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Clozapine Potentiation of N-Methyl-D-Aspartate (NMDA) Receptor Currents in the Nucleus Accumbens: Role of NR2B and Protein Kinase A/ Src Kinases

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Abbreviations:

ACSF – artificial cerebrospinal fluid; AMPA – Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BIS I – Bisindolylmaleimide I; Chloro-APB hyrdobromide – 6-

Chloro-N-allyl-SKF-38393 hydrobromide; D-AP5 – D-(-)-2-Amino-5-phosphonopentanoic acid; EGTA – Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; EPSC – excitatory postsynaptic current; HEPES – N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; SCH23390 – (R)-(+)-7-Chloro-8-hyrdoxy-3-methyl-1-phenyl-2,3,4,5-tetrahyrdo-1H-3-benzazepine hydrochloride; SEM – standard error of the mean

Recommended section: Neuropharmacology

Abstract

Clozapine is an atypical antipsychotic that has a unique clinical profile that distinguishes it from other typical and atypical antipsychotics. At present, the underlying mechanisms of action of clozapine are unclear. Recent studies in the field of schizophrenia suggest that compounds that potentiate N-Methyl-D-Aspartate (NMDA) receptor function in the appropriate brain regions might be effective antipsychotic agents. One relevant region in which NMDA receptors play a key role in mediating neurotransmission is the nucleus accumbens. We therefore investigated the regulation of NMDA receptor currents and EPSCs by clozapine in nucleus accumbens neurons. Whole cell patch clamp recordings were performed in rat brain slices. We demonstrate that bath application of clozapine but not haloperidol or the selective 5HT2A antagonist MDL100907 induces a robust potentiation of NMDA -evoked currents and of glutamatergic EPSCs and that this potentiation is dependent on dopamine release and postsynaptic activation of D1 receptors. Furthermore, the effect of clozapine is selective for NR2B subtype containing NMDA receptors and is blocked by the selective Src family kinase inhibitor PP2 and the protein kinase A selective inhibitor H-89, but not by the protein kinase C selective inhibitor BIS I. This effect of clozapine in the nucleus accumbens might underlie the unique clinical profile of this atypical antipsychotic and provides a basis for novel treatment approaches.

Introduction

Schizophrenia is a debilitating psychiatric disorder that affects 1% of the world's population. The disorder is characterized by a combination of negative (blunted affect, withdrawal, anhedonia) and positive (paranoia, hallucinations, delusions) symptoms as well as marked cognitive deficits. The dopamine hypothesis, the dominant theory of the pathophysiology underlying schizophrenia, suggests excessive dopaminergic transmission in the forebrain as a key causative factor in this disorder. This dopamine hyperfunction hypothesis is based on the fact that all clinically effective antipsychotic drugs have antagonist activity at dopamine D2 receptors and that the therapeutic efficacy of these compounds is correlated with their affinity for striatal D2 receptors (Seeman, 1987). However, the atypical antipsychotic clozapine exhibits some interesting differences suggesting actions unrelated to D2 receptor blockade. In contrast with typical antipsychotics and most other atypical antipsychotics, clozapine not only alleviates positive symptoms but also can improve the negative and cognitive symptoms of schizophrenia. In addition, clozapine produces fewer extrapyramidal side effects than typical antipsychotics. Unfortunately, the clinical use of clozapine is limited by rare yet fatal side effects. Therefore, much research has been focused on determining what properties of clozapine are responsible for its unique clinical profile.

Recent findings have led to the development of several complimentary theories on the etiology of schizophrenia. For example, the finding that acute administration of the N-Methyl-D-Aspartate (NMDA) receptor channel blocker ketamine or phencyclidine to healthy volunteers can elicit a psychosis indistinguishable from acute schizophrenia, including symptoms such as formal thought disorder and auditory hallucinations as well

as negative symptoms (Fauman et al., 1976) suggests that NMDA receptor function may play a role in this disorder. Furthermore, when NMDA antagonists are administered to schizophrenic patients, a sustained worsening of symptoms can occur, usually in the form of an acute exacerbation of preexisting symptomatology. These findings have led to the development of the NMDA hypofunction hypothesis of schizophrenia which proposes a hypo-glutamatergic state in the brain leading to a selective hypo-activation of the NMDA-subtype of ionotropic glutamate receptors (for review see (Marino and Conn, 2002). According to this model any treatment that increases NMDA receptor function in the appropriate brain regions would be expected to alleviate the positive, negative and cognitive symptoms of this disease. However, the anatomical localization of this hypothetical NMDA hypofunction is not known.

A growing body of evidence implicates the importance of the nucleus accumbens (Nucleus accumbens) in the pathophysiology of schizophrenia. In response to antipsychotic drugs, the Nucleus accumbens shows alterations in expression of mRNA (Polese et al., 2002), induction of immediate early genes (Robertson and Fibiger, 1992; Polese et al., 2002; Kinney et al., 2003) and intracellular dye coupling (Onn and Grace, 1995). In addition, the Nucleus accumbens represents a site in which NMDA receptors play a critical role in gating cortical information (for review see (Grace, 2000). Nucleus accumbens neurons receive glutamatergic afferent inputs from regions that have been associated with schizophrenia, including the neocortex, hippocampus, and the amygdala (Fuller et al., 1987; Sesack et al., 1989; Finch, 1996). Inhibitory neurons in the Nucleus accumbens project to the ventral pallidum (Heimer et al., 1991), which in turn sends inhibitory efferents to the mediodorsal thalamus (Young et al., 1984). The glutamatergic

neurons of the mediodorsal thalamus project to (Uylings and van Eden, 1990) and provide a major regulatory control over the prefrontal cortex. Thus, increased activity in the Nucleus accumbens would lead to the disinhibition of the mediodorsal thalamus and a consequent increase in glutamate release in the prefrontal cortex. Since hypofunction or dysfunction of the prefrontal cortex has been implicated in the severity of negative and cognitive symptoms in schizophrenia patients (for review see (Weinberger and Berman, 1996)), an increase in the activity of the Nucleus accumbens could ultimately lead to an activation of the prefrontal cortex. Since NMDA receptors are known to play an important role in synaptic transmission and cell excitability, one obvious mechanism whereby Nucleus accumbens activity could be increased is the potentiation of NMDA receptor function. We therefore, tested the hypothesis that clozapine potentiates NMDA receptor function in the Nucleus accumbens.

Methods

Electrophysiology

Slice preparation

All experiments involving rats were carried out in accordance with the guide for the care and use of laboratory animals and the Merck Research Laboratories institutional animal care and use committee approved all studies described in this paper. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility in strict compliance with all of the applicable regulations.

Thirteen- to 20-day-old Sprague-Dawley rats (Taconic, Germantown, NJ) were used for all patch clamp studies. After decapitation, brains were rapidly removed and submerged in an ice cold choline chloride buffer (in mM): Choline chloride, 126; KCl, 2.5; $MgSO_4$, 8; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; Glucose, 10; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2. Frontal slices (for synaptic stimulation parasagittal slices) (300µm thick) were made using a Vibraslicer (WPI) or vibratome (Leica). Slices were transferred to a holding chamber containing normal artificial cerebrospinal fluid (ACSF) (in mM): NaCl, 124; KCl, 2.5; $MgSO_4$, 1.3; NaH_2PO_4, 1.0; $CaCl_2$, 2.0, Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2 at room temperature. In all experiments, 5µM glutathione, 500µM pyruvate, and 250µM kynurenic acid were included in the choline chloride buffer and holding chamber to increase slice viability.

Electrophysiological recordings

Whole-cell patch-clamp recordings were obtained under visual control. During recordings, slices were maintained fully submerged on the stage of a brain slice chamber

at 34^0C and perfused continuously with equilibrated ACSF (2-3ml/min). Neurons in the Nucleus accumbens were visualized with a 40X water immersion lens mounted on an Axioscope (Zeiss, Germany). Patch electrodes were pulled from borosilicate glass on a two stage vertical patch pipette puller (Heka Elektronik, Lambrecht, Germany) and filled with (in mM): cesium methane sulfonate, 140; HEPES, 16; NaCl, 10; EGTA, 2; NaGTP, 0.2; MgATP, 2; pH adjusted to 7.5 with 1M CsOH. Electrode resistance was 5-8 MΩ. All recordings were performed using a HEKA EPC10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). For measurement of exogenous NMDA receptor currents, NMDA (100μM, Tocris) was applied directly to the postsynaptic cell with a modified fast application system. NMDA-evoked currents were recorded at a holding potential of –60 mV and tetrodotoxin (500nM-1μM, Sigma, Calbiochem) was present in the bath to block synaptic transmission.

Excitatory postsynaptic currents (EPSCs) were evoked with a bipolar tungsten stimulation electrode placed within the Nucleus accumbens 100-200μm rostral to the recording site. EPSCs were recorded from a holding potential of -60 mV unless otherwise stated and low Mg^{2+}-ACSF (in mM): NaCl, 124; KCl, 2.5; NaH_2PO4, 1.0; $CaCl_2$, 2.0, Glucose, 20; $NaHCO_3$, 26; equilibrated with 95% O_2/5% CO_2 at room temperature) and (-)-bicuculline (20 μM) were bath applied during all EPSC recordings to increase NMDA receptor activation and to block inhibitory synaptic transmission. For exogenous NMDA receptor currents percent potentiation was defined by using the ratio of maximum current during drug application (average of three trials during maximal drug effect) to average current amplitude of three trials immediately preceding drug application. For EPSC recordings, percent potentiation was defined by using the ratio of amplitude or area under

the curve (for charge density) during drug application (average of five trials during last 5 minutes of drug application) to average amplitude or area under the curve of five trials immediately preceding drug application. All data are expressed as mean ± SEM.

Catecholamine depletion

In order to achieve DA depletion, reserpine (5mg/kg, i.p.) was administered acutely to the rats 1-1.5h before sacrifice and the brain slices were maintained in the tyrosine hydroxylase inhibitor α-methyl-L-P-tyrosine (100μM) after the dissection to block dopamine synthesis. Reserpine was prepared fresh each day. This acute treatment with reserpine induced a marked catalepsy in all animals (data not shown).

Drugs and drug application

Drugs were made fresh into stock solutions of 10 - 100 mM every day and diluted to the desired concentration in ACSF immediately prior to bath application to the slice. Antagonists were applied at least 10-15 min prior to application of agonists. Kinase inhibitors were bath applied for at least 15min before attempting to patch onto a cell. This pretreatment was necessary since prolonged baseline recordings prior to clozapine application lead to a loss of the clozapine effect, likely due to dialysis of intracellular contents. A 10mM stock of clozapine (in DMSO) was made fresh every day and slowly diluted 1:10 in water directly before diluting down to its final concentration and bath application. Haloperidol hydrochloride stocks were made in 4 parts 1M sodium hydroxide and 6 parts 8.5% lactic acid. (S)-(-)-Sulpiride, (*R*)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluoro-phenyl)ethyl]-4-piperidine methanol (MDL100907), 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), N-[2-(*p*-

Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), Bisindolylmaleimide I (BIS I) and (1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-proponal (CP101,606) stocks were made in dimethylsulfoxide at 1000 – 10000X their final concentrations. Tetrodotoxin, (*R*)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrochloride (SCH23390 hydrochloride), 6-Chloro-N-allyl-SKF-38393 hydrobromide (SKF 82958 hydrobromide), (-)-Bicuculline methiodide, Ifenprodil hemitartrate, and D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) were prepared in distilled water. Clozapine, SCH23390, S-(-)-sulpiride, ifenprodil hemitartrate, and D-AP5 were obtained from Tocris (Ballwin, MO). H-89 was obtained from Biomol (Plymouth Meeting, PA). BIS I was obtained from Calbiochem (EMD Biosciences) and PP2 was obtained from Alexis (San Diego, CA). All other compounds were obtained from Sigma (St. Louis, MO).

Data analysis

Values are expressed as mean $\pm$ SEM. Statistical comparisons between two groups were performed by using a Student's *t*-test. Statistical comparisons between several groups were performed by one-way analysis of variance followed by Tukey's post hoc comparisons. Statistical comparisons within groups (EPSCs at different holding potentials) were performed by repeated measures analysis of variance. An effect was considered significant when $p<0.05$.

Results

Clozapine potentiates NMDA receptor currents in Nucleus accumbens neurons.

NMDA receptor currents were evoked in Nucleus accumbens medium spiny neurons by application of exogenous NMDA (see methods). These exogenous NMDA receptor responses were mediated by activation of NMDA receptors as they could be blocked completely by bath application of 20μM D-AP5 (data not shown). Ten minute bath application of the atypical antipsychotic clozapine (100nM) produced a robust increase in NMDA receptor currents in 21 of 25 cells studied in the Nucleus accumbens (42.6 $\pm$ 6.5% increase, n=25, $p<0.01$, Fig1A,E). This effect of clozapine was not mimicked by the typical antipsychotic haloperidol at a concentration selective for D2 dopamine receptors (100nM) (Seeman, 1987) (2.5 $\pm$ 9.5% increase, n=6, $p>0.05$, paired *t*-test, Fig1B,E). It has been suggested that clozapine might mediate its antipsychotic effects through inhibition of 5-HT2A receptors. We therefore tested whether a 5-HT2A-selective antagonist could mimic the potentiating effect of clozapine on NMDA receptor currents. However, bath application of the 5-HT2A-selective antagonist MDL100907 (100nM) had no significant effect on the amplitude of NMDA-evoked currents in Nucleus accumbens neurons (16.3 $\pm$ 13.9% increase, n=4, $p>0.05$, paired *t*-test, Figure 1E). In most experiments, the effect of 100nM clozapine was not reversible after 10 minutes (figure 1C). The concentration-response relationship for the clozapine-induced potentiation of NMDA receptor currents exhibited a narrow and biphasic shape (Figure 1D). Lower concentrations (30 and 100nM) increased NMDA receptor currents whereas higher concentrations (300nM) did not.

Clozapine-induced potentiation of NMDA receptor currents is mediated by activation of D1 dopamine receptors.

Previous studies have revealed that clozapine can increase dopamine release in the Nucleus accumbens (Yamada et al., 1995). Furthermore it has been shown that dopamine and D1 agonists can increase NMDA receptor function in the striatum (Cepeda et al., 1998; Flores-Hernandez et al., 2002). We therefore performed pharmacological studies using selective dopamine receptor antagonists to test whether clozapine potentiates NMDA receptor currents by a dopaminergic mechanism. Prior bath application (10-15min) of haloperidol at concentrations sufficient to block both D1 and D2 dopamine receptors (10µM) (Seeman, 1987) or the D_1-selective antagonist SCH23390 at a maximal concentration (10µM) completely blocked the potentiating effect of 100nM clozapine (haloperidol: -0.4 $\pm$ 9.2% increase, n=6, $p>0.05$; SCH23390: -0.07 $\pm$ 5.3% increase, n=3, $p>0.05$; Figure $2A_{2,3}$,C). In addition, bath application of the D2-selective antagonist sulpiride at a maximal concentration (10µM) prior to clozapine partly decreased the effect of clozapine (23.9 $\pm$ 14.4, n=5, $p>0.05$, Figure A_4,C). Bath application of sulpiride (10µM) alone mimicked the effect of clozapine and significantly increased NMDA-receptor currents (43.1 $\pm$ 13.2% increase, n=4, $p<0.05$, paired t-test, Figure 2B,C). Thus it is possible that the sulpiride-induced blockade was mediated by functional occlusion of the response. In contrast, bath application of the D1 selective antagonist SCH23390 (10µM) alone had no effect on the amplitude of NMDA receptor currents (data not shown).

Previous work has shown that clozapine, presumably via an inhibitory action on D2 receptors, can release dopamine in the Nucleus accumbens of rat brain slices

(Yamada et al., 1995). Therefore, one possible mechanism for the NMDA potentiating action of clozapine could be via a similar mechanism. Release of dopamine by inhibition of D2 receptors could then activate postsynaptic D1 receptors leading to an indirect potentiation of NMDA receptor currents.

This hypothesis predicts that it should be possible to mimic the effect of clozapine with a D1-selective agonist. Bath application of 100nM SKF82958, a highly selective full agonist for D1 receptors, significantly potentiated NMDA receptor currents in 10 of 12 Nucleus accumbens neurons recorded (26.1 $\pm$ 4.8% increase, n=12, $p<0.01$, paired *t*-test, Figure3A,D). This effect of SKF82958 (100nM), like the clozapine effect, was long lasting (Figure 3B) and showed a bell-shaped concentration-response curve (Figure 3C).

We further tested the hypothesis that the clozapine-induced potentiation of NMDA receptor currents is mediated by clozapine-induced release of dopamine acting on postsynaptic D1 receptors in the Nucleus accumbens, by repeating the clozapine experiments in slices from acutely dopamine-depleted animals. Consistent with our hypothesis, the effect of clozapine (100nM) was completely absent in dopamine depleted slices (3.2 $\pm$ 3.4% increase, n=8, $p>0.05$, paired student's *t*-test, Figure $4A_2$,B,E). Since the effect of the D1-selective agonist SKF82958 should not be influenced by dopamine depletion, we also compared its effects in dopamine-depleted slices. As predicted, the effect of SKF82958 (100nM) on NMDA receptor currents was not abolished by dopamine depletion (27.4 $\pm$ 8.6% increase, n=6, $p<0.05$, paired student's *t*-test, Figure $4C_2$,D,E). These data suggest that clozapine-induced release of dopamine is an obligatory step in the cascade of events leading to the increase in NMDA receptor currents. The data

furthermore show that the potentiation of NMDA receptor currents is not mediated by a direct action of clozapine on D1 receptors.

The potentiating effect of clozapine on NMDA receptor currents is mediated by activation of Src family kinases and protein kinase A.

It has been hypothesized that protein kinase-mediated phosphorylation of NMDA receptor subunits leads to a potentiation of NMDA receptor function (Blank et al., 1997;Ali and Salter, 2001). We therefore investigated the effect of several selective kinase inhibitors on the clozapine-induced potentiation of NMDA receptor currents. Prior bath application (at least 15min) of maximal concentrations of the selective Src kinase family inhibitor PP2 (10µM) (Hanke et al., 1996) or the protein kinase A selective inhibitor H-89 (10µM) (Chijiwa et al., 1990) blocked the effect of 100nM clozapine (3.9 $\pm$ 8.2% increase, n=5, $p>0.05$, Figure 5A_2,B and 4.5 $\pm$ 3.9% increase, n=6, $p>0.05$, Figure 5A_3,B). The selective protein kinase C inhibitor bisindolylmaleimide I (BIS I, 10µM), at a concentration that partially blocks protein kinase A (Toullec et al., 1991) also inhibited the effect of 100nM clozapine on NMDA receptor currents (0.5 $\pm$ 8% increase, n=3, $p>0.05$, Figure 5A_5,B). When Bis I was applied at a lower concentration that is selective for protein kinase C (1µM) (Toullec et al., 1991) clozapine potentiation of NMDA receptor currents was not blocked (22.6 $\pm$ 5.4% increase, n=5, $p<0.05$, Figure 5A_4,B). Together these data suggest that the mechanism of clozapine-induced potentiation of NMDA receptor function in the Nucleus accumbens involves activation of Src family kinases and protein kinase A but not protein kinase C. However, it is possible that one or both (protein kinase A and Src family kinases) may act presynaptically by altering the

release of dopamine rather than directly via a postsynaptic mechanism. We addressed this issue by repeating the above experiments and tested the effect of the Src family kinase inhibitor PP2 and the protein kinase A inhibitor H-89 on the effect of the D1 agonist SKF82958 (100nM). Consistent with a direct postsynaptic effect, the potentiating effect of SKF82958 (100nM) on NMDA receptor currents was significantly blocked by prior bath application of either the selective Src family kinase inhibitor PP2 (10µM) or the protein kinase A inhibitor H-89 (10µM) (6.0 $\pm$ 5.5% increase, n=9, $p>0.05$, and 2.4 $\pm$ 4.5% increase, n=6, $p>0.05$, Figure 6).

These data suggest that the potentiating effect of clozapine is mediated by postsynaptic activation of protein kinase A and Src family kinases which provide a mechanistic link between D1 receptor activation and the observed increase in NMDA receptor function.

Potentiation by clozapine is restricted to NMDA receptors containing the NR2B subunit

NMDA receptor subtypes are known to be modulated selectively in other systems. Therefore, we examined clozapine potentiation of NMDA receptor currents in the presence and absence of the NR2B selective antagonists CP101,606 (Chenard et al., 1995) and ifenprodil (Reynolds and Miller, 1989). Prior bath application of maximal concentrations of CP101,606 (1µM) or ifenprodil (10µM) completely blocked the potentiating effect of 100nM clozapine on NMDA receptor currents (CP101,606: 6.5 $\pm$ 5% increase, n=6, $p>0.05$; ifenprodil: -2.3 $\pm$ 2.6% increase, n=6, $p>0.05$; Figure $7A_{2,3}$,B). Next we examined what proportion of the NMDA evoked currents in the Nucleus accumbens were mediated by activation of NMDA receptors containing the NR2B

subunit. Figure 7C shows the time course of the inhibiting effect of 15min bath application of ifenprodil (10µM) on the amplitude of evoked NMDA receptor currents in the Nucleus accumbens (46.8 ± 5% decrease, n=5, $p<0.05$, paired student's t-test). The remaining NMDA receptor current could be blocked completely by additional application of the non-selective NMDA receptor antagonist D-AP5 (20µM) (Figure 7C). Taken together, these data suggest that clozapine selectively potentiates the function of NMDA receptors containing the NR2B subunit and that a significant proportion (46.8%) of the NMDA evoked current in Nucleus accumbens medium spiny neurons is mediated by activation of NR2B-subunit containing NMDA receptors.

Clozapine potentiates the function of synaptic NMDA receptors but not Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors.

There is a good possibility that direct application of NMDA (100µM) to the slices activates mostly extrasynaptic NMDA receptors and very few synaptic NMDA receptors. To test whether the effect of clozapine on NMDA receptor currents is relevant for glutamatergic synaptic transmission we compared the effects of clozapine on synaptically evoked glutamatergic EPSCs in parasagittal brain slices. Glutamatergic EPSCs were evoked by placing a bipolar stimulation electrode into the Nucleus accumbens 100-200µm rostral to the recording site and stimulating synaptic inputs every 15sec. Bath application of clozapine (100nM) for 15 min produced a small non-significant increase in EPSC amplitude (21.7 ± 12.0% increase, n=7, $p>0.05$, paired student's *t*-test, data not shown) but did induce a significant increase of charge density (42.6 ± 11.6% increase, n=7, $p<0.01$, Paired Student's *t*-test; Figure $8A_1$,B). This increase in charge density likely

is due to the selective potentiation of the kinetically slower NMDA receptor currents (Figure 8A1). We determined whether this effect of clozapine on charge density is mediated by an increase of NMDA receptor function rather than by effects on AMPA receptors, by repeating the experiment in the presence of the NMDA receptor antagonist D-AP5 (20μM). No significant effect of clozapine (100nM) on EPSC charge density in the presence of D-AP5 (20μM) (17.2 $\pm$ 13.7, n=5, $p>0.05$, Paired Student's *t*-test, Figure $8A_2$,B) was observed. We also examined whether the effect of clozapine was voltage dependent, by repeating the experiment stimulating excitatory inputs every 15 seconds while holding the cell at different holding potentials, changing from -20mV to -80mV. The results are shown in a charge density – voltage plot in Figure 8C. The charge density - voltage plot revealed a profile characteristic of NMDA receptor activation with the peak charge densities observed at a holding potential of -40mV. There was an overall significant increase of charge density induced by clozapine (100nM) at the different holding potentials ($p<0.01$, n=5, Figure 8C). However, there was no significant effect of voltage on the effect of clozapine. When we repeated the experiment in the presence of the NMDA receptor antagonist D-AP5 (20μM), no significant effect of clozapine was seen ($p>0.05$, n=5, Figure 8D). Taken together, these data suggest that clozapine potentiates the function of synaptically activated NMDA receptors, but not AMPA receptors, in a voltage-independent manner.

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Discussion

The results of the present study show that acute application of low concentrations of clozapine, but not haloperidol or the 5-HT2A antagonist MDL100907, potentiates NMDA receptor function in Nucleus accumbens neurons. This suggests an important mechanism of action for this unique atypical antipsychotic. We also demonstrate that the clozapine effect is mediated by D2-mediated dopamine release resulting in activation of postsynaptic D1 receptors. Furthermore, we show that the D1-induced potentiation of NMDA receptor currents is limited to receptors containing the NR2B subunit of NMDA receptors and requires activation of the Src and protein kinase A family of kinases.

The current data add to a growing body of evidence demonstrating that clozapine can lead to a potentiation of the function of NMDA receptors in various brain regions. We and others have previously shown that in vivo application of clozapine induces a robust increase in c-fos expression in the Nucleus accumbens and the prefrontal cortex with only minimal increase in the medial striatum and no change in the dorsolateral striatum (Robertson and Fibiger, 1992; Kinney et al., 2003). This c-fos expression in the Nucleus accumbens is blocked by pretreatment with the NMDA receptor antagonist MK801 (Leveque et al., 2000), or increased by coadministration of D-cycloserine, a partial agonist at the strychnine-insensitive glycine site of the NMDA receptor complex (Leveque et al., 2000; Polese et al., 2002). Furthermore, we reported earlier that an inhibitor of the glycine transporter glycine transporter 1 induces a similar pattern of c-fos expression as clozapine (Kinney et al., 2003) suggesting that increases in NMDA receptor function have a similar effect. Previously, several electrophysiological studies have provided additional functional evidence demonstrating that clozapine potentiates

evoked NMDA receptor currents, NMDA EPSCs and can enhance NMDA-dependent long term potentiation (LTP) in the prefrontal cortex (Arvanov et al., 1997;Chen and Yang, 2002;Gemperle et al., 2003).

There is a large body of evidence implicating the importance of the Nucleus accumbens in the pathophysiology of schizophrenia. The Nucleus accumbens furthermore represents a site in which NMDA receptors may play a critical role in gating the flow of cortical information back to the prefrontal cortex (Grace, 2000). We now show that clozapine can induce a potentiation of NMDA receptor currents resulting in an increase in glutamatergic transmission in the Nucleus accumbens. Hypofunction of the prefrontal cortex has been implicated with the severity of negative and cognitive symptoms in schizophrenia patients (Weinberger and Berman, 1996). An increase in activity in the Nucleus accumbens would be expected ultimately to lead to increased activation of the prefrontal cortex, representing one possible mechanism by which clozapine might reduce negative and cognitive symptoms in these patients.

Previous studies in the prefrontal cortex have shown a similar effect of low concentrations of clozapine but also haloperidol and the 5HT2A antagonist MDL100907 on NMDA receptor currents (Arvanov et al., 1997; Arvanov and Wang, 1998). However, in the present study, we demonstrate that only clozapine and neither haloperidol nor MDL100970 mimics this effect in Nucleus accumbens neurons, indicating that the mechanism of action of antipsychotics may differ in different brain regions and that this might be one of the factors that produce the unique clinical profile of clozapine. The reason for the lack of effect of haloperidol in the nucleus accumbens remains unknown. However it has been shown that haloperidol selectively binds to NR2B containing

NMDA receptors (Coughenour and Cordon, 1997) and inhibits NR1/NR2B-NMDA receptor currents at higher concentrations (IC50 3µM, (Ilyin et al., 1996). Therefore, haloperidol might partially inhibit NR1/NR2B-NMDA receptors in our study and thus prevent the D1 induced increase in NR2B-containing NMDA receptor currents.

We have shown that inhibition of both protein kinase A and Src family kinases blocks the potentiating effect of clozapine and D1 receptor activation on NMDA receptor currents indicating that both kinases are necessary for this effect. Both Src-family tyrosine kinases and protein kinase A have been shown to phosphorylate NMDA receptor subunits. In particular the Src-family kinase *fyn* has been identified as the main enzyme phosphorylating the NR2B subunit (Nakazawa et al., 2001). Protein kinase A also has been shown to phosphorylate the NR1 subunit of NMDA receptors leading to an increase in function (Tingley et al., 1997; Blank et al., 1997). Further, biochemical studies have demonstrated that D1-induced activation of protein kinase A leads to a protein kinase A mediated synergistic mechanism of increased phosphorylation and decreased dephosphorylation of the NR1 subunit in Nucleus accumbens slices and isolated striatal cells (Snyder et al., 1998; Flores-Hernandez et al., 2002). Our data show that only NR2B containing NMDA receptors are potentiated by clozapine, however activation of both, Src-family kinases and protein kinase A, are necessary to induce the clozapine-mediated changes. The regulatory steps between the activation of D1 receptors and the increase of NMDA receptor currents are unclear however recent studies suggest three possible mechanisms. First, protein kinase A phosphorylation of NR1 subunits in addition to NR2B phosphorylation by Src-family kinases might lead to an increase in NMDA receptor function. Second, RACK1 has recently been identified as a scaffolding protein

for *fyn* as well as the NR2B subunit of the NMDA receptor presumably providing a novel mechanism that localizes *fyn* in the proximity of NR2B subunits and at the same time prevents *fyn* phosphorylation of the NR2B subunit (Yaka et al., 2002). Interestingly, activation of the cAMP/protein kinase A pathway has been identified as an important modulator of the *fyn*/RACK1/NR2B complex leading to protein kinase A induced release of RACK1 from the NR2B subunit and its subsequent *fyn*-mediated tyrosine phosphorylation (Yaka et al., 2003). Finally, a mechanism underlying increased NMDA receptor currents may be insertion of new receptors into the membrane. There is evidence that in the striatum D1 induced tyrosine kinase phosphorylation of NR2A and NR2B subunits, possibly by Src-family tyrosine kinases, leads to a rapid trafficking of NMDA receptors containing these subunits from intracellular membranous structures to the postsynaptic membrane (Dunah and Standaert, 2001). In addition, phosphorylation of NR1 subunits at serine^{890} has been shown to produce a redistribution of NR1 subunits from intracellular sites to the cell surface (Tingley et al., 1997). Therefore phosphorylation of NR1 and NR2B subunits might lead to the trafficking of the NR1/NR2B NMDA receptor complex to the cell surface. Recent data give further support to the idea that receptor trafficking may underlie the clozapine-induced increase in NMDA receptor currents. In this study chronic treatment of rats with clozapine induced increased MK801-binding only in the Nucleus accumbens but not in other brain regions (Schmitt et al., 2003), without any changes in gene expression of NMDA receptor subunits (Schmitt et al., 2003; Hanaoka et al., 2003).

In summary the present results demonstrate that acute clozapine application potentiates NR2B-containing NMDA receptor currents in the Nucleus accumbens by a

distinct mechanism involving Src kinases and protein kinase A possibly leading to an increase of cortical information flow through the Nucleus accumbens. This effect of clozapine could be an important possible mechanism underlying the significant clinical benefit of clozapine on cognitive and negative symptoms in schizophrenic patients.

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Footnotes

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Figure legends

Figure 1. **Clozapine potentiates NMDA receptor currents in Nucleus accumbens neurons in rat brain slices**. (A) Example traces showing the effect of 100nM clozapine on the amplitude of NMDA receptor currents induced by fast application of NMDA (100μM). (B) Example traces showing the lack of an effect on the amplitude of NMDA-evoked currents by bath application of the D_1/D_2 antagonist haloperidol (100nM). (C) Average time course of the effect of 10-minute bath application of clozapine (100nM) on NMDA receptor currents (filled circles) and of "no drug" control (open circles). Each time point represents the normalized mean $\pm$ SEM of data from 22 cells and 7 cells, respectively. (D) Concentration response relationship of the clozapine-induced potentiation of NMDA receptor currents. Each point represents the mean $\pm$ SEM of the maximal effect during drug application from 3 to 24 experiments. (E) Bar graph showing the effect of clozapine (100nM) and the lack of an effect of haloperidol (100nM) and of the 5HT2A-selective antagonist MDL100907 (100nM) on NMDA receptor currents. Each bar represents the mean $\pm$ SEM of data from 24, 5 and 4 experiments, respectively. (* $p<0.05$, significant effect compared to predrug baseline, Student's Paired *t*-test).

Figure 2. The clozapine-induced potentiation of NMDA receptor currents is blocked by D_1-selective dopamine antagonists and mimicked by D_2-selective antagonists. (A_{1-4}) Example traces showing the effect of clozapine (100nM) in the absence (A_1) and presence (A_{2-4}) of selective dopaminergic antagonists. (B) Example traces illustrating the effect of bath application of the D_2-selective antagonist sulpiride (10μM). (C) Bar graph

showing the effect of clozapine (100nM) in the absence and presence of selective dopaminergic antagonists and the effect of sulpiride (10μM) on NMDA receptor currents. Each bar represents the mean ± SEM of data from 3 to 12 experiments. (*$p<0.05$, significant effect compared to predrug baseline).

Figure 3. The effect of clozapine is mimicked by the D1 selective dopamine agonist SKF82958. (A) Example traces showing the effect of bath application of clozapine (100nM) and SKF82958 (100nM) on the amplitude of NMDA receptor currents. (B) Average time course of the effect of 10-minute bath application of SKF82958 (100nM) on NMDA receptor currents (filled circles) and of "no drug" control (open circles). Each time point represents the normalized mean ± SEM of data from 11 cells and 7 cells, respectively. (C) Concentration response relationship of the SKF82958-induced potentiation of NMDA receptor currents. Each point represents the mean ± SEM of the maximal effect during drug application from 4 to 12 experiments. (D) Bar graph showing the average effects of clozapine (100nM) and the D_1-selective agonist SKF82958 (100nM). Each bar represents the mean ± SEM of the maximal effect during drug application from 24 and 12 experiments, respectively. **$p<0.01$, significant effect compared to predrug baseline, Student's Paired *t*-test.

Figure 4. Acute dopamine depletion induced by in vivo reserpine treatment prevents the potentiating effect of clozapine on NMDA receptor currents in Nucleus accumbens neurons. ($A_{1,2}$) Example traces showing the effect of 100nM clozapine in slices obtained from control rats (A_1) and in slices obtained from reserpine treated

animals (A_2). (B) Average time courses showing the effect of clozapine (100nM) on NMDA receptor currents in slices from control animals (filled circles) and from reserpine treated animals (open triangles). The potentiating effect of clozapine is absent in dopamine depleted slices. Each time point represents the normalized mean $\pm$ SEM of data from 6 cells and data from 7 cells (3 animals), respectively. ($C_{1,2}$) Example traces showing the effect of SKF82958 (100nM) in slices obtained from control rats (C_1) and in slices obtained from reserpine treated animals (C_2). (D) Average time courses showing the effect of SKF82958 (100nM) on NMDA receptor currents in slices obtained from control rats (filled circles) and from reserpine treated rats (open triangles). Each time point represents the normalized mean $\pm$ SEM of data from 11 cells and data from 5 cells (3 reserpinized animals), respectively. (E) Bar graph showing the average effects of 100nM clozapine (black bars) and 100nM SKF82958 (grey bars) on NMDA receptor currents in control and reserpine treated animals. Each bar represents the mean $\pm$ SEM of data from 10 and 8 experiments in 3 animals (clozapine) and 11 and 6 experiments in 3 animals (SKF82958), respectively. (** $p<0.01$, *$p<0.05$, significant effect compared to predrug baseline, Student's Paired *t*-test).

Figure 5. Acute inhibition of protein kinase A and of Src family kinases, but not protein kinase C, blocks the potentiating effect of clozapine on NMDA receptor currents in Nucleus accumbens neurons. (A_{1-5}) Example traces showing the effect of clozapine (100nM) in the absence (A_1) and presence of the Src-family kinase inhibitor PP2 (10µM) (A_2), the protein kinase A inhibitor H-89 (10µM) (A_3), and the protein kinase C inhibitor BIS I (1µM and 10µM) ($A_{4,5}$). (B) Bar graph showing the effect

clozapine in the absence and presence of the selective kinase inhibitors PP2 (10μM), H-89 (10μM) and BIS I (1μM and 10μM). Each bar represents the mean ± SEM of data from 3 to 12 experiments. (**p<0.01, significant effect compared to predrug baseline).

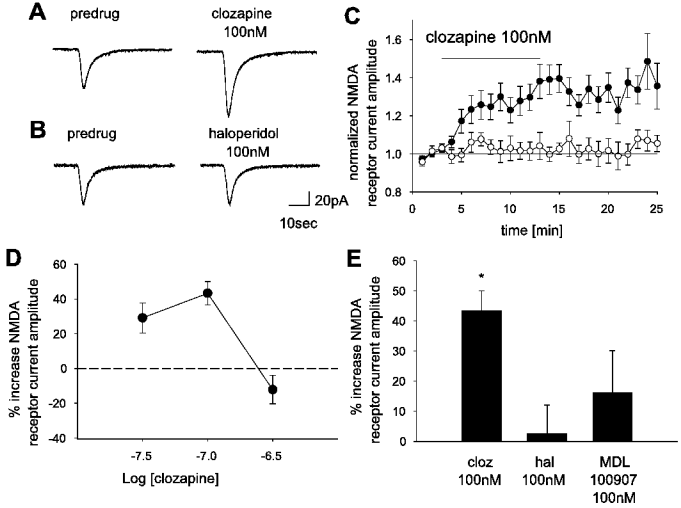
Figure 6. Acute inhibition of protein kinase A and of Src family kinases blocks the potentiating effect of SKF82958 on NMDA receptor currents in Nucleus accumbens neurons. (A_{1-3}) Example traces showing the effect of SKF82958 (100nM) in the absence (A_1) and presence of the Src-family kinase inhibitor PP2 (10μM) (A_2), and the protein kinase A inhibitor H-89 (10μM) (A_3). Bar graph showing the average effect of SKF82958 (100nM) in the absence and presence of the selective kinase inhibitors PP2 (10μM) and H-89 (10μM). Each bar represents the mean ± SEM of data from 6 to 11 experiments. (**p<0.01, significant effect compared to predrug baseline).

Figure 7. Prior bath application of NMDA receptor subunit NR2B-selective antagonists blocks the potentiating effect of clozapine on NMDA receptor currents. (A_{1-3}) Example traces showing the effect of clozapine (100nM) in the absence (A_1) and presence of the NR2B-selective NMDA receptor antagonists CP101,606 (1μM) (A_2) and ifenprodil (10μM) (A_3). (B) Bar graph showing the effect clozapine in the absence and presence of the NR2B-selective antagonists CP101,606 (1μM) and ifenprodil (10μM). Each bar represents the mean ± SEM of data from 6 to 12 experiments. (C) Average time course showing the effect of 15 min bath application of the NR2B-selective antagonist ifenprodil (10μM) on NMDA receptor currents and the additional effect of the NMDA

receptor antagonist D-AP5 (20μM). Each point represents the mean ± SEM of data from 5 experiments. (**p<0.01, significant effect compared to predrug baseline).

Figure 8. Clozapine increases the charge density but not amplitude of electrically evoked glutamatergic EPSCs in Nucleus accumbens. EPSCS were recorded in the presence of 20μM bicuculline and in low Mg^{2+} ACSF. ($A_{1,2}$) Example traces showing the effect of bath application of clozapine (100nM) on the amplitude and charge density of glutamatergic EPSCs before (predrug) and during clozapine application in the absence (A_1) and presence of the NMDA-selective antagonist D-AP5 (20μM) (A_2) at a holding potential of -60mV. The third traces represent an overlay of the predrug (black trace) and drug condition (grey trace) in the absence (A_1) and presence (A_2) of D-AP5 showing the effect of clozapine on the slower NMDA component of the EPSCs (A_1). (B) Bar graph showing the average effect of clozapine (100nM) on EPSC charge density in the absence and presence of D-AP5 (20μM) at a holding potential of -60mV. Each bar represents mean ± SEM of data from 5 experiments each. (*p<0.05; significant effect compared to predrug baseline) (C) Normalized average data of charge densities at different holding potentials (-80mV to -20mV) before drug (filled circles) and after 15min bath application of clozapine (100nM) (open triangles). Data was obtained from 5 experiments and each point represents the mean ± SEM of charge density at a particular holding potential. (D) Normalized average data of charge densities at different holding potentials in the presence of D-AP5 (20μM), before (filled circles) and after 15min of clozapine (100nM) application (open triangles). Data was obtained from 5 experiments and each point represents the mean ± SEM of charge density at a particular holding potential. (C,D)

Each point was normalized to maximum control charge density (for C control value at -40mV, for D control value at -80mV).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haloperidol 100nM
20pA
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clozapine 100nM
normalized NMDA receptor current amplitude
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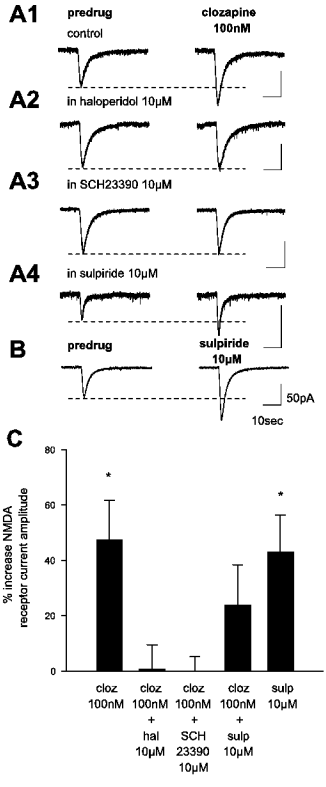
hal 100nM
MDL 100907 100nM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80
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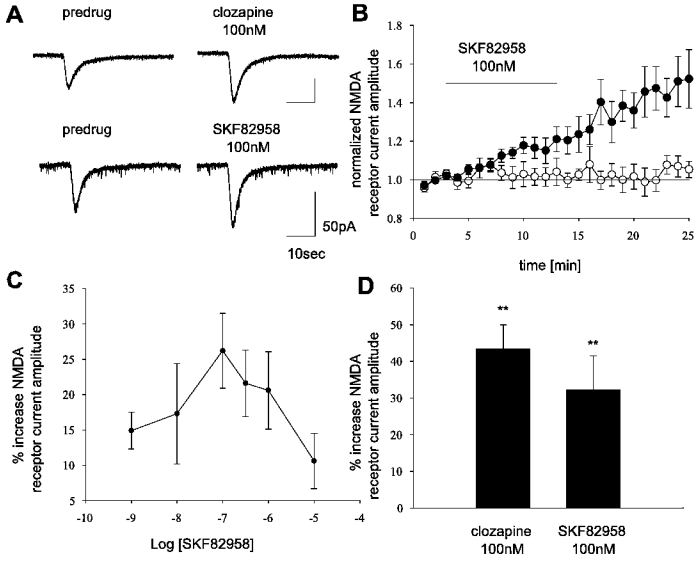
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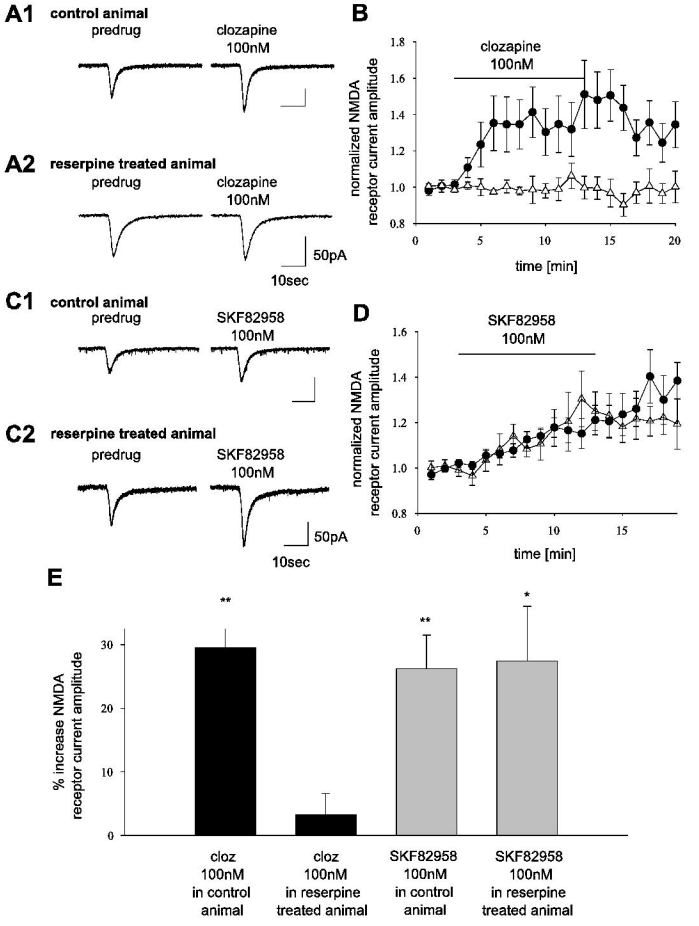
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reserpine treated animal
predrug
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normalized NMDA
receptor current amplitude
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receptor current amplitude
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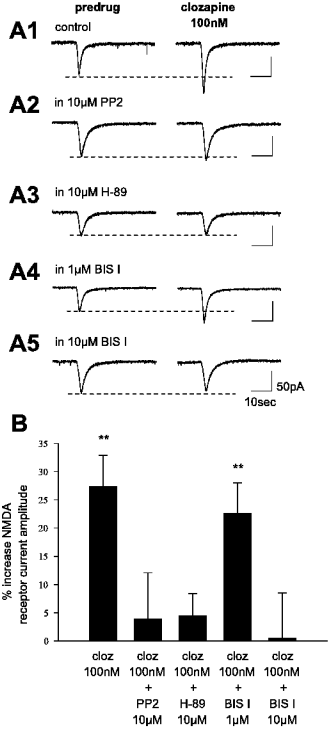
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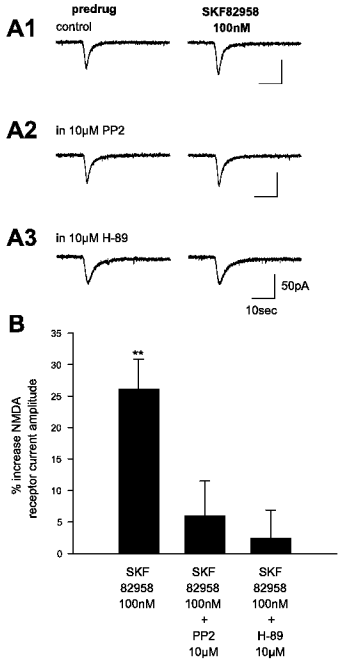
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SKF
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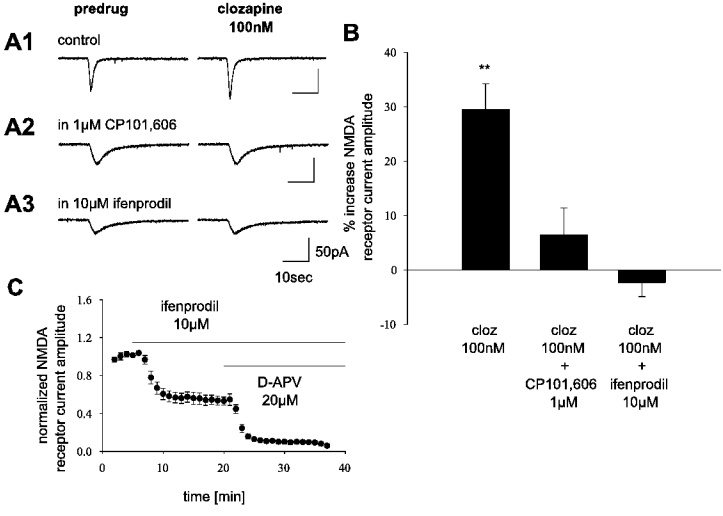
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normalized NMDA receptor current amplitude
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D-APV 20μM
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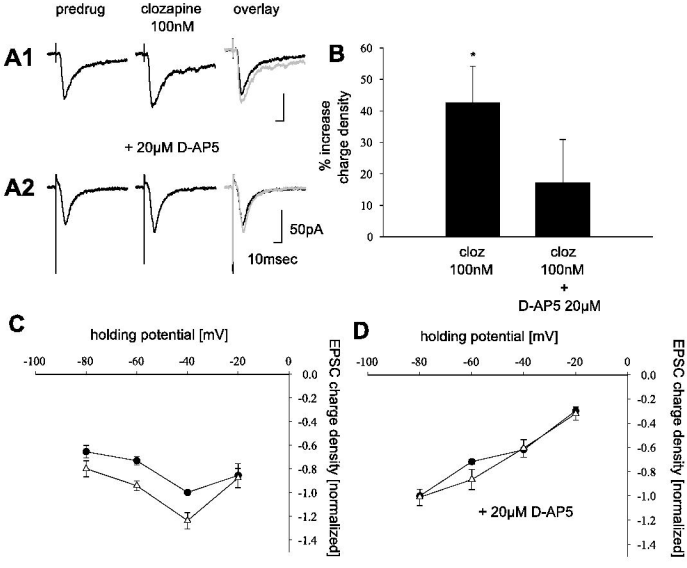
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clozapine 100nM
overlay
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+ 20μM D-AP5
A2
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10msec
B
% increase charge density
cloz 100nM
cloz 100nM + D-AP5 20μM
C
holding potential [mV]
EPSC charge density [normalized]
D
holding potential [mV]
+ 20μM D-AP5
EPSC charge density [normalized]