or repeatedly stressed (B) rats. Scale bars, 20 mV, 2 s. *, *p < 0.01, t test. C and D, representative spontaneous firing recordings and scatter plots of firing rates of PFC pyramidal neurons showing the effect of PD168077 (40 μM) in stressed (acutely or repeatedly) rats. Scale bars, 20 mV, 2 s. *, *p < 0.01, t test.

**FIGURE 2.** The stress-induced alteration of glutamatergic transmission is restored by D₄ activation. A and B, plot of normalized AMPAR-EPSC showing the effect of PD168077 (40 μM) in nonstressed versus animals exposed to acute stress (A) or repeated stress (B). C, Bar graphs (mean ± S.E. (error bars)) showing the amplitude of EPSC in PFC neurons from nonstressed, acutely stressed, or repeatedly stressed animals before and after PD168077 application. #, *p < 0.05; *, *p < 0.001, ANOVA. D and F, cumulative distribution plot showing the effect of PD168077 (40 μM) on miniature EPSC amplitude and interevent interval in animals exposed to acute stress (D) or repeated stress (F). Inset, representative mEPSC traces. Scale bar, 20 pA, 1 s. E and G, bar graphs (mean ± S.E.) showing the amplitude and frequency of mEPSC in PFC neurons from different groups (NS, AS, RS) before and after PD168077 application. *, *p < 0.001, ANOVA.

pA, n = 12; AS: 237.9 ± 15.1 pA, n = 16; p < 0.001, ANOVA) and was substantially decreased by repeated stress (NS: 151.4 ± 10.5 pA, n = 16; RS: 80.4 ± 4.4 pA, n = 19, p < 0.001, ANOVA). Application of PD168077 (40 μM) slightly reduced AMPAR-EPSC in nonstressed animals (23.0 ± 3.1% reduction, n = 7, p < 0.05, ANOVA), and this reducing effect of PD168077 was significantly augmented in acutely stressed animals (49.2 ± 4.5% reduction, n = 16, p < 0.001, ANOVA). On the other hand,
PD168077 significantly enhanced AMPAR-EPSC in repeatedly stressed animals (53.6 ± 6.5% enhancement, n = 14, p < 0.001, ANOVA). Thus, the excitatory synaptic strength has been brought back to the control level by D₄ in stress conditions (AS+PD: 120.8 ± 8.3 pA, n = 16; RS+PD: 123.6 ± 7.3 pA, n = 14). We next measured AMPAR-mediated mEPSC, a response from quantal release of single glutamate vesicles. Acute stress caused a significant increase of the mEPSC amplitude (NS: 12.1 ± 0.6 pA; AS: 17.2 ± 2.0 pA, p < 0.001, ANOVA), which was recovered to the nonstressed level by PD168077 (10.9 ± 0.3 pA, 2.1 ± 0.2 Hz, n = 8, Fig. 2, D and E). The mEPSC frequency was not significantly changed by acute stress, consistent with our previous report (27). On the other hand, the mEPSC amplitude and frequency were significantly reduced in animals exposed to repeated stress (9.5 ± 0.3 pA, 1.5 ± 0.3 Hz, n = 7), and PD168077 restored mEPSC similar to that observed in nonstressed animals (14 ± 0.5 pA, 2.8 ± 0.3 Hz, n = 7, Fig. 2, F and G). Taken together, these results suggest that D₄ activation provides a homeostatic regulation of excitatory synaptic transmission in stressed animals.

The Homeostatic Effect of D₄ Targets Mainly GluA2-lacking AMPA Receptors—Because GluA2-containing and GluA2-lacking AMPAR channels have distinct channel conductance, open probability, Ca²⁺ permeability and rectification, we examined whether D₄ receptors differentially affect AMPAR subunits in stress conditions. It has been found that GluA2-lacking AMPARs have prominent rectification (33), and changes in GluA2-lacking AMPARs alter the inward rectification due to voltage-dependent blockade of intracellular polyamine (34). Thus, rectification index (RI) of AMPAR responses (ratio of the current amplitude at +60 mV to that at +40 mV) was measured with a spermine (100 μM)—containing intracellular solution (35). As shown in Fig. 3A, the RI in acutely stressed animals was much bigger than that in nonstressed control animals (NS: 2.3 ± 0.34, n = 7; AS: 4.0 ± 0.39, n = 11; p < 0.001, ANOVA), suggesting that acute stress may predominantly increase GluA2-lacking AMPARs at the synapse. Application of PD168077 (40 μM) reduced the RI back to the control level (2.4 ± 0.2, n = 11), indicating that D₄ activation removes the synaptic GluA2-lacking AMPARs that are previously delivered by acute stress. On the other hand, in repeatedly stressed animals, D₄ was unchanged (NS: 2.1 ± 0.2, n = 9; RS: 2.2 ± 0.2, n = 9, p > 0.05, ANOVA) but was markedly increased by PD168077 (4.1 ± 0.6, n = 8), suggesting that D₄ increases the synaptic recruitment of GluA2-lacking AMPARs.

To investigate further the target of D₄, we used NASPM (100 μM), a selective blocker of GluA2-lacking AMPA receptors (36). As shown in Fig. 3, C and D, NASPM had a minimal effect on basal AMPAR-EPSC in nonstressed rats (−12.9 ± 4.2%, n = 8, p > 0.05, t test), consistent with the low level of synaptic GluA2-lacking AMPARs at the baseline condition as reported previously (37). The NASPM sensitivity was significantly increased in acutely stressed animals (−30.3 ± 3.2%, n = 9, p < 0.001, t test) and was unchanged in repeatedly stressed animals (−11.2 ± 2.2%, n = 11, p > 0.05, t test), consistent with the delivery of GluA2-lacking AMPARs by acute stress.

In acutely stressed animals, PD168077 significantly reduced AMPAR-EPSC (Fig. 3E, 52.4 ± 7.2% reduction, n = 9), and subsequent application of NASPM failed to alter AMPAR-EPSC (−11.1 ± 1.7%, n = 9). On the other hand, in repeatedly stressed animals, PD168077 significantly enhanced AMPAR-EPSC (Fig. 3F, 47.7 ± 9.4% enhancement, n = 11), and subsequent application of NASPM markedly reduced AMPAR-EPSC (−37.9 ± 5.1%, n = 11). This suggests that the D₄-sensitive EPSC is mediated by GluA2-lacking AMPARs in both stress conditions.

To test further whether the change in AMPAR trafficking is involved in D₄ regulation of synaptic transmission at stress conditions, we carried out surface biotinylation assays. As shown in Fig. 3, G and H, PD168077 caused a marked reduction of surface GluA1 expression in PFC slices from acutely stressed animals, but significantly increased the level of surface GluA1 in PFC slices from repeatedly stressed animals (NS: −29.6 ± 2.6%, n = 11; AS: −55.9 ± 7.0%, n = 7; RS: 46.9 ± 11.2%, n = 10; p < 0.01, ANOVA). The biochemical evidence is consistent with what was observed in electrophysiological recordings.

CaMKII Is Involved in the D₄ Regulation of AMPAR Transmission in Stressed Animals—Our previous studies have shown that D₄ exerts an activity-dependent bi-directional regulation of CaMKII (38), which is critical for the D₄-induced homeostatic regulation of AMPARs in vitro (23, 24). Thus, we tested the role of CaMKII in the D₄ actions in stressed animals. As shown in Fig. 4, A and B, in PFC slices from nonstressed animals, application of PD168077 (40 μM, 10 min) reduced the level of activated (Thr286-phosphorylated) CaMKII (32.1 ± 7.1% reduction, n = 5). In acutely stressed animals, the basal level of activated CaMKII was elevated (1.59 ± 0.19-fold of control, n = 5), and the reducing effect of PD168077 was significantly augmented (71.4 ± 7.3% reduction, n = 5). Conversely, in repeatedly stressed animals, the basal level of activated CaMKII was unchanged (1.12 ± 0.12-fold of control, n = 6), whereas PD168077 significantly increased the level of activated CaMKII (61.4 ± 10.5% increase, n = 6). This bi-directional regulation of CaMKII activity by D₄ receptors provides a basis for the dual effects of D₄ on AMPAR transmission in PFC pyramidal neurons from acutely versus chronically stressed animals.

To examine whether the D₄ reduction of AMPAR-EPSC in acutely stressed animals is through suppression of CaMKII, we diazylated neurons with an EGTA-free internal solution containing purified active CaMKIIα protein (0.6 μg/ml), calmodulin (30 μg/ml) and CaCl₂ (0.3 mM). The active CaMKII protein itself did not significantly alter the basal EPSC (−7.8 ± 1.7%, n = 6), which is likely due to the high level of CaMKII in these cells. As shown in Fig. 5A, the reducing effect of PD168077 on AMPAR-EPSC was largely blocked by intracellular infusion of the active CaMKII protein (6.2 ± 2.1% reduction, n = 9, Fig. 5C), but not the heat-inactivated CaMKII protein (46.4 ± 3.9% reduction, n = 11; p < 0.001, t test, Fig. 5B).
As shown in Fig. 5B, the enhancing effect of PD168077 on AMPAR-EPSC was blocked by KN-93 (3.0 ± 3.9% enhancement, n = 9, Fig. 5C), but not the inactive analog KN-92 (20 μM, 52.3 ± 6.1% enhancement, n = 9; p < 0.001, t test, Fig. 5C).

Finally, we examined the role of CaMKII in D₄ regulation of neuronal excitability in stressed animals. As shown in Fig. 6, in animals exposed to acute stress, the reducing effect of PD168077 on spontaneous action potential firing was largely...
blocked by intracellular infusion of the active CaMKII protein (0.6 μg/ml, control: 2.16 ± 0.18 Hz, PD: 1.07 ± 0.08 Hz, n = 7), but not the GST control (control: 2.18 ± 0.14 Hz, PD: 1.07 ± 0.08 Hz, n = 7; p < 0.01, t test). On the other hand, in animals exposed to repeated stress, the enhancing effect of PD168077 on the firing rate was blocked by KN-93 (20 μM, control: 0.44 ± 0.03 Hz, PD: 0.49 ± 0.04 Hz, n = 7), but not the inactive analog KN-92 (control: 0.45 ± 0.04 Hz, PD: 1.02 ± 0.07 Hz, n = 6; p < 0.01, t test).

Taken together, these results suggest that D₄ restores glutamatergic transmission in stress conditions through bi-directional regulation of CaMKII activity, which contributes the dual regulation of neuronal excitability by D₄ receptors.

**DISCUSSION**

Stress serves as a key controller for neuronal responses that underlie behavioral adaptation, as well as maladaptive changes that lead to cognitive and emotional disturbances in stress-related mental disorders (26). Corticosterone, the major stress hormone, has been found to exert a complex effect on PFC excitatory synapses and PFC-mediated behaviors (25, 39). Acute stress induces synaptic potentiation by increasing surface delivery of AMPARs and NMDARs via glucocorticoid/SGK/Rab4 signaling, resulting in enhanced working memory performance (27, 28, 40). Conversely, chronic stress induces dendritic shortening, spine loss, and impairment in cognitive flexibility and perceptual attention (41–43). Our recent study has found that the detrimental effect of repeated stress on cognition is causally linked to the ubiquitin/proteasome-mediated degradation of AMPAR and NMDAR subunits and the suppression of glutamatergic transmission in PFC (44). In addition, the alteration of RNA editing of AMPAR subunits (45, 46) is potentially another mechanism underlying the effects of stress.

In this study, we have found that the spontaneous action potential firing is bi-directionally changed by acute versus repeated stress, suggesting that the synaptic drive onto PFC pyramidal neurons is altered by stress, leading to the change in PFC circuit excitability. Activation of D₄ reduces the potentiated AMPAR responses in acutely stressed animals and enhances the depressed AMPAR responses in repeatedly stressed animals. The restoration of synaptic strength to the control level by D₄ in stress conditions supports the notion that D₄ serves as a homeostatic synaptic factor to stabilize cortical excitability (23, 24).

AMPA receptors are tetramers assembled by GluA1–4 subunits. Different populations of AMPARs have distinct properties and regulatory mechanisms. GluA2-containing AMPARs are dominant in glutamatergic synapses of neocortex and hippocampus (47, 48), whereas GluA2-lacking AMPARs can be...
**D₄ Regulation of AMPA Receptors in Stressed Rats**

**A** and **C** represent spontaneous action potential recordings showing the effect of PD168077 (40 μM) in PFC neurons infused with active CaMKII versus GST from acutely stressed rats (**A**) or KN-93 versus KN-92 from repeatedly stressed rats (**C**). Scale bars, 20 mV, 2 s. **B** and **D**, scatter plots of firing rates before (con) and after PD168077 in the presence of various reagents affecting CaMKII activity in PFC pyramidal neurons from acutely stressed (**B**) or repeatedly stressed (**D**) rats. *, p < 0.01; t test.

One of the most important regulators of AMPARs and synaptic plasticity is CaMKII (54). CaMKII activation has been found to increase channel conductance (53), compared with GluA2-containing AMPARs. The unique Ca²⁺ permeability of GluA2-lacking AMPARs plays an important role in homeostatic plasticity (49) and LTP induction in hippocampus (35). Here, we show that GluA2-lacking (GluA1 homomeric) AMPARs is the major target of D₄. D₄ reduces the GluA2-lacking AMPARs that are delivered by acute stress, and D₄ delivers more GluA2-lacking AMPARs to the synapse in repeatedly stressed animals. Thus, by redistributing GluA2-lacking AMPARs, D₄ restores glutamatergic transmission in stress conditions. It is expected that GluA2 knock-out animals will show differential effects of D₄R activation when acutely stressed and repeatedly stressed animals are compared.

In summary, we conclude that D₄ is a synaptic gatekeeper that stabilizes glutamatergic transmission in prefrontal cortex. When neuronal activity changes in situations such as acute or repeated stress, D₄ redistributes GluA2-lacking AMPARs at synapses through the bi-directional control of CaMKII. This homeostatic action of D₄ provides a potential mechanism for the unique role of D₄ in many stress-related neuropsychiatric disorders.

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**REFERENCES**


