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Acute Clozapine Suppresses Synchronized Pyramidal Synaptic Network Activity by Increasing Inhibition in the Ferret Prefrontal Cortex

Wen-Jun Gao^{1,2}

¹Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, Pennsylvania; and ²Department of Neurobiology, Yale University School of Medicine, New Haven, Connecticut

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Gao W.-J. Acute clozapine suppresses synchronized pyramidal synaptic network activity by increasing inhibition in the ferret prefrontal cortex. *J Neurophysiol* 97: 1196–1208, 2007. First published December 20, 2006; doi:10.1152/jn.00400.2006. Recent studies have indicated that impaired neural circuitry in the prefrontal cortex is a prominent feature of the neuropathology of schizophrenia. Clozapine is one of the most effective antipsychotic drugs used for this debilitating disease. Despite its effectiveness, the mechanism by which clozapine acts on prefrontal cortical circuitry remains poorly understood. In this study, *in vitro* multiple whole cell recordings were performed in slices of the ferret prefrontal cortex. Clozapine, which effectively inhibited the spontaneous synchronized network activities in the prefrontal neurons, achieved the suppressive effect by decreasing the recurrent excitation among pyramidal neurons and by enhancing the inhibitory inputs onto pyramidal cells through a likely network mechanism. Indeed, under the condition of disinhibition, the depressing effects were reversed and clozapine enhanced the recurrent excitation. These results suggest that the therapeutic actions of clozapine in alleviating the positive symptoms of schizophrenia are achieved, at least partially, through the readjustment of synaptic balance between the excitation and inhibition in the prefrontal cortical circuitry.

INTRODUCTION

Schizophrenia is a severe, chronic, and debilitating psychotic disorder that disrupts a wide range of cognitive and emotional brain functions (Duncan et al. 1999a; Glenthøj and Hemmingsen 1999; Lewis 1995; Lewis and Lieberman 2000; Miyamoto et al. 2003; Weinberger et al. 1986). Throughout the history of medicine, the pathophysiological processes underlying schizophrenia remains largely unknown. However, recent studies have proposed that the cognitive and affective impairments of this disorder may be attributed to the failure to integrate the activities of both local and distributed neural circuitry in the prefrontal cortex (PFC) (Benes 2000; Benes and Berretta 2000; Friston and Frith 1995; Grunze et al. 1996; Lewis 2000; Lidow et al. 1998; Pralong et al. 2002; Sarter et al. 2005; Seimon and Goldman-Rakic 1999; Yang and Chen 2005; Yang et al. 1999). This hypothesis is supported by the demonstrated abnormalities in the morphology and distribution of certain cortical neurons, particularly GABAergic inhibitory interneurons, in postmortem schizophrenic brains (Benes 2000; Benes and Berretta 2001; Benes et al. 1992; Hashimoto et al. 2003; Lewis et al. 2003; Volk and Lewis 2002; Woo et al. 1998). GABAergic interneurons in the cerebral cortex are known to be important for the concerted interactions between inhibitory and excitatory circuitries and constitute a critical

substrate of synchronous neural activity in the brain (McBain and Fisahn 2001; Spencer et al. 2003, 2004; Whittington and Traub 2003; Whittington et al. 2000). Recent studies showed that gamma-band oscillations and synchronization, especially in the 40-Hz range, are severely disrupted in patients with schizophrenia (Calhoun et al. 2004; Lee et al. 2003a; Spencer et al. 2003, 2004; van der Stelt et al. 2004). The disrupted synchronization exhibited a decrease of gamma responses with negative symptoms or a significant increase of gamma activity with positive symptoms (Herrmann and Demiralp 2005; Lee et al. 2003a; Lee et al. 2003b).

As an atypical antipsychotic drug, clozapine has been found to be effective in alleviating positive symptoms, and possibly negative symptoms as well (Remington and Kapur 2000), of the treatment-resistant schizophrenia (Ashby and Wang 1996; Duncan et al. 1999b; Lidow and Goldman-Rakic 1994; Lieberman et al. 1989; Maguire 2002; Miyamoto et al. 2005; Safferman et al. 1994). However, the underlying mechanisms of these therapeutic actions on prefrontal cortical neuronal activity have not been fully elucidated. Previous studies suggested that clozapine enhances glutamatergic excitatory transmission in the PFC (Arvanov et al. 1997; Chen and Yang 2002; Jardemark et al. 2003; Ninan 2003) and nucleus accumbens (Wittmann et al. 2005) under conditions of disinhibition, *i.e.*, in the presence of γ -aminobutyric acid type A (GABA_A) antagonist bicuculline. This enhancement seems to require the activation of *N*-methyl-D-aspartate receptors (NMDARs) by various mechanisms (Arvanov et al. 1997; Chen and Yang 2002; Jardemark et al. 2003; Ninan 2003; Wittmann et al. 2005). In this study, electrophysiological recordings from acute prefrontal cortical slices were used to examine the synaptic and cellular actions of clozapine on recurrent excitation and inhibition in the PFC. These experiments were designed to address the following questions. 1) How does clozapine act on the excitatory and inhibitory synaptic transmission? 2) How does clozapine affect network activities in the PFC?

METHODS

Physiological recording

This study used 36 ferrets from 2 to 4 mo old (Marshall Farms, North Rose, NY). The animals were cared for under National Institutes of Health animal use guidelines and the experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Yale and Drexel (Gao et al. 2001). The ferrets were

Address for reprint requests and other correspondence: W.-J. Gao, Department of Neurobiology and Anatomy, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129 (E-mail: wen-jun.gao@drexel.edu).

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beheaded with a guillotine after being administered intraperitoneally (ip) with an overdose of sodium pentobarbital (100 mg/kg) to achieve deep anesthesia. The brains were quickly removed and 300- μ m-thick horizontal slices containing medial PFC (mPFC, an area equivalent to rat mPFC; personal communication with Dr. Alvaro Duque) were cut on a vibratome (Vibratome, St. Louis, MO). The slice cuttings were maintained in oxygenated ice-cold Na⁺-free sucrose solution containing (in mM) 2.5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 0.5 CaCl₂, 7.0 MgSO₄, and 213 sucrose. The slices were continuously incubated at 35°C in a Ringer solution [artificial cerebrospinal fluid (ACSF)] containing (in mM) 124 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgSO₄, 26 NaHCO₃, and 10 dextrose, pH 7.4. The temperature of the slices in the recording chamber was kept at approximately 35–37°C. Dual or quadruple whole cell recordings were used to detect the layer 5 pyramidal-to-pyramidal unitary synaptic connections. For current clamp, the recording pipettes were filled with intracellular solution containing (in mM) 120 K-gluconate, 6 KCl, 0.5 CaCl₂, 0.2 EGTA, 4 ATP-Mg, 10 HEPES, and 0.3% biocytin, with a final pH of 7.25 (Molecular Probes, Eugene, OR). Resistances of the patch pipettes ranged from 5 to 10 M Ω . For the voltage clamp, a Cs⁺-based intracellular solution (110 mM Cs-gluconate, 10 mM CsCl₂, 0.2 mM EGTA, 10 mM HEPES, 1 mM ATP-Mg²⁺, 5 mM QX-314, and 0.3% biocytin, pH 7.3) was loaded into recording pipettes (3–6 M Ω) to block sodium and potassium channels. To record the spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs), a different Cs⁺-based intracellular solution (134 mM CsCl₂, 2 mM MgCl₂, 2 mM Na₂-ATP, 0.5 mM Na₂-GTP, 5 mM Na₂-phosphocreatine, 1 mM EGTA, 10 mM HEPES, and 0.3% biocytin, pH 7.25) was used. The signals were amplified and filtered at 2 kHz with Multi-Clamp 700A amplifiers (Axon Instruments, Union City, CA) and acquired at sampling intervals of 20–100 μ s through a DigiData 1322 interface with program pCLAMP 8.2 (Axon Instruments). The membrane potentials were not corrected for liquid junction potential. The access resistance was monitored during recordings and the data were excluded from analysis if the series resistance changed >20% from control levels (10–25 M Ω). Synaptic strengths under conditions of control (baseline) and drug application were recorded at trial intervals of 5 s (0.2 Hz).

Pharmacological treatments

All drugs were applied to the bath through gravity. Stock solutions of all tested chemicals were stored at –70°C and were diluted to proper concentration before each experiment. The sodium channel blocker tetrodotoxin (TTX, 1 μ M), the NMDA receptor antagonist D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μ M), and the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μ M) were purchased from Sigma-RBI (St. Louis, MO). Clozapine (0.2–10 μ M, 10 mM stock in dimethyl sulfoxide), the GABA_A receptor antagonist bicuculline methiodide (10 μ M), and dopaminergic D4 receptor agonist PD 168077 maleate (10 μ M) were obtained from Tocris Cookson (Ellisville, MO).

Data analysis

The spontaneous and miniature excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) recorded in voltage-clamp mode were analyzed with Clampfit 9.2 (Axon Instruments). A typical EPSC/IPSC was selected to create a sample template for event detection within a data period. The frequency (event number) and amplitude of individual events were examined with Clampfit. For monosynaptic pyramidal–pyramidal connections recorded in the current-clamp mode, the amplitudes of evoked (by stimulating presynaptic cell) unitary EPSPs were measured from an average of 40–80 sweeps. To identify the synaptic mechanism, the rate of synaptic failure (percentage of stimulations that did not result in an EPSP), the

coefficient of variation (CV) of individual synaptic responses and the paired-pulse ratio (PPR = 2nd EPSP/1st EPSP amplitude) between two pulses at 20 Hz (50-ms interval) were calculated. The percentage of synaptic failure to the evoked presynaptic spike was determined individually for each recording. Failure was defined as an event in which the EPSP amplitude was <1.5 \times noise. The mean amplitude and SD of the EPSPs were obtained from 40 to 80 successive sweeps and the CVs of EPSP amplitude for control and clozapine application conditions were computed as SD divided by mean (CV = SD/mean).

The input resistances of the tested neurons were calculated off-line from the voltage produced by negative current injection before the step currents (Fig. 1). The number of spikes was counted from recordings of control, during clozapine application, and washout period. The threshold, first spike latency, first interspike interval (1st ISI) of action potentials (APs), and the fast afterhyperpolarization (fAHP) were manually measured from the series step recordings. The amplitude and half-peak duration of APs were determined from the single AP recordings evoked by step current injection. The data were evaluated with paired *t*-tests and presented as means \pm SE.

Histology

The PFC slices were immediately fixed in 4% paraformaldehyde for \geq 3–5 days after the physiological recordings. After thorough rinsing with 0.1 mM phosphate buffer (PBS, pH 7.4), the cortical

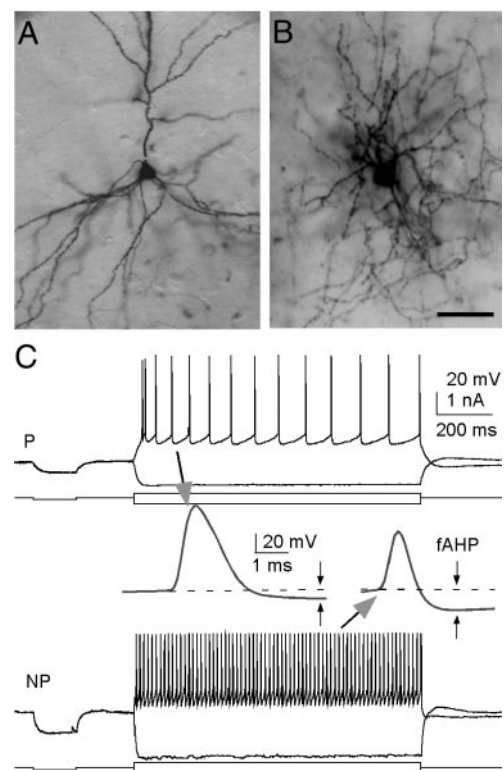


FIG. 1. Morphological characteristics and firing patterns of layer 5 pyramidal cells and a fast-spiking interneuron. *A* and *B*: microphotographs of a biocytin-labeled pyramidal cell (*A*) and a multipolar interneuron (*B*). Interneuron has multiple dendritic processes with characteristics of a GABAergic basket cell. Scale bar = 100 μ m for *A* and 50 μ m for *B*. *C*: current-clamp recordings from a pyramidal neuron (*P*) and a fast-spiking interneuron (*NP*) during injection of 2-step current pulses. *Middle*: single spikes in an expanded period. Before the step currents, a negative current (0.1 nA) was applied to monitor the input resistance of the recorded cell. Note the large fast afterhyperpolarization (fAHP) following a spike and the repetitive high-frequency firing without adaptation in NP. In contrast, the regular spiking pyramidal neuron has prominent firing adaptation, wider action potential, lower firing frequency, and smaller fAHP (see insets, middle panel).

slices were treated with 3% hydrogen peroxide for 25 min to reduce the endogenous horseradish peroxidase. The ABC reactions (Vectastain ABC standard kit, Vector Laboratories, Burlingame, CA) were then conducted, followed by a Ni-DAB reaction. The slices were resectioned into 100- to 150- μ m sections, mounted in 0.1 mM PB, and covered with water-soluble mounting media. Neurons were identified under a Zeiss Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany) and photographed with a Optronics digital camera (Optronics, Goleta, CA) (Gao et al. 2003).

RESULTS

All recordings were conducted in layer 5 of the mPFC and the recorded neurons were initially identified by their morphologies under infrared-differential interference contrast (IR-DIC) visualization. The interneurons were small cells with round or oval cell bodies, whereas the pyramidal neurons were larger cells with triangular soma and pia-oriented apical dendrites. The neuronal identities were then determined by their characteristic firing patterns. The fast-spiking interneuron has repetitive high-frequency firing without adaptation, narrow action potential, smaller spike amplitude, and a characteristic large fAHP (Fig. 1; Table 1), whereas the regular-spiking pyramidal neuron has prominent firing adaptation, wider action potential, lower firing frequency, and much smaller fAHP (Kawaguchi 1993, 1995). Based on these morphological and physiological characteristics, pyramidal cells and interneurons can be easily distinguished.

Origin of the spontaneous synchronized activity in the ferret prefrontal cortical slices

Neurons in acute brain slices are usually quiescent and stable when incubated and recorded at room temperature or, alternatively, first incubated at room temperature then recorded at higher temperature (<35°C) with regular ACSF solution. Under this condition, spontaneous excitatory and inhibitory postsynaptic currents (sEPSCs and sIPSCs) were frequently observed but exhibited asynchronous characteristics (Fig. 2A). However, when the brain slices were continuously maintained at a temperature of 35–37°C after slice cutting and perfused with a prewarmed (37°C) ACSF solution during recordings (chamber temperature maintained at about 36°C), synchronized barrages of synaptic activities could be observed in simultaneously recorded pyramidal neurons (held at –70 mV) with the Cs⁺-based intracellular solution (Figs. 2, B and C and 3A). The synchronous activities, which usually consisted of

both individual and large complex PSC discharges (Fig. 3A), were defined as simultaneously detected oscillatory postsynaptic currents (PSCs) in multiple recorded neurons. These synchronous activities could be detected in about one third of the prefrontal slices under our recording conditions. The remaining two thirds of the cortical slices were relatively stable with very little or no synchronization, although the spontaneous activities appear to be more vigorous than those of the slices incubated at room temperature. We believe that the spontaneous synchronized activity observed here is not like a rhythmic up- and down-state (Sanchez-Vives and McCormick 2000) nor an epileptic activity (Gao and Goldman-Rakic 2006) because the amplitudes are much smaller and the durations of the PSCs are much shorter than those of the up-states and epileptic discharges observed under these two conditions. These activities may represent patterns of overexcited hypersynchrony of synaptic activities in the neuronal network. Indeed, bath perfusion of AMPA receptor blocker CNQX (20 μ M) and NMDAR antagonist D-AP5 (50 μ M) completely blocked the synchronized activities in all three pairs of pyramidal cells tested (Fig. 2D), suggesting the involvement of synaptic activation of glutamate receptors in the synchronized events. This proposition is supported by numerous previous studies indicating that synchronized oscillations in neocortex are generated by recurrent synaptic activity mediated by glutamate receptors (Castro-Alamancos and Rigas 2002; Fisahn et al. 1998; Sanchez-Vives and McCormick 2000).

Clozapine abolishes synchronized activities in the prefrontal slices

To understand how clozapine affects the synchronized activity, pyramidal neurons from prefrontal slices that exhibited spontaneous synchronized activities were recorded before and after bath application of clozapine. Figure 3B shows an example of triple recording in which three pyramidal neurons were recorded simultaneously. Two of them (P1 and P2) were held in voltage-clamp mode (–70 mV), whereas the third one (P3) was recorded in current-clamp mode to monitor the neuronal excitability change during the clozapine application. A train of five-pulse (50-Hz, 20-ms) ISIs with positive current injection at a rate of 0.1 Hz was given to evoke action potentials in the P3 cell. Spontaneous synchronizations emerged among the pyramidal cells, although these neurons were not monosynaptically connected. In all five triplets tested, clozapine (1 μ M, 3 min) effectively abolished the synchronized PSCs, although it

TABLE 1. *Effects of clozapine on properties of action potentials (APs)*

Property	Pyramidal Cells (n = 6)		Fast-Spiking Interneurons (n = 17)	
	Control	Clozapine	Control	Clozapine
RMP, mV	–68.8 \pm 2.05	–69.7 \pm 2.03	–72.3 \pm 1.59†	–69.9 \pm 1.48**
Input resistance, M Ω	115.0 \pm 15.44	121.2 \pm 15.08	170.6 \pm 12.72††	192.0 \pm 14.42**
AP amplitude, mV	66.1 \pm 3.48	65.8 \pm 3.41	50.1 \pm 4.23††	50.4 \pm 2.33
AP half-width, ms	1.18 \pm 0.20	1.21 \pm 0.22	0.58 \pm 0.02††	0.62 \pm 0.03
Threshold, mV	–39.9 \pm 1.46	–40.3 \pm 1.44	–45.9 \pm 1.65††	–47.4 \pm 1.49*
1st spike latency, ms	14.5 \pm 3.18	14.6 \pm 3.24	7.4 \pm 0.51††	5.2 \pm 0.53**
ISI, ms	27.3 \pm 4.46	31.6 \pm 6.69	8.7 \pm 0.31††	6.2 \pm 0.32*
AHP, mV	3.1 \pm 1.01	2.9 \pm 0.85	13.2 \pm 1.17††	11.4 \pm 1.19**

Values are means \pm SE. †P < 0.01, ††P < 0.001 between pyramidal cell and interneuron. *P < 0.01, **P < 0.001 between control and clozapine. RMP, resting membrane potential; ISI, interspike interval; AHP, afterhyperpolarization.

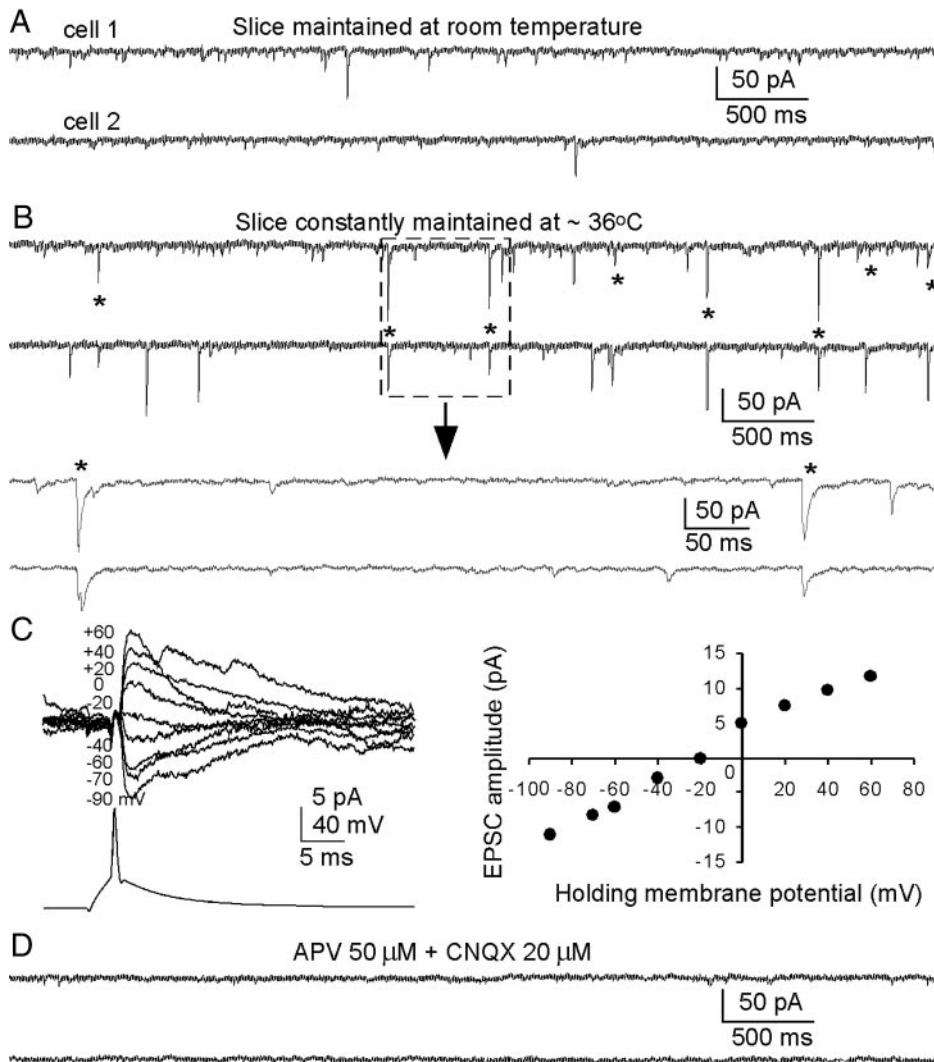


FIG. 2. Involvement of glutamatergic receptors in the spontaneous synchronized activity. *A*: examples of dual whole cell recording from 2 layer 5 pyramidal neurons recorded under voltage-clamp mode (held at -70 mV). Neurons were quiescent and stable when incubated at room temperature. Spontaneous synaptic activity were observed but not synchronized. *B*: when slices were continuously maintained at higher temperature at 35 – 37°C , the pyramidal neurons exhibited well-synchronized spontaneous activities (asterisks). *C*: a synaptic pair recorded from 2 monosynaptically connected pyramidal neurons. Presynaptic cell was recorded with K^+ -gluconate intracellular solution under current-clamp mode, whereas the excitatory postsynaptic currents (EPSCs) were evoked at different holding potentials with Cs^+ solution. Note the voltage dependency of the synaptic activities under this condition. Reversal potential of the EPSCs is about -20 mV. *D*: bath application of the *N*-methyl-D-aspartate (NMDA) receptor antagonist D-(–)-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μM), and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) completely blocked the synchronization and most of the synaptic activities.

had no significant effect on the excitabilities of pyramidal cells (see Fig. 3*B*, insets P3). These data also indicate that the synchronized activity is associated with the neuronal network among many neurons and, once this network activity erupted, it could not be interrupted by evoked action potentials in a single neuron. Further analysis indicated that clozapine significantly decreased PSC amplitude, especially by abolishing the synchronized PSC events (24.9 ± 0.61 pA in control vs. 16.5 ± 0.48 pA in clozapine, $n = 11$, $P < 0.0001$, Fig. 4*A*). In addition, clozapine also significantly reduced the frequencies of PSCs (5.55 ± 0.44 Hz in control vs. 4.35 ± 0.77 Hz in clozapine, $P = 0.038$, Fig. 4*B*). Counting the synchronous PSCs before and during clozapine application provided further evidence that the synchronized events were totally abolished by clozapine (Fig. 4, *C* and *D*). This effect was not recovered after a 10- to 15-min washout period.

Clozapine effectively depresses recurrent excitation among pyramidal neurons by network mechanisms

Previous studies indicated that the synchronous activities largely depended on the network connections among the cortical neurons, especially recurrent connections in local neuro-

nal circuitry (Singer et al. 1990; Traub et al. 2004). To examine how clozapine regulates recurrent synaptic transmission in the PFC, multiple whole cell recordings of the layer 5 pyramidal cells were performed. Clozapine consistently and significantly decreased the amplitude of unitary EPSPs in seven unidirectional pyramidal–pyramidal connections when the postsynaptic neurons were held in their resting membrane potentials (-66.3 ± 1.25 mV, $n = 7$, Fig. 5, *A* and *B*). The EPSP amplitude change was significant with an average reduction of 27.9% (range 13.7–52.3%, 0.88 ± 0.09 mV in the control and 0.66 ± 0.11 mV in clozapine; $n = 7$, $P < 0.00015$, Fig. 5*B*). The depressing effects of clozapine were partially reversed after a 10- to 15-min wash (Fig. 5*B*). To determine the mechanism of this depression, we examined several parameters. We found that the rates of synaptic failure were essentially unaltered (1.6% in control vs. 3.3% in clozapine, $P = 0.215$). The coefficients of variation of EPSP amplitude were not changed (CV, 0.28 ± 0.04 in control conditions vs. 0.33 ± 0.04 in clozapine, $P = 0.192$). Moreover, the paired-pulse ratios were unaffected (PPR, 0.79 ± 0.03 in control conditions vs. 0.75 ± 0.04 in clozapine, $P = 0.433$, Fig. 5*C*). All of these parameters suggest that clozapine's action on recurrent excitatory synaptic transmission does not arise from the reduction of

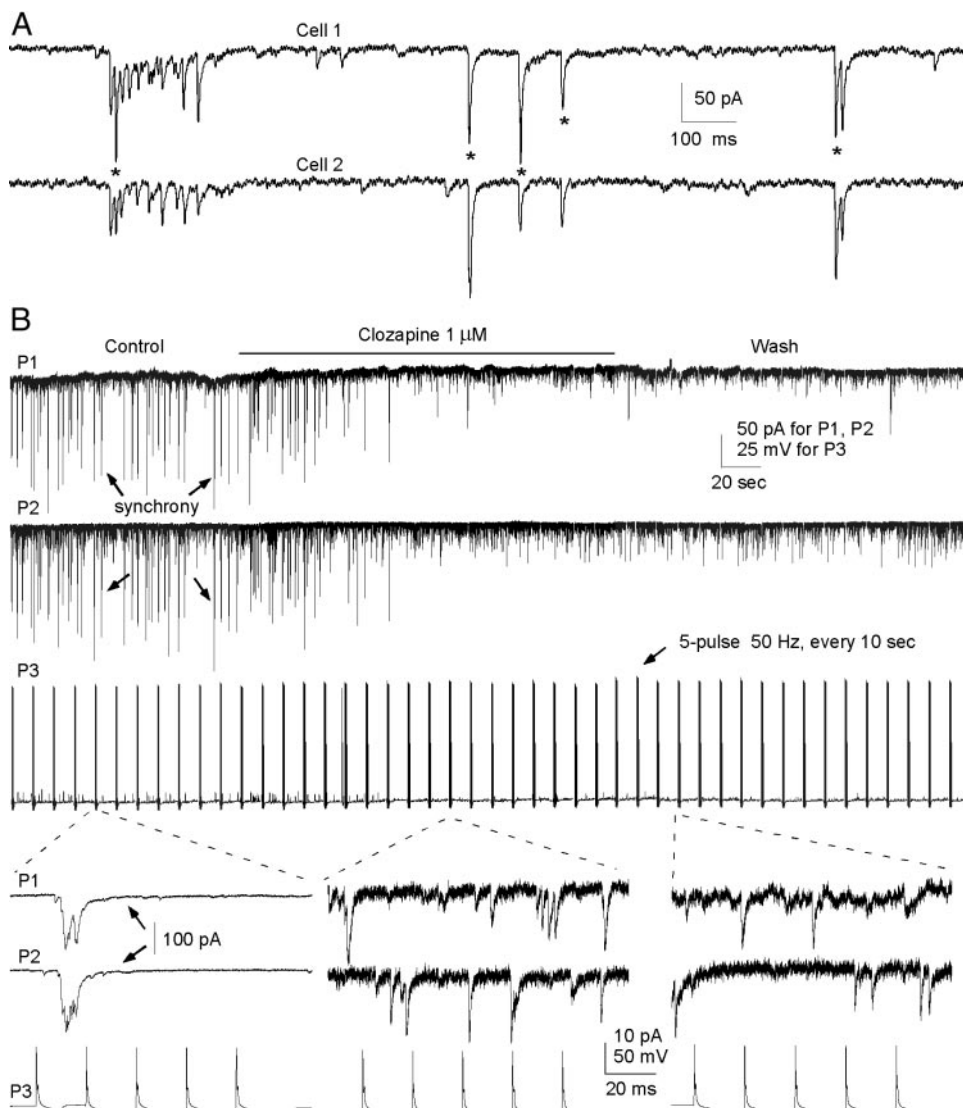


FIG. 3. Clozapine effectively abolished the spontaneous synchronized activities without affecting the neuronal excitabilities of pyramidal cells in medial prefrontal cortex (mPFC). *A*: 2 pyramidal neurons exhibited well-synchronized spontaneous activities (asterisks) that usually consisted of both individual and large complex PSC discharges. *B*: 3 pyramidal neurons recorded simultaneously, in which 2 of them (P1 and P2) were recorded in voltage-clamp mode (held at -70 mV), whereas the third one (P3) was documented in current-clamp mode. Note that there were synchronized PSCs in the bursting patterns among these neurons (arrows). A 50-Hz, 5-pulse train at 10-s interval was applied to P3 to monitor excitability change during clozapine application. Bath application of clozapine abolished the synchronized activities (as exhibited in P1 and P2) with little detectable effects on the neuronal excitability of P3. Three expanded panels in the bottom were from the recordings of control, during clozapine application and washout, respectively. There were no differences in the firing patterns of P3 recordings before and after clozapine application, whereas the large synchronized events in P1 and P2 [and the small excitatory postsynaptic potentials (EPSPs) in P3 as well] are completely blocked (note the scale difference in control).

presynaptic glutamate release, but likely involves postsynaptic or network mechanisms. This speculation was further supported by the following experiments. When the GABA_A receptor antagonist bicuculline ($10 \mu\text{M}$) was bath applied to block inhibition, the depressing effect of clozapine on recurrent excitation was completely eliminated (Fig. 6). Under this disinhibited condition, although the elicited unitary synaptic responses between two monosynaptically connected pyramidal neurons remained similar to the control, spontaneous paroxysmal "giant" EPSPs occasionally emerged, suggesting potential epileptic activities in the network of neuronal circuitry. Clozapine, in contrast, significantly enhanced the unitary EPSP amplitude by 24.4% ($n = 5$, $P < 0.05$) under this condition. This intriguing result is in agreement with numerous previous reports conducted under similar conditions (Arvanov et al. 1997; Chen and Yang 2002; Jardemark et al. 2003; Ninan 2003). The mechanism involving these differential effects of clozapine on glutamatergic EPSPs under conditions of control and of disinhibition is not clear. However, it may be attributed to the induced epileptic activities after blockade of inhibition (Keros and Hablitz 2005b). Many previous studies showed that under the disinhibited state, the ionic and receptor properties of

cortical neurons are greatly changed (Avoli et al. 2002; McCormick and Contreras 2001) and thus clozapine may effect, through very different mechanisms, D1-NMDA receptor interactions, such as suggested by Chen and Yang (2002). These results, however, further support the proposition that the depressing effect of clozapine on synchronized activity is by network actions, possibly through the activation of inhibitory neurons. It also hints of the complexities of clozapine actions in the prefrontal neurons.

Clozapine selectively and mildly increases the excitability of fast-spiking interneurons but not pyramidal cells in layer 5 of PFC

To further study the possibility that clozapine has direct effects on the inhibitory neurons, we examined the direct effect of clozapine on neuronal excitabilities of the prefrontal interneurons. We focused on the fast-spiking interneurons because these cells preferentially project to the soma/initial segment/proximal dendritic portions of cortical pyramidal cells and these kinds of cells constitute roughly 70% of the cortical interneurons (Kawaguchi 1995, 2001). Seventeen fast-spiking

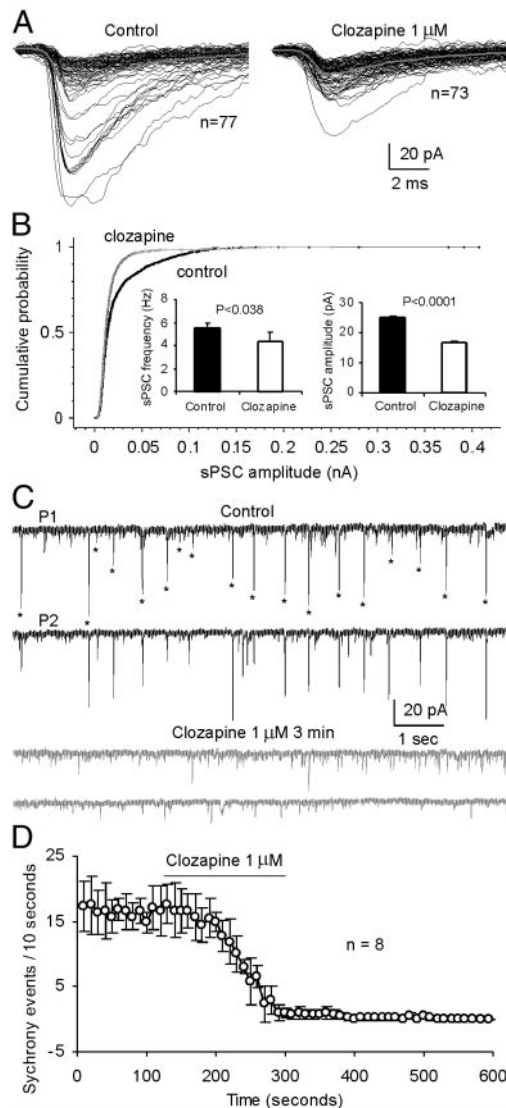


FIG. 4. Clozapine depressed the synchronized activities and reduced the amplitudes of the spontaneous PSCs. *A*: sample spontaneous (s)PSCs detected within a period of 10 s. Clozapine was very effective in reducing the sPSCs. *B*: clozapine significantly reduced the amplitude of sPSCs ($n = 8$, $P < 0.0001$), as well as the frequency ($P < 0.05$). *C*: samples of arbitrarily determined synchronized events (asterisks) in P1 and P2 (black traces) and the absence of these events after the application of clozapine (gray traces). *D*: time course of clozapine's effectiveness in reducing the synchronized events.

interneurons in deep layers of PFC were recorded in current-clamp mode. These neurons were initially selected under direct infrared-DIC visualization based on their multipolar morphologies and their high-frequency nonadaptive firing patterns. The identities of these neurons were confirmed by their physiologic properties, including unique action potential properties such as amplitude, half-width, and afterhyperpolarization (AHP) (Fig. 1, Table 1) (Gao et al. 2003). Most (11/17, 64.7%) of the tested cells were morphologically recovered with biocytin-labeling and all of these cells were confirmed as interneurons according to their characteristic somatodendritic morphologies (i.e., smooth and multipolar dendrites without spines). The other subtypes of interneuron, although interesting as well, were excluded from the analysis because of insufficient data to make consistent conclusions. The fast-spiking interneurons had an

average resting membrane potential of -72.3 ± 1.59 mV (Table 1). At this resting potential, the input resistance averaged 170.6 ± 12.72 Ω M. Spontaneous action potentials (spikes) were rare during the resting control condition. Injection of suprathreshold depolarizing current pulses (1,000-ms duration) evoked a train of nonadaptive high-frequency spikes with short durations (half-width of 0.58 ± 0.02 ms), followed by strong fast afterhyperpolarizations (fAHPs, average 13.2 ± 1.17 mV, Fig. 1). In contrast, pyramidal neurons ($n = 6$) from the same cortical area fired long-duration (half-width 1.18 ± 0.20 ms) spikes with profound adaptation, followed by significant weaker fAHPs (3.1 ± 1.01 mV, $P < 0.001$, Fig. 1). In addition, the spike amplitudes of interneurons were also significantly smaller (50.1 ± 4.23 mV) than those of pyramidal neurons (66.1 ± 3.48 mV, $P < 0.001$; Figs. 1 and 7).

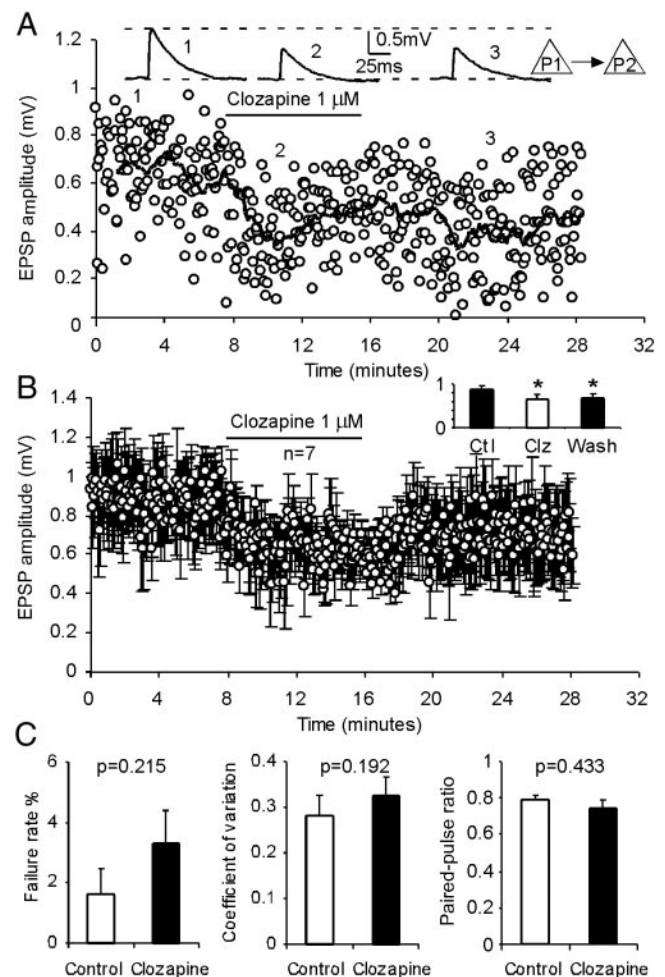


FIG. 5. Clozapine was effective in regulating the recurrent excitation between layer 5 pyramidal neurons, possibly through postsynaptic or network mechanisms. *A*: bath application of clozapine (1 μ M) depressed the amplitude of monosynaptic EPSPs of one pair of pyramidal–pyramidal (P–P) cells. Traces of EPSPs before and after the application of clozapine (top) and a graph of the amplitudes of EPSPs (bottom) from this same pair are shown. Traces are average of 20 EPSPs recorded in the marked periods (numbers in the graph). *B*: summary graph showing the significant depressing effects of clozapine on EPSP amplitudes of 7 P–P pairs ($P < 0.001$). Monosynaptic EPSP amplitude was partially recovered after a 10- to 15-min washout. *C*: there were no significant changes in the synaptic failure rate, the coefficient of variation (CV) of synaptic release, and the paired-pulse ratio (PPR), suggesting a postsynaptic effect.

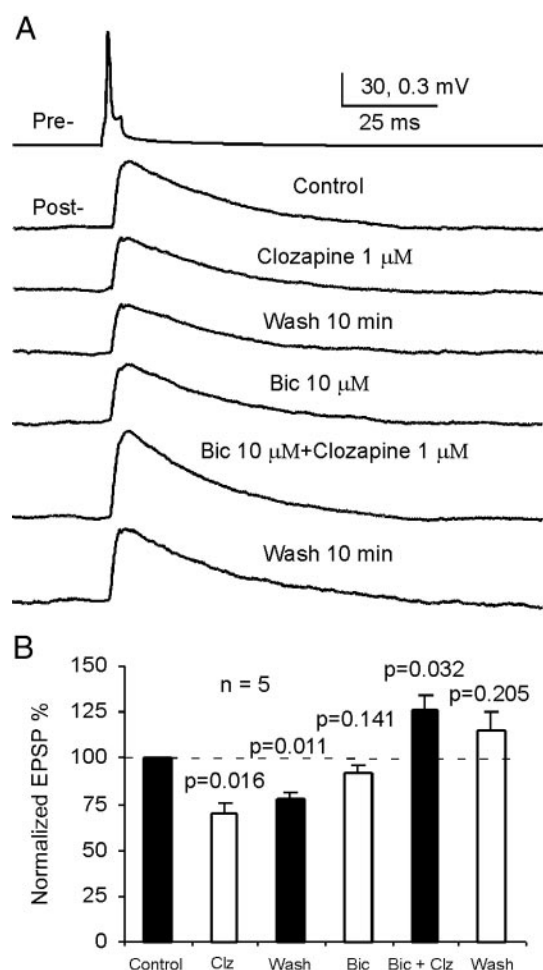


FIG. 6. Clozapine enhanced recurrent excitation under the conditions of disinhibition. *A*: recordings illustrate a monosynaptic connection between 2 pyramidal cells. A stimulus was applied to one pyramidal cell (Pre-) and recordings were made in the monosynaptically connected postsynaptic pyramidal cell (Post-). Clozapine reduced the EPSP amplitude. However, when it was coapplied with the γ -butyric acid type A (GABA_A) receptor antagonist bicuculline (Bic), and it increased the amplitude of the EPSP. *B*: graph summarized the data from the dual recordings of 5 P-P pairs showing the depression of EPSPs with application of clozapine alone and the augmentation of EPSPs when both clozapine and bicuculline were added to the bath. Significant enhancement of EPSPs in the disinhibited conditions suggests that clozapine may effectively modulate the inhibitory circuitry of prefrontal cortex.

Bath application of clozapine for 3 min (1–10 μ M) caused concentration-independent depolarizations in all 17 interneurons tested, with an average membrane potential increase of 2.43 ± 0.40 mV. The same amount of injected positive current thus evoked more spikes (increase $41.7 \pm 6.42\%$, $n = 17$, $P = 0.0001$, Fig. 7, *B–E*). Dose–response relations were examined by the application of clozapine at different concentrations. As shown in Fig. 7*D*, clozapine slightly increased the spike number at a low concentration of 0.2 μ M and the effect turned out to be more significant at higher concentrations of 1–10 μ M (0.2 and 1 μ M were for the same group of neurons). In fact, 1 μ M seems to be a saturated concentration because higher concentrations did not produce more spikes. This effect was long-lasting, without a clear recovery after a 10- to 20-min washout (Fig. 7*E*). This is consistent with the aforementioned partial recovery of recurrent excitation reduction. The input

resistances of these cells were also increased to 192 ± 14.42 Ω M (12.6%, $P < 0.001$) by clozapine application. In addition, clozapine also significantly reduced the spike threshold ($P < 0.01$, Table 1) and fAHP ($P < 0.0001$) and shortened the first spike latency ($P < 0.001$) and the first ISI ($P < 0.005$) of the tested interneurons (Fig. 7*F*). In contrast, clozapine had much weaker and less-consistent effects on pyramidal cells. In three of the six layer 5 pyramidal neurons tested, clozapine induced a small hyperpolarization (-1.57 ± 0.53 mV), but in the remaining three clozapine had no clear effects, with an average reduction of -0.87 ± 0.67 mV ($n = 6$, $P = 0.129$). The average spike number decreased slightly ($11.7 \pm 5.47\%$), without statistical significance ($P = 0.178$, Fig. 7*C*). These results indicate that clozapine selectively and slightly increases the excitability of fast-spiking interneurons in the mPFC, whereas it has no clear effects on pyramidal neurons, as previously reported (Pietraszek et al. 2002).

Clozapine enhances sIPSCs in pyramidal cells

To test the hypothesis that clozapine could selectively enhance action potential-dependent GABAergic inhibitory transmission, spontaneous IPSCs (sIPSCs) were recorded under voltage-clamp conditions in the presence of NMDA receptor antagonist D-AP5 (50 μ M) and AMPA receptor antagonist CNQX (10 μ M). To increase the driving force, the neurons were loaded with a 134 mM CsCl₂ intracellular solution (see METHODS). The reversal potential of this solution in our recording condition was about +10 mV. Voltage-clamp recordings from six layer 5 pyramidal neurons (membrane potentials held at -70 mV) showed that bath application of clozapine (1–2 μ M, 3–5 min) increased the frequencies (the events numbers) of sIPSCs in pyramidal cells by 22.9% (average 6.15 ± 0.76 Hz in control vs. 7.56 ± 0.71 Hz in clozapine, $n = 6$, $P < 0.05$; Fig. 8, *A* and *B*). In the presence of clozapine, there were also more large-amplitude events, presumably because of the increased spontaneous action potentials. The amplitude of averaged sIPSCs was also significantly increased by 37.2% (average 14.02 ± 0.35 pA in control vs. 19.24 ± 0.34 pA in clozapine, $n = 6$, $P < 0.0001$). Dose–response relations were not studied here because neuronal responses to different concentrations of clozapine (1–10 μ M) were invariant (Fig. 7*D*).

Clozapine increases the frequency but not the amplitude of mIPSCs in pyramidal cells

From evidences suggesting that clozapine is an antagonist of dopamine D2 receptors, and that these receptors are mainly localized on GABAergic interneurons (Mrzljak et al. 1996), clozapine could potentially modulate the release of GABA or affect the properties of GABA_A receptors. To test this hypothesis, mIPSCs were recorded in six pyramidal neurons in the presence of 1 μ M TTX to block the sodium channel-mediated action potentials and 50 μ M D-AP5 and 10 μ M CNQX to block the ionotropic glutamate receptors. In all neurons tested, bath application of 1 μ M clozapine for 3–5 min significantly increased the frequencies of mIPSCs (0.78 ± 0.14 Hz in control vs. 1.23 ± 0.24 Hz in clozapine, $n = 6$, $P = 0.023$); however, it had no clear effects on the mIPSC amplitudes (16.3 ± 0.36 pA in control vs. 16.6 ± 0.43 pA in clozapine, $P = 0.153$, Fig. 8, *C* and *D*) and waveforms (not shown). These data suggest

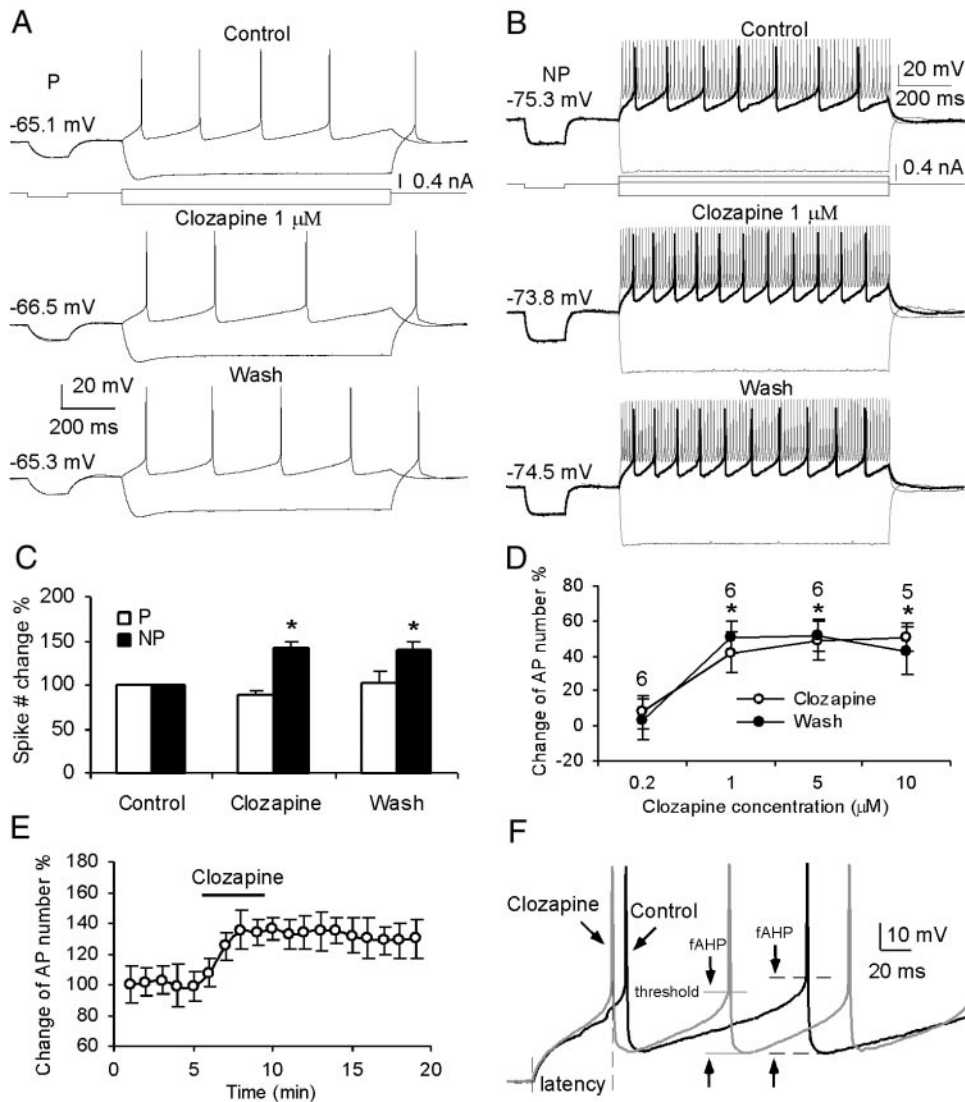


FIG. 7. Clozapine increased the excitabilities of fast-spiking interneurons but not those of pyramidal cells. A–C: sample recording and summary graphs showing that clozapine had no significant effects on the excitabilities of pyramidal neurons ($n = 6$, $P = 0.178$). In contrast, clozapine mildly increased the spike numbers of fast-spiking interneurons at concentrations of $\geq 1 \mu\text{M}$ (NP, $n = 17$, $P = 0.0001$; $*P < 0.001$). D: dose-independent response of action potential (AP) change in the presence of clozapine ($*P < 0.01$). E: time course of AP changes in bath application of clozapine at concentrations of 1–10 μM . F: clozapine significantly reduced the threshold ($P < 0.01$) and fAHP ($P < 0.0001$), shortened the first spike latency ($P < 0.001$) and the first interspike interval (ISI; $P < 0.005$) of the tested interneurons.

that clozapine is effective in regulating GABA release from presynaptic axonal terminals of interneurons but it may not involve the gating of postsynaptic GABA receptors.

Clozapine's effects on interneuron excitability and mIPSCs are occluded by coapplication of dopamine D4 agonist

To further elucidate the receptor specificity of clozapine actions on interneuron excitabilities, we conducted experiments to assess the potential mechanism(s) of receptor interactions. We chose to focus on dopamine D4 receptor because clozapine seems to have the highest binding affinity with this receptor and this receptor is mainly localized on the cortical GABAergic interneurons (Mrzljak et al. 1996). Figure 9 exhibits the effects of D4 agonist on interneuron excitabilities and mIPSCs on the pyramidal neurons. The spike numbers of the fast-spiking interneurons were reduced in seven of the eight cells tested with an average reduction of $18.9 \pm 6.78\%$ when PD 168077 (10 μM) was bath applied ($n = 8$, $P = 0.035$; Fig. 9, A and B). However, when clozapine (1 μM) was coapplied with PD 168077 (10 μM), the changes of spike number were variable, with four cells having no changes in spike numbers

and the other four cells having either an increase ($n = 2$) or a decrease ($n = 2$) of spike numbers between 10 and 20% compared with PD 168077 application alone. The overall spike numbers were essentially unchanged in the eight interneurons tested ($n = 8$, $P = 0.599$). These results suggest that clozapine's action on neuronal excitability was antagonized by the D4 agonist.

To further examine the D4 agonist effect, we also recorded the mIPSCs on the layer 5 pyramidal cells. PD168077 (10 μM) slightly but not significantly reduced the amplitudes of mIPSCs (17.0 ± 2.45 pA in control vs. 15.7 ± 0.80 pA in PD 168077, $n = 6$, $P = 0.583$; Fig. 9, C and D). In contrast, the frequencies of mIPSCs were significantly decreased an average of $27.5 \pm 9.52\%$ (0.63 ± 0.17 Hz in control vs. 0.37 ± 0.10 Hz in PD 168077, $n = 6$, $P = 0.034$). However, in the presence of PD 168077, clozapine appeared not to have effects on either the amplitude (15.7 ± 0.80 pA in PD 168077 vs. 16.7 ± 1.75 pA in PD 168077 + clozapine, $n = 6$, $P = 0.504$) or the frequency (0.37 ± 0.10 Hz in PD 168077 vs. 0.43 ± 0.11 Hz in PD 168077 + clozapine, $n = 6$, $P = 0.405$) of the mIPSCs on layer 5 pyramidal cells. These results are consistent with previous studies that suggested a dopamine D4-receptor-me-

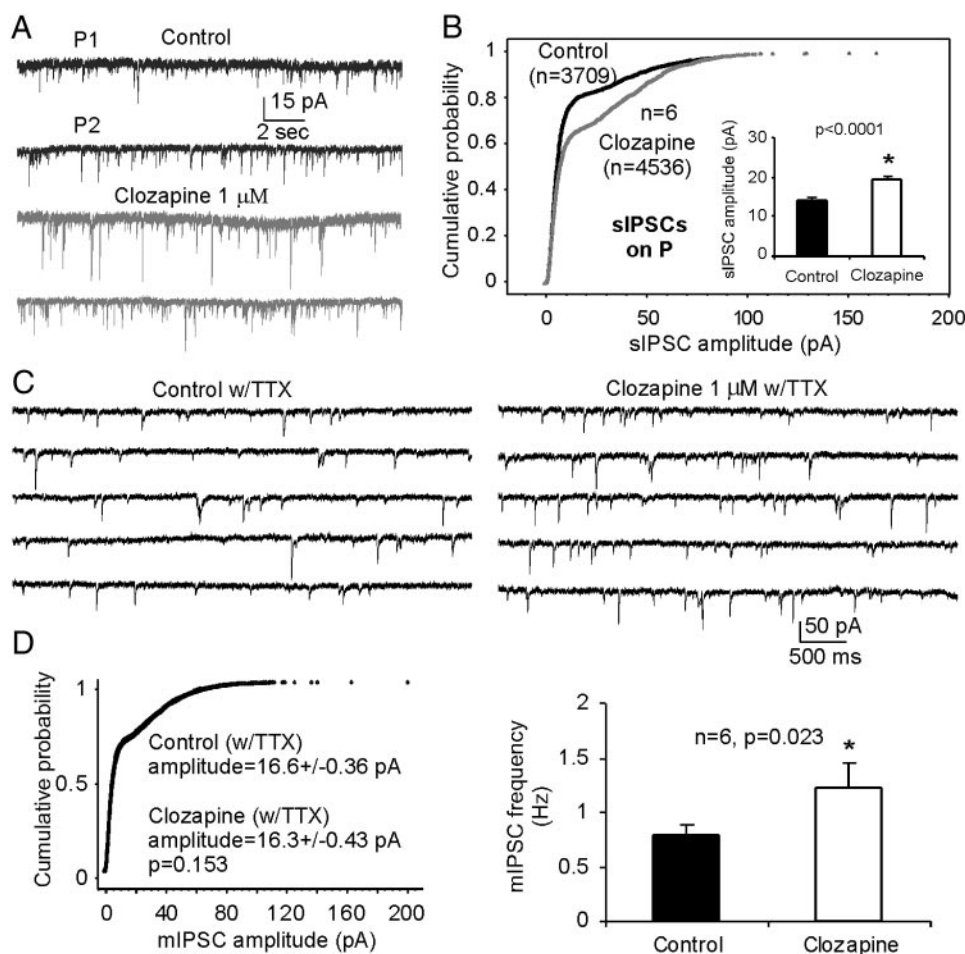


FIG. 8. Clozapine increases the sIPSCs and mIPSCs on pyramidal cells. *A*: sample of 2 pyramidal cells (P1 and P2) recorded simultaneously in voltage-clamp mode. Clozapine significantly increased the amplitude and the frequency of sIPSC on pyramidal cells. *B*: cumulative graph from 6 pyramidal neurons showing that clozapine significantly increased the amplitudes of sIPSCs in P ($P < 0.0001$). sIPSC frequencies were also significantly increased in P ($P < 0.05$), suggesting an action potential-dependent effect of clozapine on inhibitory circuitry. *C* and *D*: in the presence of tetrodotoxin (TTX), CNQX, and D-AP5, clozapine significantly increased the frequency ($n = 6$, $P = 0.023$) but not the amplitude of miniature (m)IPSCs ($P = 0.153$) in layer 5 pyramidal neurons.

diated presynaptic inhibition of GABAergic transmission in the rat supraoptic nucleus (Azdad et al. 2003), dorsolateral septal nucleus (Asaumi et al. 2006), and hippocampus (Romo-Parra et al. 2005), but differ from the effects on postsynaptic GABA_A receptors in PFC (Wang et al. 2002) and globus pallidus neurons (Shin et al. 2003). In the presence of D4 agonist, clozapine has no clear effect on either mIPSC frequency or mIPSC amplitude, suggesting that clozapine's increase of mIPSC may be mediated by D4 receptor.

DISCUSSION

This study used multiple whole cell recordings in ferret prefrontal cortical slices to examine the synaptic and cellular mechanisms of clozapine action on cortical network activity and synaptic transmission. Clozapine effectively suppresses the spontaneous synchronized network activities by depressing the recurrent excitation by a network mechanism related to the enhanced excitability of interneurons and the enhanced GABA release of inhibitory synapses.

The suppressive effect seemingly contradicts previous studies that suggested a NMDA-receptor-mediated enhancement of excitatory transmission in the PFC (Arvanov et al. 1997; Chen and Yang 2002; Gemperle et al. 2003; Jardemark et al. 2003; Ninan 2003) and nucleus accumbens (Wittmann et al. 2005). All of those studies, however, were conducted under conditions designed to isolate AMPA- and NMDA-receptor-mediated currents, i.e., in the presence of GABA_A antagonist

bicuculline. We also found that, under this same disinhibition condition, clozapine, instead of depressing recurrent excitation, significantly enhances the excitatory transmission. The mechanism involved in these differential results under normal control and disinhibition conditions remains unclear. A dopamine D1-receptor-mediated interaction with NMDA receptor was previously suggested as a possible explanation (Chen and Yang 2002) but further exploration is probably needed. The condition of the bicuculline-treated tissue may be akin to the loss of GABAergic synapses onto PFC pyramidal neurons and the associated changes in GABA_A receptors presented in postmortem schizophrenic brains. The enhancement of recurrent excitation under this condition may thus be the cause of the proconvulsant effects of clozapine in human patients (Denney and Stevens 1995; Minabe et al. 1998; Stevens et al. 1996) because seizure activity is usually accompanied with impaired inhibitory functions (Avoli et al. 2002; Cossart et al. 2005; Hablitz 2004; Wong et al. 1986). Indeed, disinhibition of *in vitro* cortical slice by blockade of GABA_A receptors could disrupt the neuronal network and induce synchronized epileptiform burst. This preparation was commonly used as a study model of epileptic activities (Avoli et al. 2002; Castro-Alamancos and Rigas 2002; Chagnac-Amitai and Connors 1989; Cohen et al. 2002; Connors 1984; Gao and Goldman-Rakic 2006; Keros and Hablitz 2005a; McCormick and Contreras 2001). Moreover, the acute suppressive effects of clozapine shown in normal brain tissue from ferret PFC are consistent with the acute sedative effect

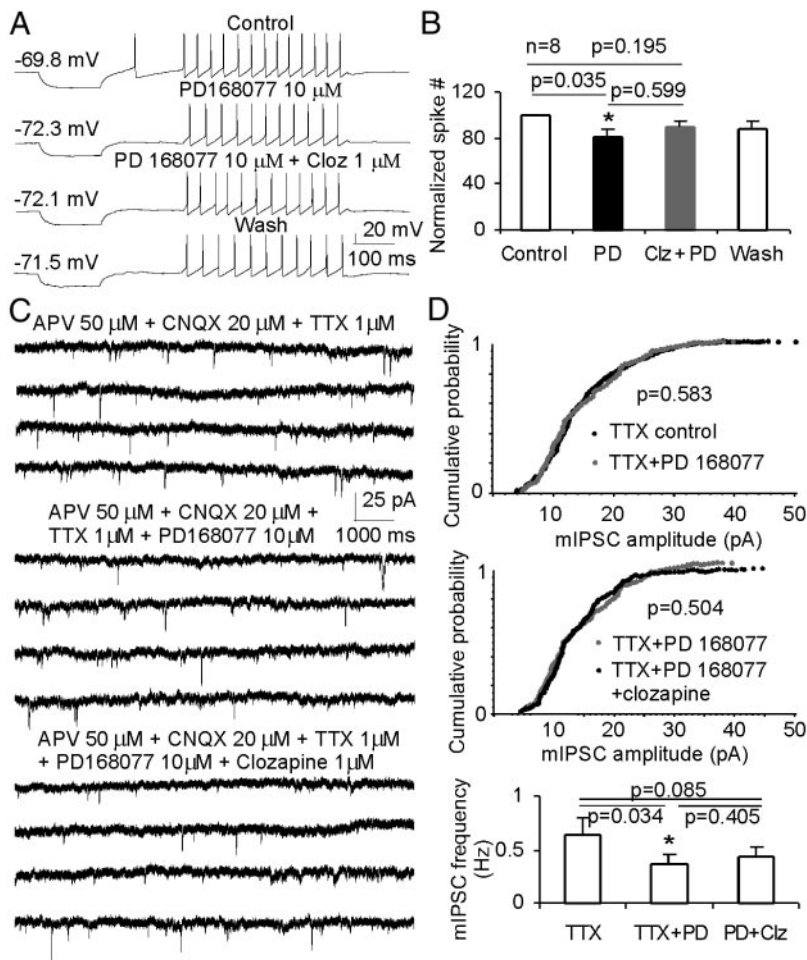


FIG. 9. D4 agonist occluded clozapine's actions on the excitability of fast-spiking interneurons and the mIPSCs on pyramidal neurons. *A*: bath application of dopamine D4 against PD 168077 significantly reduced the spike number of fast-spiking interneurons ($P = 0.035$). This effect was antagonized by coapplication of clozapine and PD 168077 ($P = 0.599$). *C* and *D*: PD 168077 reduced the frequency ($P = 0.034$) but not amplitude ($P = 0.583$) of the mIPSCs on layer 5 pyramidal neurons. These effects, however, were occluded by coapplication of clozapine with PD 168077. Both frequency ($P = 0.405$) and amplitude ($P = 0.504$) exhibited no clear changes under this condition.

of clozapine when given to humans in both the normal control and schizophrenic patients (Ereshefsky et al. 1989; Matz et al. 1974; Ortega-Alvaro et al. 2006; Weizman and Weizman 2001). Nonetheless, whether this effect is part of the ultimate therapeutic result remains unknown.

On the other hand, the electrophysiological responses to acute clozapine may involve multiple indirect effects. A large body of neurochemical literature shows that acute clozapine can cause the release of all three major monoamines as well as amino acid transmitters in the brain (Ashby and Wang 1996; Ichikawa et al. 2002; Moghaddam and Bunney 1990; Zhang et al. 2000). For the time being, the question of whether schizophrenia is a hyperglutamatergic or hypoglutamatergic state remains unresolved. Various studies reported different results of either an increase (Daly and Moghaddam 1993), a decrease (Pietraszek et al. 2002), no change (Yamamoto and Cooperman 1994), or a chronic (9 wk) increase (Melone et al. 2001) of glutamate in the PFC after using clozapine. When clozapine is administered chronically to match the therapeutic regimens given to human schizophrenic patients, the profile of these transmitters released in the brain might be quite different. Thus the acute change in neuronal excitability and GABA transmission is likely a result of complex interactions between or among several of these transmitters, although D4 agonist exhibited partial occlusion of the acute clozapine effects on the interneuron excitabilities and the GABA release in the presynaptic terminals.

Recent studies demonstrated that interactions between inhibitory interneurons and excitatory pyramidal cells constitute a critical neural substrate for the high-frequency (15–80 Hz, beta and gamma) oscillatory activity in the cortex (McBain and Fisahn 2001; Whittington and Traub 2003; Whittington et al. 2000). Accumulating evidence bolsters the notion that such synchronous oscillations may underlie cognitive functions such as object perception, selective attention, and working memory (Calhoun et al. 2004; Lee et al. 2003a; Spencer et al. 2003, 2004; van der Stelt et al. 2004). It was hypothesized that the severely disturbed synchronous neural activity in schizophrenia, largely in the 40-Hz range gamma-band oscillation, might be attributable to the dysfunctional inhibitory networks (Calhoun et al. 2004; Lee et al. 2003a; Lewis et al. 2005; Spencer et al. 2003, 2004; van der Stelt et al. 2004; Volk and Lewis 2002). In schizophrenic patients, negative symptoms correlate with a decrease of gamma responses, whereas a significant increase in gamma amplitudes is observed during positive symptoms such as hallucinations (Herrmann and Demiralp 2005; Lee et al. 2003b).

Schizophrenia is a chronic disorder that develops gradually over time (Javitt 2004). When the disease is fully developed, obvious abnormalities in interneurons can be detected in post-mortem schizophrenia brains (Addington et al. 2005; Benes et al. 1992, 1997; Gisabella et al. 2005; Hashimoto et al. 2003; Lewis et al. 2003; Volk and Lewis 2002). Clozapine was previously shown to be very effective in alleviating both the

positive symptoms (e.g., hallucinations and delusions) and the negative symptoms (e.g., social and emotional withdrawal) of schizophrenia, although its effectiveness against the latter treatment-resistant psychosis remains controversial (Remington and Kapur 2000). Clozapine's capacity to block abnormal overexcited synchronization of prefrontal neuronal network, as observed in this study, allows it to counteract the abnormal state of disrupted inhibitions that are associated with increased gamma-band oscillation in patients of schizophrenia with positive symptoms. Further supporting this hypothesis is the direct action of clozapine on the excitability of fast-spiking interneurons and its specific regulation of inhibition on pyramidal cells. Clozapine's opposing actions on excitatory and inhibitory transmission has a synergistic effect in increasing the functionality of inhibitory circuitry of PFC. The observation of clozapine's action in enhancing inhibitory transmission is also consistent with its putative role as an antagonist of dopamine D4 receptor and the localized D4 receptor on GABAergic interneurons (Mrzljak et al. 1996). Indeed, D4-receptor activation was found to reduce the excitability of interneurons in the prefrontal cortex and this reduction is antagonized by clozapine, consistent with previous study (Wang et al. 2002). Nonetheless, this result is not altogether congruent with other findings, suggesting that clozapine might moderately block GABA_A receptors (Squires and Saederup 1998) and lower GABA release in PFC (Bourdelaïs and Deutch 1994). Based on the experimental results in the present study, I speculate that clozapine can readjust the balance of excitation and inhibition in cortical neuronal networks in the schizophrenic cortex by suppressing the abnormal excitatory activity exhibited in the disease (Javitt 2004). Clozapine's acute physiologic actions in inhibitory neurons explain, at least partially, the clinical effectiveness of this antipsychotic drug on positive symptoms.

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