Interaction of Dopamine D1 and NMDA Receptors Mediates Acute Clozapine Potentiation of Glutamate EPSPs in Rat Prefrontal Cortex

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INTRODUCTION

Blockade of the N-methyl-D-aspartate (NMDA) receptor channel complex in the brain (e.g., by phencyclidine, ketamine) results in cognitive deficits and psychiatric symptoms indistinguishable from schizophrenia (Javitt and Zukin 1991; Krystal et al. 1999). Dysfunction of NMDA receptor and/or glutamate-mediated synaptic transmission in the prefrontal cortex (PFC) are implicated in the pathophysiology of schizophrenia (Olney et al. 1999; Tamminga 1998). The atypical antipsychotic clozapine significantly improves many cognitive and behavioral deficits manifested in schizophrenia and in animals treated with NMDA antagonists (Breier 1999; Hauber 1993; Malhotra et al. 1997). Unlike typical antipsychotics like haloperidol, clozapine has low liability for extrapyramidal side effects, although it can induce seizure activity at higher therapeutic doses (Devinsky and Pacia 1994; Korpi et al. 1995; Stevens et al. 1996). Neural activation by clozapine, determined by immediate early gene expression (e.g., *c-fos*), occurred mainly in limbic and PFC areas but not striatum (Deutch and Duman 1996; Nisenbaum and Fibiger 2000; Robertson and Fibiger 1992; Semba et al. 1996). Poor activation of striatum by clozapine may account for its low extrapyramidal side effects, while seizure susceptibility of clozapine in limbic and PFC areas could play an important, though controversial, role in its therapeutic actions (Denney and Stevens 1995; Koukkou et al. 1979; Stevens et al. 1996).

Clozapine exerts part of its "pro-convulsant" effects via its interaction with multiple ion channels and ligand gated-receptor systems (Ashby and Wang 1996). Clozapine has moderate blocking actions on inward rectifier K+ channels (Kobayashi et al. 2000), and GABA_A receptors (IC_{50} = 8.2 μM) (Michel and Trudeau 2000), thus resulting in an increase in neuronal excitability. Furthermore, clozapine can lower the basal level of GABA released in PFC in vivo (Bourdelais and Deutch 1994). Acute clozapine also potentiates the NMDA component of glutamatergic synaptic responses in striatal and PFC slices (Arvanov and Wang 1997; Banerjee et al. 1995), and field potential responses in hippocampus in vivo (Kubota et al. 1996, 2000). At higher doses, clozapine suppresses the Schaffer-collateral-evoked field potentials in hippocampal slices (Baskys et al. 1993). A combination of these actions may serve to augment neuronal excitability following acute clozapine.

Neurochemically, dopamine and glutamate are closely linked to many major actions of clozapine. First, acute or chronic clozapine increases PFC, but not striatal, extracellular glutamate levels (Daly and Moghaddam 1993; Yamamoto and Cooperman 1994). *c-Fos* expression induced by clozapine is reduced by NMDA antagonist MK-801 (Levesque et al. 2000), suggesting that glutamate release by clozapine may activate NMDA receptor to mediate *c-Fos* induction. Second, acute and chronic clozapine has been shown to induce a greater DA...
release in PFC over striatum in vivo (Bymaster et al. 1999; Daly and Moghaddam 1993; Nomikos et al. 1994; Yamamoto et al. 1994; Youngren et al. 1999). This dopamine may act via D1 receptor to stimulate c-fos expression in a manner that is dependent on Ca2+ influx via co-activated NMDA receptors (Konradi et al. 1996; Nakazato et al. 1998). A postsynaptic interaction of D1 and NMDA receptors (with the associated intracellular biochemical and genomic changes) can lead to a sustained enhancement of excitatory synaptic responses or neuronal excitability, in the striatum, PFC, and hippocampus (Cépeda and Levine 1998; Greegarrd et al. 1999; Huang and Kandel 1995; Seamans et al. 2001; Wang and O’Donnell 2001; Yang 2000; Zheng et al. 1999). Hence, given that clozapine selectively enhance glutamate and dopamine release mainly in the PFC but not in subcortical area, we aim to examine the effects of acute clozapine on NMDA receptor-mediated synaptic responses in PFC neurons. We hypothesize that acute clozapine actions in PFC may involve presynaptic glutamate and dopamine release and postsynaptic D1 receptor modulation of NMDA receptor in PFC neuronal networks. Preliminary findings have been presented as an abstract (Chen and Yang 1999).

**METHO DS**

**Brain slice preparations**

The experiments were performed in brain slices prepared from young adult (27–36 days) male Sprague-Dawley rats. The euthanasia method used was approved by the Lilly Animal Use Committee, the policies of which adhere closely with the U. S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS policy) and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. Following decapitation by a guillotine (using a plastic Decapicone rat restrainer; Braintree Scienti fi c, FL), the brain was quickly removed and placed for 1–2 min in ice-cold oxygenated (95% O2–5% CO2) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 sodium chloride, 26 NaHCO3, 2.5 KCl, 0.5 CaCl2, 4 MgCl2, 1.3 ascorbic acid, and 25 glucose. The temporal lobes of the brain from both hemispheres were trimmed away, leaving the medial cortex from both hemispheres. The ACSF, contained (in mM) 120 CsOH, 120 D-gluconic acid, 10 HEPES, 1.1 EGTA, 2 MgCl2·6H2O, 0.1 CaCl2, 20 NaCl, 2 Na2ATP, and 0.5 Tris-GTP, and the pH was adjusted by CsOH to 7.3 (±290 mOsM). A Cs+-containing recording pipette was used to block outward rectifying K+ current, thus allowing current clamp of membrane at very positive potentials of +20 to +30 mV. At a steady-state maintained membrane depolarization, all Na+ channels would be inactivated, and so no Na+ channel blocker (XQ-314) was included in the pipettes. Voltage and current signals in current- and voltage-clamp modes, respectively, were amplified by an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). All signals were digitized with a 12 bit A/D converter (Digitidata 1200B) and stored in the computer hard disk for off-line analysis. In current-clamp mode, series resistance (10–20 Ω after “break-in”) was 80% compensated and was monitored constantly during the entire experiment by “bridge”—balancing of the instantaneous voltage responses to hyperpolarizing current pulses (200 ms, −20 to −100 pA) prior to each electrical stimulus delivery. Recordings were terminated, and the data were discarded if the series resistance changed by ≥6 MΩ. In voltage-clamp experiments, spontaneous- and mini-EPSCs [in the presence of 1 μM tetrodoxin (TTX)] were recorded at Vhold = −80 mV in the presence of 10–15 μM bicuculline to block all GABA_A receptor-mediated current.

**Synaptic stimulations**

Electrical stimulation was delivered via a concentric bipolar metal stimulating electrode (MCE-100X, David Kopf) placed in layer V, ~200–300 μm from the adjacent recorded neuron to activate local afferents synaptically. Programmed monophasic square pulses (0.2 ms, 50–200 μA, at 0.25–0.5 min inter-stimulus intervals) were delivered via a Master-8 programmable pulse-generator to an optically isolated stimulator (Isoflex, AMPI, Israel).

**Drug applications**

All drugs used were bath-applied by gravity. Stock solutions of 6,7-Dinitroquinoxaline-2,3-dione (DNQX), 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydropyridine-7-sulfonamide (NBQX), 2-amino-5-phosphonovaleric acid (APV, Tocris, UK), clozapine, SCH 23390, SCH50911, SKF81297, bicuculline methiodide, bupropion (Sigma/ RBI), TTX (Alomone Laboratory, Israel) were prepared in de-ionized water and stored as frozen aliquots at −20°C. Stock solution of clozapine (1 mM) was made using a drop of 0.1 N HCl and with the final volume made up in de-ionized water. Appropriate dilution of clozapine (e.g., to 1 μM, pH 6.5) was made in ACSF before bath application. In some experiments, clozapine was also dissolved in dimethylsulfoxide (DMSO). Identical responses were induced by either of this preparation of clozapine. Nimodipine or nifedipine (Sigma/ RBI) was made up fresh in 100% ethanol (1 mM stock) and then diluted to the appropriate final concentration (1 μM) in the perfusate.
Ambient and microscope lights were dimmed during perfusion of these Ca$^{2+}$ channel antagonists.

Unless stated otherwise, a low concentration of bicuculline (1–2 μM) was used to reveal the late NMDA receptor-mediated synaptic component (Kanter et al. 1996; Luhman and Prince 1990). In some experiments when prominent GABA$_A$ response was evoked, a GABA$_A$ receptor antagonist SCH50911 (10 μM) was also added.

**Data analyses**

The integrated areas of EPSPs were measured using pClamp 8.0 software (Axon Instruments). Mean integrated EPSP area from five to eight traces from each cell was used. Spontaneous- and mini-EPSCs were analyzed using MiniAnalysis program (www.synaptosoft.com). Total counts of sEPSCs and mEPSCs were binned and cumulative histograms were plotted using SigmaPlot (Version 6.0). Paired Student’s t-tests were used to compare differences between group data. All group data are expressed as means ± SE. Multiple group comparisons were performed using a one-way ANOVA with post hoc Tukey’s or Dunnett’s test applied for comparison of individual group data (GraphPad Prism Version 3.02). Differences between control and experimental responses with $P < 0.05$ were deemed significant.

**RESULTS**

**Glutamatergic EPSPs evoked by stimulation of layers V–VI**

Ninety-five layer V–VI pyramidal PFC neurons, visually identified under DIC-IR, were recorded using whole cell patch-clamp recordings in current-clamp mode. These PFC neurons had a mean input resistance of 92.36 ± 24 MΩ and an initial spike height >80 mV (with KMeSO$_4$-based internal solution). Electrical stimulation of the deep layers V–VI typically evoked an EPSP–inhibitory postsynaptic potential (IPSP) sequence in the majority of the pyramidal neurons studied (Fig. 1A).

At holding potentials between −60 to −65 mV, the glutamatergic EPSPs of interest in this study were often masked by an IPSP (Fig. 1, A–C). We used a low concentration of bicuculline (1–2 μM) to partially block the GABA$_A$ receptor-mediated IPSPs. After 20–30 min of continuous bath application of bicuculline, the same stimulation then evoked mainly EPSPs (Fig. 1D). Under this condition, and with the membrane potentials current clamped at −60 to −65 mV, the EPSPs typically consisted of two components. An early short-duration component, which can be blocked by the AMPA/kainate receptor antagonist DNQX (10 μM), was typically followed by a more prolonged late component that could be blocked by the NMDA antagonist APV (50 μM) (Seamans et al. 2001). Preliminary experiments show that stable control EPSPs could be held in a typical recording period for ≤80 min (Fig. 2, A and B, open circles).

**Clozapine potentiated NMDA-receptor mediated polysynaptic EPSPs in a local network of PFC neurons**

Bath application of clozapine (0.1–1 μM) potentiated the late component of the EPSPs. This late EPSP potentiation resulted in spike burst firing with no change in input resistance ($n = 12$, Fig. 1, E–G). In addition, there was a considerable shift in the onset latency of the burst firing arising from the late EPSP component, suggesting that this might be mediated polysynaptically.

In a separate series of experiments, the lidocaine-derivative Na$^+$ channel blocker QX-314 was included in the internal pipette solution to block Na$^+$ spike firing. Bath application of clozapine (1 μM) mainly enhanced late EPSPs, thus yielding a significant increase of the integrated area and half-width of the entire EPSP (Fig. 2, A–D). The onset of the potentiating effects of clozapine occurred slowly and it frequently took 10–15 min to reach steady-state response (Fig. 2, A and B). However, once the augmentation of the EPSP by clozapine was achieved, the effect was long lasting (Fig. 2, A and B). Group data in this series of experiment showed that clozapine at 1 μM most reliably augmented the integrated area of the mixed glutamatergic EPSP significantly ($P < 0.05$; Fig. 2D).
In rodents and primates, many cortical pyramidal neurons form long recurrent collateral connections (≤500 μm apart) with neighboring pyramidal cells (Douglas et al. 1995; Gao et al. 2001; Gonzalez-Burgos et al. 2000; Levitt et al. 1993; Markram 1997; Pucak et al. 1996; Szentagothai 1978; Thomson and Deuchars 1997). We stimulated electrically the forcep minor (white matter) to evoke antidromically the intrinsic local axonal outputs of the layer V–VI neurons. The antidromic stimulation activated a small network of interconnected pyramidal neurons (Fig. 3A; n = 5). Nevertheless, because forcep minor also contain axonal inputs extrinsic to the PFC, the same stimulation also evoked orthodromically some of these inputs. Ten to 15 min after exposure to clozapine (1 μM), multiple EPSPs occurred at varying onset latencies on forcep minor stimulation (Fig. 3B). The variable latencies may represent a different axonal conduction time between interconnected neurons. Some of the potentiated EPSPs reached threshold for spike burst firing. These findings suggest that clozapine can promote polysynaptic network interactions between intrinsically interconnected PFC neurons, although potentiation of the co-activated excitatory extrinsic inputs also contributed to the overall responses.

To examine the polysynaptic nature of clozapine potentiation of evoked EPSP and spike bursts, we have elevated extracellular Ca\(^{2+}\) concentration (from 2.3 to 7 mM) to reduce polysynaptic activation (Berry and Pentreath 1976). With higher [Ca\(^{2+}\)]\(_o\) (5–7 mM), the monosynaptic early EPSP remained unchanged while the late EPSP and spike bursts with variable onset latencies were abolished (n = 4; Fig. 3, C–E). Furthermore, the clozapine-induced potentiation of the late EPSP and the associated burst firing were also blocked by NMDA receptor antagonist APV (50 μM; n = 5; Fig. 3, F–H), suggesting that acute clozapine enhanced these NMDA receptor-mediated polysynaptic network interactions. On the other hand, application of L-type Ca\(^{2+}\) channel antagonists nifedipine or nifedipine (0.5 μM) failed to block the potentiation of the EPSP by clozapine (n = 5, not shown). These data suggest that the augmented late NMDA-EPSP is not associated with synaptic activation of L-type Ca\(^{2+}\) channel-mediated dendritic Ca\(^{2+}\) spike potentials (Seamans et al. 1997).

Clozapine potentiates mixed glutamatergic reversed EPSPs but not pharmacologically isolated AMPA or NMDA reversed EPSPs

As mentioned in the last series of experiments, augmentation of NMDA-EPSP may also co-activate Ca\(^{2+}\) spike potentials generated in proximal dendrites. This dendritic Ca\(^{2+}\) potential will mix with the NMDA-EPSP and complicates the measurement of a potentiated NMDA component (Seamans et al. 1997). On the other hand, if the holding potential is held too negatively, a strong Mg\(^{2+}\) block of the NMDA-R channel complex will occur. To overcome both of these problems, we have deliberately current-clamped the neurons beyond the reversal potentials of the NMDA-R-mediated EPSPs (V\(_R\) ≈ 0 mV). We used a more positive holding potential (e.g., +20 to +30 mV) to ensure that there was an adequate activation of the NMDA receptor (by relieving voltage-dependent Mg\(^{2+}\) block) and that the steady-state positive holding potential would minimize activation of dendritic Ca\(^{2+}\) spike potentials (Seamans et al. 1997). We accomplished this by using Cs\(^+\)-containing recording pipettes to block all outwardly rectifying K\(^+\) currents. In addition, a steady-state depolarization of the membrane continuously clamping at +20 mV also inactivates all Na\(^+\) channels, thus no additional Na\(^+\) channel blocker QX-314 was necessary in the recording pipette.

PFC neurons were current-clamped (at a V\(_{\text{Hold}}\) = +20 to +30 mV) in bicuculline (1–2 μM) and normal ACSF. Under this condition, a negative-going reversed EPSP (rEPSP) was evoked by synaptic stimulation (Fig. 4A). Bath application of clozapine (1 μM) significantly (P < 0.01) enhanced the integrated area of the rEPSP, especially the late NMDA receptor-mediated synaptic component (n = 7; Fig. 4, B and C). The time course of this rEPSP enhancement was enduring with the effects outlasting the period of clozapine application (Fig. 4B).

We then isolated the AMPA and NMDA receptor-mediated components of the rEPSP pharmacologically and examined the
direct effect of clozapine on these separated synaptic components. Following APV (50 μM) blockade of the NMDA component to reveal only the AMPA-rEPSP, clozapine (1 μM) caused a small but insignificant reduction in peak (P > 0.4) and integrated area (P > 0.7) of the isolated AMPA-rEPSP (n = 7; Fig. 4, D–F). Under voltage clamp (V_{hold} = −80 mV), clozapine also failed to induce a significant change in the peak and integrated area of the synaptically evoked AMPA current (n = 4; not shown).

In a separate group of PFC neurons, the AMPA/kainate receptor antagonist DNQX (n = 6) or NBQX (n = 3; 10 μM) was used to block the AMPA component to isolate the NMDA-eEPSP. Clozapine (1 μM) failed to alter the pharmacologically isolated NMDA rEPSP (Fig. 4, G–I). These findings suggest that clozapine did not directly potentiate the AMPA or the NMDA component of the evoked rEPSPs. Additional factor(s) are involved in this potentiation effect of clozapine.

**Dopamine transporter inhibitor bupropion further augmented the rEPSPs potentiated by clozapine**

Clozapine can cause considerable release of endogenous dopamine in the PFC in vivo via as yet unknown mechanisms (Daly and Moghaddam 1993; Hértel et al. 1996; Kuroki et al. 1999; Nomikos et al. 1994; Pehek and Yamamoto 1994; Yamamoto et al. 1994; Youngren et al. 1999). Consequently, this released dopamine can modulate postsynaptic NMDA receptor functions (Levesque et al. 2000). Our electrophysiological technique cannot directly detect the possible release of endogenous dopamine in the slice. Hence, we made use of the dopamine transporter blocker bupropion (Richelson and Pfenninger 1984; Tatsumi et al. 1997) to examine the hypothesis that clozapine augmentation of NMDA-EPSP can be further potentiated when reuptake of the released dopamine is blocked. If clozapine induces presynaptic release of dopamine in the brain slices, then the presence of dopamine reuptake blocker would allow more of the released dopamine to be available for synaptic modulation of NMDA receptor.

Evoked rEPSPs were recorded at +20 to +30 mV using Cs⁺-filled recording pipette in bicuculline (1–2 μM). Ten minutes following bath application of bupropion (1 μM) alone, there was a small but insignificant (+19.4 ± 12%; P > 0.05) increase of the late component of the rEPSP. The addition of clozapine (1 μM) enhanced significantly (+66 ± 50%;
Voltage traces before and after buproprion and clozapine were then subtracted digitally (Fig. 5, A and B). The resulting subtracted traces, together with the grouped data of the rEPSP integrated area, show a significant (P < 0.05) increase of the NMDA receptor-mediated late rEPSP when compared with the control (Fig. 5, A–C), as well as to the potentiated rEPSP by clozapine alone (as shown in Fig. 4C). Application of the NMDA receptor antagonist APV (50 μM) blocked this clozapine-enhanced component of the rEPSP (in the presence of bicuculline), suggesting that it was NMDA receptor-mediated (Fig. 5C). In three of nine PFC neurons, both the amplitude and integrated area of the rEPSP was reduced markedly by APV (reduced to 10% of control). In another three neurons, digital trace subtraction showed that although the slow late component of the rEPSP was reduced significantly by APV, the remaining early fast component was sensitive to blockade by a higher concentration of GABA_α antagonist bicuculline (10 μM; not shown).

Potentiating effects of clozapine on glutamatergic EPSPs were blocked by dopamine D1 antagonist SCH23390

In the continuous presence of bicuculline (2 μM), bath application of the D1 antagonist SCH23390 (1 μM) did not induce significant changes regardless of whether the glutamatergic EPSP or rEPSPs were evoked at −60 or +20 mV, respectively (Fig. 5, D–G). However, in the presence of SCH23390, the clozapine-induced potentiation of EPSP or rEPSPs was completely abolished. This resulted in no significant difference between EPSPs or rEPSPs in SCH23390 alone and in SCH23390 plus clozapine (P > 0.05; Fig. 5, D–G). These results suggest that clozapine’s ability to potentiate the rEPSP is dependent on D1 receptor activation. Similar to results obtained by Seamans et al. (2001) on NMDA-excitatory
postsynaptic currents (EPSCs), our study showed D1 agonists dihydrexidine or SKF81297 (5–10 μM) potentiated the rEPSPs (n = 11; not shown). Collectively, data in this and the preceding section suggest that clozapine is likely to release dopamine in the slices. The released dopamine then activates postsynaptic D1 receptors to augment NMDA component of the glutamatergic EPSPs.

Clozapine enhanced spike-activity-dependent glutamate release: effects of clozapine on AMPA receptor-mediated m- and sEPSCs

To examine whether clozapine acts directly on presynaptic glutamatergic axonal terminals to augment glutamate release, we have recorded miniature excitatory postsynaptic currents (mEPSCs) in 11 layer V–VI pyramidal cells in the presence of TTX (1 μM). TTX blocks synaptic transmission and spike-dependent release of transmitters, thus ensuring that any change in glutamate release (detected as changes in mEPSC frequency) is due to a direct action of clozapine on the presynaptic terminals. In contrast, a change in the amplitude of mEPSCs would be due to actions of clozapine on postsynaptic glutamate receptors (Del Castillo and Katz 1954; Katz and Miledi 1969; Redman 1990).

In this series of experiments, a higher concentration of bicuculline (10 μM) was used to block all GABA_A-mediated inhibitory postsynaptic currents (IPSCs). Spontaneous presynaptic glutamate release was monitored postsynaptically as DNQX-sensitive fast mEPSCs, which were recorded in PFC neurons. Neurons were voltage-clamped at −80 mV to maximize the inward driving force of the cationic AMPA-mEPSCs. Under these conditions, bath application of clozapine (0.1 and 1 μM) failed to induce any significant increase in either the overall amplitude (control mEPSCs: 14.15 ± 1.64 pA; in 0.1

FIG. 5. Clozapine enhancement of the slow late rEPSPs involves dopamine release and D1/D5 receptor activation. A: control rEPSP evoked in the presence of 2 μM bicuculline and 10 μM SCH50911 (gray trace). Each displayed voltage trace was an average from 8 to 10 rEPSPs sweeps. Bath application of dopamine reuptake blocker buproprion (1 μM) slightly augmented the slow component of the rEPSP. Electronic subtraction of the 2 voltage traces resulted in a subtracted trace (indicated by an open arrow head arrow) showing a net increase of the rEPSP by buproprion. B: in the same PFC neuron, when clozapine (1 μM) was applied in the presence of buproprion, there was a significant augmentation of the slow late APV-sensitive component of the rEPSP. The gray trace (averaged from 8 to 10 sweeps) represents the buproprion response trace and is identical to that shown in A. Electronic subtraction of the buproprion trace with the buproprion plus clozapine trace resulted in a subtracted trace (indicated by an open arrow head arrow) showing a net increase of the rEPSP with this treatment. As indicated in the flat subtracted trace before stimulus was applied, there was no change in the voltage responses induced by the hyperpolarizing (−50 pA) prepulse, and this suggests that there was little or no change in input resistance. C: histograms summarizing group data from the experiments illustrated in the preceding text. ANOVA: [F(3,33)] = 5.5; P < 0.01]. Tukey’s test: *P < 0.05; **P < 0.01. D and E: representative evoked EPSP from a separate group of 5 PFC neurons. Dopamine D1 antagonist SCH23390 (1 μM) alone did not change the EPSP evoked at −60 mV. However, pretreatment of SCH23390 completely blocked the ability of clozapine to potentiate the evoked EPSPs. Each displayed voltage trace was an average from 8 to 10 EPSPs sweeps. F and G: reversed EPSPs were evoked at a steady-state holding potential of −20 mV. Pretreatment of SCH23390 (1 μM) failed to cause a significant (P > 0.05) change to the rEPSP, but the D1 antagonist blocked the potentiating effects of clozapine on the rEPSPs. Collectively, these data suggest that the enhanced NMDA receptor-mediated component of the EPSP or the rEPSP requires activation of D1 receptors by endogenous dopamine that was released presynaptically by clozapine.

postsynaptic currents (EPSCs), our study showed D1 agonists dihydrexidine or SKF81297 (5–10 μM) potentiated the rEPSPs (n = 6; not shown). Collectively, data in this and the preceding section suggest that clozapine is likely to release dopamine in the slices. The released dopamine then activates postsynaptic D1 receptors to augment NMDA component of the glutamatergic EPSPs.
μM clozapine, 9.3 ± 1 pA; in 1 μM clozapine, 14.1 ± 2 pA) or the frequency (control intervals: 124.8 ± 16.7 ms; in 0.1 μM clozapine, 171.5 ± 27 ms; 1 μM clozapine 106.4 ± 18.7 ms) of the mEPSCs. Total event counts recorded in control and in clozapine, grouped in bins of 5-pA or 10-ms intervals, show no quantitative difference or in temporal shifts (n = 11; Fig. 6, A and C).

In six PFC neurons tested in current-clamped mode, bath application of clozapine alone transiently increases spontaneous EPSPs in all these neurons but did not change the overall resting membrane potentials with time (Fig. 7, A and B). Using voltage clamp, spontaneous EPSCs (sEPSCs) recorded without TTX (n = 8 neurons) showed that clozapine at 0.1 μM was sufficient to reduce inter-event intervals significantly (control intervals of sEPSCs: 166.7 ± 37.5; in 0.1 μM clozapine, 85.9 ± 25.4 ms; P < 0.01; Fig. 7, C and E). This overall frequency increase (i.e., a reduction in inter-event intervals) was accompanied by no change in the overall amplitude of the sEPSCs (control sEPSC amplitude, 16.5 ± 1.1 pA; in 0.1 μM clozapine, 16.9 ± 0.96 pA) recorded in PFC pyramidal neurons (Fig. 7F). The sEPSC frequency enhancement occurred within the first 10–15 min of exposure to clozapine. Continuous exposure to the same concentration of clozapine did not induce a continuous increase in the frequency of sEPSCs. Hence, it is likely that the readily releasable pool of presynaptic glutamate might have been depleted in the slices or that the AMPA receptor is desensitized. This initial increase in AMPA-sEPSC is insufficient to cause a steady-state membrane depolarization over time (as shown in current-clamp recordings in Fig. 7, A and B).

Different sEPSC amplitudes summed and binned in a cumulative event plot (Fig. 7D) showed that clozapine increases the occurrence of the number of low-amplitude (5–10 pA) sEPSCs (Fig. 7D). This was accompanied by a large increase of the sEPSCs in the shorter inter-event intervals (20–60 ms; i.e., higher frequency sEPSCs; Fig. 7E). These data suggest that clozapine has both pre- and postsynaptic effects on glutamatergic transmission. Because the increased AMPA-sEPSPs by clozapine were not associated with any changes in overall membrane potentials (as shown in current-clamp recordings in Fig. 7, A and B), any voltage-dependent activation of dendritic NMADA receptors by the increased AMPA-sEPSPs are likely to take place mainly in dendritic regions distal from our recording pipette.

**DISCUSSION**

Findings from the present study show that in layer V–VI PFC pyramidal neurons clozapine potentiates the late, NMDA receptor-mediated component of evoked EPSPs to elicit spike burst firing. rEPSPs were used to provide a clearer separation of the NMDA component of the EPSP from co-activated dendritic Ca2+ spike potentials. Clozapine potentiated evoked EPSPs and rEPSPs but failed to potentiate pharmacologically isolated AMPA- and NMDA-rEPSPs. The potentiated evoked rEPSPs were augmented further when dopamine reuptake was blocked by buproprion. Pretreatment with the D1 antagonist SCH23390 blocked clozapine potentiation of the late rEPSPs, suggesting that dopamine released by clozapine activates D1 receptor to achieve a sustained enhancement of the NMDA receptor-mediated synaptic responses within the PFC neuronal network.

**Clozapine potentiates the NMDA components of the evoked EPSPs and rEPSPs in PFC neurons**

Electrophysiological findings from the striatum and PFC have shown that clozapine at clinically relevant serum levels (≤1 μM) (Baldessarini et al. 1988; Conley 1998) increased spontaneous glutamate EPSPs and enhanced the NMDA-com
ponent of glutamatergic synaptic responses (Arvanov and Wang 1999; Banerjee et al. 1995). Other electrophysiological studies also emphasized that clozapine could activate, or block, serotonin receptor subtypes to potentiate inward current induced by exogenous microdrop NMDA applications in PFC slices (Arvanov and Wang 1998, 1999). It is notable that the cited in vitro electrophysiological studies used elevated [glycine]o and reduced [Mg²⁺]o levels in extracellular media to maximize detection of NMDA-mediated responses. In our present study using normal ACSF with no added [glycine]o or reduced [Mg²⁺]o, we found that clozapine induced a sustained potentiation of SCH23390- and APV-sensitive late EPSPs, and rEPSPs. Our data suggest that both D1 and NMDA receptors are functionally engaged in the actions of clozapine.

Clozapine may exert several different mechanisms to activate NMDA receptors. Our data show that clozapine increases the frequency and the occurrence of low-amplitude (5–10 pA) AMPA receptor-mediated spontaneous EPSCs, without changing miniature EPSCs or causing depolarizing changes to the steady-state membrane potential. These findings suggest that clozapine can increase spike-dependent presynaptic glutamate release (as shown in vivo) (Daly and Moghaddam 1993; Yamamoto and Cooperman 1994), which resulted in the subsequent increased sEPSPs. Functional AMPA receptors are primarily located in distal dendrites, while NMDA receptors are mainly located near soma-proximal dendritic regions in single neocortical pyramidal neurons (Dodt et al. 1998; Schiller et al. 2000). In our study, it is likely that the increased presynaptic glutamate release by clozapine (as detected by a brief increase of AMPA spontaneous EPSP/CS) activated postsynaptic dendritic AMPA receptors distal to the somatic recording electrode. The brief increase in AMPA-EPSPs (e.g., ~10 min) may reflect a rapid AMPA receptor desensitization (Mosbacher et al. 1994; Otis et al. 1996), coupled with strong filtering/shunting of spatial-temporal summation of distal dendritic AMPA-EPSPs that were recorded by the somatic electrode (Inoue et al. 2001). However, any AMPA receptor activation may already be suf-

![ FIG. 7. Clozapine effects on membrane potential and spontaneous EPSCs (sEPSCs). A: bath-application of clozapine failed to change the steady-state resting membrane potential of PFC neuron (n = 6). B: voltage trace displayed at a faster time scale showed that there was an increase in sEPSPs in the presence of clozapine (1 μM, right). C, left and middle: under voltage-clamp (V_Hold = −80 mV), clozapine enhanced both the amplitude and frequency of sEPSCs. Right: the sEPSCs are primarily mediated by AMPA receptor because they are completely blocked by selective AMPA receptor antagonist DNQX (10 μM). D and E: when the group data from 8 neurons were binned, the distribution histograms shows that clozapine increases the occurrence of low-amplitude short interval sEPSCs (i.e., increase the frequency of low-amplitude sEPSCs). F: overall, the increase of the sEPSC amplitude was not significant (P > 0.05) but the decrease in the intervals (= increase frequency) was significant (*P < 0.05).]
CLOZAPINE MODULATES GLUTAMATE AND DOPAMINE INTERACTIONS

Presynaptic dopamine release contribute to clozapine potentiation of the NMDA component of the evoked EPSP

Additional mechanisms contribute to the potentiation of NMDA receptor by clozapine. Our present data also suggest that presynaptic dopamine release and postsynaptic D1 receptor mechanisms are involved in mediating a sustained augmentation of glutamatergic synaptic transmission by clozapine. Systemic administration of clozapine releases PFC dopamine transynaptically via subcortical pathways in vivo (Daly and Moghaddam 1993; Hérel et al. 1996; Kuroki et al. 1999; Nomikos et al. 1994; Pehek and Yamamoto 1994; Yamamoto et al. 1994; Youngren et al. 1999). Local application of clozapine can also enhance DA release directly within PFC without the influence of VTA DA neuronal firing activity (Gessa et al. 2000). The late onset (~10 min) and prolonged duration (~30 min) of clozapine potentiated evoked EPSPs was strikingly similar to the potentiated synaptic or excitatory responses following dopamine or D1 agonist application in PFC, striatal, and hippocampal neurons (Cépa and Levine 1998; Greengard et al. 1999; Huang and Kandel 1995; Seamans et al. 2001; Wang and O’Donnell 2001; Yang 2000; Zheng et al. 1999). Furthermore, application of the D1 receptor antagonist SCH23390 prevented the acute clozapine-mediated potentiation of the glutamate synaptic response, suggesting the involvement of D1 receptor in clozapine’s actions.

How clozapine enhances endogenous DA (and glutamate) release in the brain slices is unknown. There is a possibility that clozapine can block inward rectifier K+ channels (Kobayashi et al. 2000) and/or presynaptic terminal D2 autoreceptors on DA neurons to promote spike activity-dependent dopamine release. Endogenous dopamine in the PFC has a high capacity for evoked release, coupled with a slow clearance (Garris et al. 1993), perhaps due to the presence of a small number of dopamine transporters on the mesocortical dopamine terminals (Sesack et al. 1998). However, clozapine does not bind directly to dopamine transporters, thus it is not likely that clozapine increases extracellular dopamine levels by blocking the dopamine transporter (Reader et al. 1998; Rothblat and Schneider 1997). A selective blockade of dopamine transporter by buproprion prevented the reuptake of endogenous dopamine released by clozapine in the PFC slices. This elevation of dopamine will stimulate postsynaptic D1 receptor to modulate the converging glutamate synapses. This will functionally result in a sustained potentiation of the evoked glutamatergic EPSPs.

Post synaptic D1/NMDA receptor interaction mediates acute clozapine effects on glutamate synaptic transmission

Functional NMDA potentiation following acute D1 receptor activation has been demonstrated in striatum, hippocampus, and PFC (Cépa and Levine 1998; Greengard et al. 1999; Huang and Kandel 1995; Seamans et al. 2001; Wang and O’Donnell 2001; Yang 2000; Zheng et al. 1999). For acute clozapine to trigger a D1/NMDA receptor-mediated potentiation of glutamatergic transmission in PFC, the following mechanisms are likely to occur. In addition to its capability to release DA presynaptically, clozapine is also known to be a weak D1 receptor antagonist (Ki ~ 100 nM) (Bymaster et al. 1999; F. Bymaster, personal communication). Hence, a large level of dopamine released by clozapine will stimulate a weakly blocked population of postsynaptic D1 receptors. This D1 receptor activation by dopamine (as a result of a simultaneous large presynaptic release by clozapine and a weak postsynaptic blockade of D1 receptor by clozapine) may enable an optimal D1 dependent potentiation of NMDA receptor.

Optimal D1 and NMDA receptor stimulation, perhaps resulting in a D1 potentiation of NMDA receptor functions, is critical for higher cognitive processes in PFC (Aura and Riekkinen 1999; Granon et al. 2001; Pontecorvo et al. 1991; Sawaguchi and Goldman-Rakic 1994; Seamans et al. 1998). Too little or too great a D1 receptor stimulation in PFC will disrupt cognition (Murphy et al. 1996; Williams and Goldman-Rakic 1995; Zahr et al. 1997). As discussed in the preceding text, a weak blockade of D1 receptors by clozapine may prevent the cognitive impairing effects of a high level of dopamine (e.g., released presynaptically by acute clozapine). The released dopamine may then optimally “tune” D1 potentiation of NMDA receptor.

Acute postsynaptic interaction of D1 and NMDA receptor induced by clozapine can trigger a series of late intracellular biochemical and genomic changes. Although not tested in this study, neurochemical findings have suggested the importance of several intracellular signals (including protein kinase A and intracellular Ca2+) that may be critical in mediating genomic changes. The protein products of these changes are likely to be critical for long-term changes in the synaptic plasticity (Abraham et al. 1991; Blank et al. 1997; Deutch and Duman 1996; Dragunow 1996; Impye et al. 1996; Konradi 1998; Levesque et al. 2000; Nisenbaum and Fibiger 2000; Rajadhyaksha et al. 1999; Schultz et al. 1999; Sebens et al. 1995; Semba et al. 1996; Snyder et al. 1998; Tully 1997; Walton et al. 1999; Westphal et al. 1999; Worley et al. 1993). On-going studies are examining these important aspects.

Consistent with the negative finding of clozapine on AMPA-EPSC reported by others in the PFC (Arvanov and Wang 1997), we also failed to detect a significant change in evoked AMPA-rEPSPs following clozapine application. Nevertheless, in neostriatal neurons, dopamine or D1 agonists can functionally potentiate AMPA receptor-mediated evoked synaptic responses and phosphorylate GluR1 subunit of the AMPA receptor (Price et al. 1999; Snyder et al. 2000; Umemiya and Raymond 1997; Yan et al. 1999). It is likely that the AMPA subunit composition in PFC differs from that in the neostriatum because D1 receptor stimulation in PFC neurons has been shown to result in moderate AMPA-EPSC suppression (Gao et al. 2001; Seamans et al. 2001; present study).

Collectively, the combined acute actions of clozapine on presynaptic DA release, subsequent D1 receptor modulation of activated NMDA receptors, and a weak postsynaptic D1/D5 receptor blockade to restrict excessive D1 receptor stimulation
enables optimal “tuning” of D1 receptors for acute interaction with the NMDA receptor (see Fig. 8 for a summary schematic).

Finally, it has been suggested that clozapine’s “pro-convulsant” effect may be one of the important mechanisms responsible for its therapeutic action (Denney and Stevens 1995; Minabe et al. 1998; Stevens 1995; Stevens et al. 1996). Clozapine has the collective abilities to block GABA_A receptors moderately (Korpi et al. 1995; Michel and Trudeau 2000; Squires and Saezol 1998); to lower the level of GABA released in PFC (Bourdelais and Deutch 1994); to promote activity-dependent release of glutamate and dopamine in PFC (Daly and Moghaddam 1993; Herbel et al. 1996; Kuroki et al. 1999; Nomikos et al. 1994; Pehek and Yamamoto 1994; Yamamoto et al. 1994; Youngren et al. 1999); and to potentiate postsynaptic D1 receptor-dependent NMDA receptor functions (present study). Current preclinical and clinical evidence suggests that there may be a NMDA receptor hypofunction in schizophrenia (Javitt and Zukin 1991; Krystal et al. 1999). The initiation of these sequences of synaptic events in the PFC neural network by acute clozapine may trigger cellular mechanisms that mediate lasting enhancement of NMDA receptor-mediated synaptic transmission. This may represent the initial mechanisms for later cognitive improvement in schizophrenia.

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