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Effects of clozapine and *N*-desmethylclozapine on synaptic transmission at hippocampal inhibitory and excitatory synapses

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Abstract

Clozapine is the first atypical antipsychotic, and improves positive and negative symptoms of many patients with schizophrenia resistant to treatment with other antipsychotic agents. Clozapine induces minimal extrapyramidal side effects, but is more often associated with seizures. A large number of studies have been conducted to elucidate pharmacological profiles of clozapine and its major active metabolite, *N*-desmethylclozapine (NDMC). However, there are only a limited number of electrophysiological studies examining their effects on synaptic transmission. In this study, we examined effects of clozapine and NDMC on synaptic transmission by measuring inhibitory and excitatory postsynaptic currents in rat cultured hippocampal neurons. We found that clozapine and NDMC have qualitatively similar actions. They depressed the inhibitory transmission at 1-30 μM, and the excitatory transmission at 30 μM, the former being much more sensitive. The depression of IPSCs by 30 μM of these drugs was associated with an increase in the paired-pulse ratio. The GABA-induced currents were suppressed by these drugs, but less sensitive than IPSCs. The AMPA-induced currents were slightly potentiated by these drugs at 30 μM. At 30 μM, clozapine and NDMC slightly suppressed Ca^{2+} and Na^{+} channels. These results strongly suggest that clozapine and NMDC depress the inhibitory synaptic transmission mainly by antagonizing postsynaptic $GABA_A$ receptors, but at higher concentrations additionally by acting on presynaptic site, possibly in part through inhibition of presynaptic Ca^{2+} and Na^{+} channels. Preferential depression of inhibitory synaptic transmission by clozapine and NDMC might contribute to therapeutic actions and/or side-effects of clozapine.

Key Words:

N-desmethylclozapine, clozapine, synaptic transmission, IPSC, EPSC, hippocampus

1. Introduction

Clozapine is the first atypical antipsychotic, and improves positive and negative symptoms of many patients with schizophrenia whose illness is resistant to treatment with other antipsychotic agents (Kane et al., 1988; Leucht et al., 2003). Clozapine induces minimal extrapyramidal side effects, but is more often associated with seizures (Devinsky and Pacia, 1994; Haddad and Dursun, 2008; Pacia and Devinsky, 1994; Wong and Delva, 2007). It exhibits complex pharmacological properties, and interacts with various types of metabotropic receptors for neurotransmitters including dopamine, serotonin, norepinephrine, histamine and acetylcholine (Correll, 2010; Weiner et al., 2004). It has also been reported that clozapine interacts with $GABA_A$ receptor in a receptor-subtype-dependent manner (Korpi et al., 1995; Squires and Saederup, 1998), suggesting a possible role of clozapine as a direct modulator of synaptic transmission. Actually, an electrophysiological study demonstrated that clozapine depressed GABAergic inhibitory synaptic transmission through antagonism of $GABA_A$ receptors at synapses on ventral tegmental area (VTA) neurons (Michel and Trudeau, 2000). Because this effect was observed at concentrations that could occur in the brain of the patients with clozapine treatment, the authors suggested that this depression of GABAergic transmission might contribute to the therapeutic actions of clozapine and/or its

side-effects, such as seizures. However, it is not clear whether the sensitivity of GABAergic transmission to clozapine is unique to VTA neurons or a common feature throughout the brain.

N-desmethylclozapine (NDMC) is a major active metabolite of clozapine, and has unique pharmacological profiles (Mendoza and Lindenmayer, 2009). Recent studies with animal models have suggested that NDMC might contribute to the unique efficacy of clozapine (Bishara and Taylor, 2008). NDMC can interacts with various types of metabotropic receptors for monoamines and acetylcholine (Lameh et al., 2007). In addition, NDMC is also reported to interact with $GABA_A$ receptors (Wong et al., 1996), suggesting that GABAergic transmission might be sensitive to NDMC as well as clozapine. Despite a large number of pharmacological studies on NMDC, there is no electrophysiological evidence for direct action of NDMC on synaptic transmission.

In the present study, we examined effects of clozapine and NDMC on excitatory and inhibitory synaptic transmissions in cultured hippocampal neurons. The hippocampus contains glutamatergic and GABAergic neurons, but not monoaminergic neurons. Thus, the basal levels of monoamines in this culture system should be negligible. Using this preparation, we minimized possible indirect effects of the drugs on synaptic transmission through well-known antagonistic effects on monoaminergic receptors, which might be tonically active and involved in regulation of synaptic transmission. Here we provide electrophysiological evidence for direct actions of clozapine and NDMC on synaptic transmission through the interactions with postsynaptic $GABA_A$ receptors and presynaptic mechanisms. Our data clearly show that clozapine and NDMC depress the

inhibitory synaptic transmission more effectively than the excitatory one, suggesting that the preferential depression of GABAergic transmission might contribute to therapeutic actions and/or side-effects of clozapine.

2. Results

Neurons were whole-cell voltage clamped with patch pipettes. Access resistance, input resistance, and holding current at -80 mV in the presence and absence of 3 and 30 μM of clozapine or NDMC are shown in Table 1. Neurons treated with these drugs exhibited no signs of cell damage. To measure synaptic currents, one neuron of a pair was stimulated, and inhibitory or excitatory postsynaptic currents (IPSCs or EPSCs) were measured from the other neuron at -80 mV. IPSCs and EPSCs show slow and fast decay kinetics, respectively, and can be easily discriminated (Fig. 1A). In some experiments, the relevance of this criteria was confirmed by examining reversal potentials and sensitivities to $GABA_A$ and AMPA receptor antagonists, bicuculline and CNQX (Fig. 1B, C).

2.1 Effects on synaptic transmission

First we examined whether clozapine effectively depresses the inhibitory synaptic transmission in hippocampal neurons, as reported in VTA neurons. When the external solution containing 30 μM of clozapine was bath-applied for 1 min, IPSCs were depressed in a reversible manner (Fig. 2A). On average, the IPSC amplitude was decreased to 27.8 ± 3.2 % (n=7, $p<0.001$), and recovered to 91.3 ± 5.0 % of control (n=7, $p=0.28$) (Fig. 2B, 30 Clo). Because synaptic currents of cultured cells have a tendency to

rundown even in the absence of drugs, we normalized the value in the presence of drug (T) to the mean of the values of control (C) and recovery (R) in each experiment to avoid overestimation of drug effect. The data presented in Fig. 2C and similar graphs in the following figures were obtained by this means. Each open symbol in Fig. 2C shows the data from each neuron pair, and squares and diamond marks indicate the data from excitatory and inhibitory postsynaptic neurons, respectively. Open circles are the date from unidentified neurons. On average, the IPSC amplitude was decreased to 29.5 ± 3.8 % of control by 30 μM clozapine ($p<0.01$). As shown in Fig. 2C, clozapine was effective irrespective of the type of postsynaptic neurons. Further experiments with lower concentrations (1 and 3 μM) of clozapine demonstrated that clozapine was effective even at 1 μM (Fig. 2B, 2C). On average, 1 and 3 μM clozapine significantly decreased the IPSC amplitude to 89.8 ± 4.0 ($n=7$, $p<0.05$) and 82.2 ± 4.0 % ($n=9$, $p<0.05$), respectively (Fig. 2C). Similar experiments were performed with NDMC, and we found that NDMC also suppressed IPSCs in a dose-dependent, and reversible manner (Fig. 2, D-F). On average, NDMC decreased the IPSC amplitude to 88.7 ± 2.9 % ($n=8$, $p<0.05$) at 1 μM, 77.5 ± 2.4 % ($n=7$, $p<0.01$) at 3 μM, and 14.6 ± 3.0 % ($n=6$, $p<0.05$) at 30 μM (Fig. 2F). Like clozapine, NDMC was effective irrespective of the type of postsynaptic neurons (Fig. 2F).

Next we examined effects of clozapine and NDMC on EPSCs. Because NMDA-type receptors are expected to be blocked by Mg^{2+} under our recording conditions (-80 mV, 1 mM Mg^{2+}), it is conceivable that EPSCs depend largely on AMPA-type glutamate receptors. As expected, we observed that the AMPA receptor

antagonist CNQX (25 μM) suppressed EPSCs completely (Fig. 1C, 0.4 ± 1.1 %, n=8). Compared to IPSCs, EPSCs were much less sensitive to clozapine (Fig. 3, A-C) and NDMC (Fig. 3, D-F). Clozapine depressed EPSCs slightly, but significantly, at 30 μM (83.1 ± 5.7 %, n=9, $p<0.05$), but ineffective at 3 μM (97.5 ± 1.8 %, n=9, p=0.478) (Fig. 3C). Similarly, NDMC decreased the EPSC amplitude to 55.4 ± 6.3 % (n=12, $P<0.01$) at 30 μM, but was without effect at 3 μM (100.6 ± 2.1 %, n=9, p=0.65) (Fig. 3F). These results clearly show that the inhibitory synaptic transmission is more sensitive to these drugs than the excitatory transmission.

2.2 Effects on postsynaptic neurotransmitter receptors

To test the possibility that these effects on synaptic transmission are caused by direct interaction with postsynaptic receptors for neurotransmitters, that is, $GABA_A$ and AMPA receptors, we examined effects of clozapine and NDMC on these receptors. For monitoring the activity of $GABA_A$ receptors, the external solution containing 10 μM GABA was locally applied for 3 sec to the recorded neurons through a puff pipette with an interval of 2 min. The GABA-induced currents recorded in these conditions were confirmed to be mediated mostly by $GABA_A$ receptors by simultaneously treating with bicuculline (Fig. 4A, 6.1 ± 1.1 %, n=6). When GABA was locally applied in the presence of clozapine or NDMC, GABA-induced currents were significantly reduced (Fig. 4). The peak amplitude was decreased to 95.8 ± 1.8 % (n=7, $p<0.05$), 92.3 ± 3.1 % (n=6, $p<0.05$) and 52.3 ± 5.0 % (n=5, $p<0.05$) by 1, 3 and 30 μM clozapine, respectively (Fig. 4D), and 93.1 ± 1.8 % (n=6, $p<0.05$), 87.7 ± 1.0 % (n=7, $p<0.01$) and 35.2 ± 3.9 % (n=9, $p<0.01$)

by 1, 3 and 30 μM NDMC, respectively (Fig. 4H). These data indicate that the suppressing effects of clozapine and NDMC on IPSCs can be partly explained by their actions on $GABA_A$ receptors. However, the effects on GABA-induced currents were significantly weaker than the effects on IPSCs for 30 μM clozapine ($p<0.01$) (Fig. 4E), 3 μM NDMC ($p<0.01$) and 30 μM NDMC ($p<0.01$) (Fig. 4I), suggesting additional contribution of presynaptic mechanisms.

Effects of the drugs on AMPA receptors were similarly examined by applying 50 μM AMPA. The AMPA-induced currents were confirmed to be blocked by the AMPA receptor antagonist CNQX (Fig. 5A, 6.5 ± 3.1 %, n=6). When AMPA was locally applied in the presence of 30 μM clozapine or NDMC, AMPA-induced currents were slightly, but significantly, potentiated (Fig. 5B, D). The current amplitude was 112.8 % ± 1.5 % (n=8, $p<0.01$) and 118.2 ± 4.2 % of control (n=7, $p<0.01$) in the presence of 30 μM clozapine (Fig. 5C) and NDMC (Fig. 5E), respectively. These results indicate that suppressing effects of clozapine and NDMC on EPSCs cannot be explained by their actions on postsynaptic AMPA receptors, suggesting involvement of presynaptic mechanisms.

2.3 Effects on the paired-pulse ratio

Involvement of presynaptic mechanisms was further demonstrated by measuring the paired-pulse (PP) ratio, which is widely used as an index of presynaptic changes. We first confirmed that presynaptic suppression induced by the $GABA_B$ agonist baclofen, but not postsynaptic suppression by the $GABA_A$ antagonist bicuculline, is associated with an increase in the PP ratio (Fig. 6A). Similarly, we observed that the suppression of IPSCs by

30 μM clozapine (Fig. 6B) or NDMC was associated with a significant increase in the PP ratio (n=6, $p<0.05$ for clozapine, n=8, $p<0.01$ for NDMC) (Fig. 6C). At 3 μM, clozapine and NDNC induced a slight increase in the PP ratio, but this change was not significant (n=7, p=0.207 for clozapine, n=8, p=0.066 for NDMC). The suppression of EPSCs by 30 μM NDMC, but not clozapine (n=6, p=0.271), was also associated with a significant increase in the PP ratio (n=6, $p<0.05$) (Fig. 6D). These data strongly suggest that presynaptic mechanisms contribute to the suppression of IPSCs by 30 μM clozapine and NDMC and the suppression of EPSCs by NDMC.

2.4 Effects on voltage-gated ion channels

Presynaptic suppression might be causes by their action on presynaptic receptors, ion channels or some other components involved in transmitter release itself or its modulation. In the present study, we examined only their effects on ion channels. We measured Ca^{2+}, Na^{+} and K^{+} currents activated by depolarizing voltage pulses. The Cd^{2+}-sensitive component of the currents induced by depolarizing voltage pulses (from -80 mV to -10 mV, 50 ms, 0.1 Hz) under the blockade of Na^{+} and K^{+} channels was assumed to be Ca^{2+} current (Fig. 7A, the rightmost trace). Clozapine and NDMC significantly decreased the amplitude of Ca^{2+} currents to 92.8 ± 2.0 % (n=11, $p<0.01$) and 82.3 ± 3.8 % (n=11, $p<0.01$), respectively, at 30 μM, but not at 3 μM (n=5, p=0.306 for clozapine, n=5, p=0.493 for NDMC) (Fig. 7).

Inward Na^{+} currents were measured by applying depolarizing voltage pulses (from -80 mV to 0 mV, 5 ms, 0.25 Hz). At 30 μM, clozapine and NDMC slightly, but

significantly, decreased the peak amplitude of Na^+ currents to 91.3 ± 1.5 % (n=6, $p<0.001$) and 86.6 ± 2.1 % (n=6, $p<0.001$), respectively (Fig. 8). Outward K^+ currents were measured by applying voltage pulses (from -80 mV to 0 mV, 30 ms, 0.25 Hz) under the blockade of Na^+ channels. The peak amplitude of outward currents was also slightly suppressed by 30 μM clozapine (93.9 ± 1.6 %, n=6, $p<0.05$) and NDMC (96.5 ± 1.2 %, n=8, $p<0.05$) (Fig. 9). We cannot simply assume that these effects on somatic ion channels are directly representative of effects on presynaptic ion channels. However, if these effects are caused by direct interaction with ion channels, it seems likely that clozapine and NDMC act on presynaptic Ca^{2+} and Na^+ channels and thereby influence the transmitter release. Of course, we cannot exclude any other possibilities, including indirect actions through metabotropic receptors.

3. Discussions

In the present study, we found that clozapine and its active metabolite NDMC have qualitatively similar actions on synaptic transmission in rat cultured hippocampal neurons. These two substances depress both inhibitory and excitatory transmission, the former being much more sensitive. Our electrophysiological data strongly suggest that these effects on synaptic transmission can be accounted for by their interactions with postsynaptic $GABA_A$ receptors and some actions on presynaptic site.

Depression of inhibitory transmission by clozapine was previously reported in rat cultured VTA neurons (Michel and Trudeau, 2000). The study reported that clozapine decreased inhibitory autaptic currents (IACs), which were recorded in single neuron

micro-dot cultures, and exogenous GABA-induced currents to approximately the same extent, that is, 10 μM clozapine decreased IACs by 34 % and GABA-induced currents by 28 %. Thus, the authors concluded that the effect on GABAergic transmission is attributable to its action on postsynaptic $GABA_A$ receptors. There is another electrophysiological study examining effects of clozapine on inhibitory transmission (Gemperle et al., 2003). Using rat brain slices including prefrontal area, inhibitory postsynaptic potentials (IPSPs) were recorded from layer V neurons following stimulation of layer II. Clozapine failed to affect IPSPs at 30 μM, although higher concentrations of clozapine (100-300 μM) showed a dose-dependent tendency to reduce IPSPs without statistical significance. One possibility explaining this apparent discrepancy in effect of 10-30 μM clozapine is that the clozapine-sensitivity of GABAergic synapses is region-specific, and especially high in the VTA. Another possibility is that the apparent difference in clozapine sensitivity is due largely to the methodological difference, namely culture versus slice preparations. In the present study, we used cultured hippocampal neurons, and observed that 3 μM and 30 μM clozapine decreased IPSCs by 18 % and 71 %, respectively (Fig. 2), which are comparable to that seen with cultured VTA neurons. Thus, clozapine-induced depression of GABAergic transmission at micromolar concentrations is not limited to the VTA, and may be rather general phenomenon throughout the brain.

There are no electrophysiological studies reporting effects of NDMC on inhibitory synaptic transmission, although its interaction with $GABA_A$ receptors have been suggested by pharmacological studies (Wong et al., 1996). In the binding study

using rat brain membranes, NDMC was shown to antagonize $GABA_A$ receptors with a similar potency to clozapine. Both NDMC and clozapine required micromolar concentrations to affect cerebrocortical and hippocampal $GABA_A$ receptors. These results are consistent with our electrophysiological data for effects of NDMC and clozapine on GABA-induced currents. Furthermore, the present study provides the first electrophysiological evidence for depression of inhibitory synaptic transmission by NDMC. Like clozapine, NDMC was confirmed to depress the inhibitory transmission much more effectively than the excitatory one.

Effects of clozapine on excitatory synaptic transmission have also been examined using electrophysiological methods. In the CA1 area of rat hippocampal slices, 50 μM clozapine induced a transient depression (21 % of control) of extracellular field potentials followed by a small augmentation (10 %) (Baskys et al., 1993). In the prefrontal cortex of rat brain slices, 10-300 μM clozapine had no significant effect on excitatory postsynaptic potentials (EPSPs) (Gemperle et al., 2003). In rat prefrontal cortical neurons, 50-100 nM clozapine increased the NMDA component of EPSPs (Arvanov et al., 1997). The reason for these diverse effects is unclear, but it is likely that in slice preparations clozapine might influence the glutamatergic transmission indirectly, that is, through modulation of signaling of other neurotransmitters such as dopamine (Ninan and Wang, 2003). In the present study, we minimized such an indirect effect by using the culture system, and found that clozapine depresses glutamatergic transmission. Consistent with our data, inhibition of glutamate release by clozapine is reported in synaptosomes purified from rat prefrontal cortex (Yang and Wang, 2005).

We found that higher levels of clozapine and NDMC inhibit voltage-gated Ca^{2+} and Na^{+} channels, which could cause presynaptic inhibition of synaptic transmission at both inhibitory and excitatory synapses. There are some studies reporting effects of clozapine on voltage-gated Ca^{2+} channels. Clozapine inhibited voltage-gated Ca^{2+} channels in adrenal chromaffin cells (Park et al., 2001), native T-type Ca^{2+} channels in human thyroid C cells (Enyeart et al., 1992), and recombinant $Ca_V3.1$ T-type Ca^{2+} channels (Choi and Rhim, 2010). The present study demonstrated that native Ca^{2+} channels expressed in central neurons are also sensitive to clozapine, and that NDMC again mimics the clozapine's action. Hippocampal neurons express multiple types of Ca^{2+} channels including P/Q-, N-, R-, L- and T-types. Whether clozapine and NDMC inhibit all these channels or certain types selectively remains to be elucidated.

The median (10th and 90th percentiles) clozapine and N-desmethylclozapine concentrations of plasma samples from clozapine-treated patients were reported to be 0.41 (0.13-0.98) mg/l and 0.25 (0.0-0.53) mg/l, respectively (Couchman et al., 2010). Considering these values and the observations that the concentrations of clozapine and NDMC in brain tissue are higher than in serum (Gershkovich et al., 2010; Weigmann et al., 1999), the brain concentrations in patients might reach the concentrations used in the present study. Thus, it is likely that excitatory/inhibitory balance is disturbed by both clozapine and NDMC through rather selective depression of inhibitory synaptic transmission, which might be implicated in the therapeutic actions and/or seizures as side-effects associated with clozapine-treatment.

4. Experimental Procedure

4.1 Preparation of neurons.

All experiments were performed according to the guidelines laid down by the animal welfare committees of Kanazawa University. Cultured hippocampal neurons were prepared from newborn Sprague-Dawley rats, as described previously (Ohno-Shosaku et al., 2001). Briefly, cells were mechanically dissociated from the hippocampi and plated onto culture dishes (35 mm) pretreated with poly L-ornithine (0.01 %). The cultures were kept at 36 °C in 5 % CO_2 for 8-17 days before use.

4.2 Electrophysiology.

For measurements of synaptic currents, each neuron of a pair was whole-cell voltage clamped at –80 mV using a patch pipette (3 –5 MΩ) filled with a standard internal solution containing (in mM) 100 K-gluconate, 15 KCl, 10 HEPES, 10 EGTA, 6 $MgCl_2$, 4.65 $CaCl_2$, 40 KOH, 5 Na_2ATP and 0.2 Na_2GTP (pH 7.3, adjusted with KOH). The presynaptic neuron was stimulated by applying positive voltage pulses (to 0 mV, 2 ms) at 0.2 or 0.5 Hz, and inhibitory or excitatory postsynaptic currents (IPSCs or EPSCs) were measured from the postsynaptic neuron at -80 mV with a patch-clamp amplifier (EPC9/3, HEKA Electronics, Germany). IPSCs and EPSCs are easily distinguished by their decay time constants, reversal potentials and sensitivities to $GABA_A$ and AMPA receptor antagonists (Fig. 1). When postsynaptic neurons formed synapses on presynaptic neurons (reciprocal connections) or themselves (autapses), we could identify the type of postsynaptic neurons (i.e., excitatory or inhibitory). A standard external solution

contained (in mM) 140 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.3, adjusted with NaOH). To minimize spontaneous synaptic currents, 0.5-1 mM kynurenic acid was added to the standard external solution. The recording chamber was perfused with the kynurenic acid-containing external solution with or without a drug (clozapine or NDMC) at a flow rate of 1-3 ml/min. In each experiment, five consecutive synaptic currents were averaged before, during and after drug application, and the averaged value obtained during application was divided by the mean of the values before and after application (Fig. 2C, 2F, 3C, 3F).

For measurements of postsynaptic sensitivities to GABA and AMPA, 0.1 μM tetrodotoxin (TTX) was added to the standard external solution. The TTX-containing solution with either 10 μM GABA or 50 μM AMPA was locally applied for 3 sec through a capillary tube (250 μm inner diameter) located near the tested neurons with an interval of 2 min using a perfusion valve controller (VC-6M, Warner Instruments, CT) (Fig. 4A, 5A). The applied agonist was rapidly washed out by continuous bath perfusion. The agonist-induced inward currents were recorded at -80 mV, using patch pipettes filled with the standard internal solution.

For measurements of Ca^{2+} currents, an external solution containing (in mM) 130 NaCl, 10 TEA-Cl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 4-aminopyridine (4-AP) and 10 HEPES (pH 7.3, adjusted with NaOH) was used. Patch pipettes were filled with a solution containing (in mM) 120 CsCl, 30 CsOH, 10 HEPES, 10 EGTA, 5 Na_2ATP, 5 $MgCl_2$ and 1 $CaCl_2$ (pH 7.3, adjusted with CsOH). Voltage-gated Ca^{2+} channels were activated by changing the holding potential from -80 mV to -10 mV for 50 ms with an

interval of 10 sec, and the inward currents were recorded. Under these conditions, voltage-gated Na^+ channels and K^+ channels were blocked by TTX, TEA, 4-AP and Cs^+, and thus the recorded inward currents can be expected to be, for the most part, Ca^{2+} currents. In each experiment, 0.1 mM Cd^{2+} was added to the bath solution at the end of the experiment, and the remaining Cd^{2+}-resistant component was subtracted from the depolarization-activated currents to obtain the true Ca^{2+} currents (Fig. 7A). For measurements of Na^+ currents, the standard external and internal solutions were used as the bath and pipette solutions, respectively. The holding potential was changed from -80 mV to 0 mV for 5 ms with an interval of 4 sec, and the peak amplitude of inward currents was measured. For measurements of K^+ currents, 1 μM TTX was added to the standard external solution. Patch pipettes were filled with the standard internal solution. The holding potential was changed from -80 mV to 0 mV for 30 ms with an interval of 4 sec, and the peak amplitude of outward currents was measured. To estimate the drug effects, three consecutive currents were averaged before, during and after drug application, and the averaged value during application was divided by the mean of the values before and after application.

All experiments were performed at room temperature. Recordings were discarded when series resistance was >20 ΩM or >25 ΩM at the beginning or end of experiments, respectively, or recovery of current amplitude after drug application was less than 70 % of the basal level. Statistical significance of drug actions was assessed by Student's paired or unpaired *t*-test. Single, double and triple asterisks in figures indicate $p<0.05$, $p<0.01$, $p< 0.001$, respectively. All data are expressed as mean ± SEM.

4.3 Drugs.

Clozapine, NDMC, CNQX and baclofen were purchased from Tocris Cookson (Bristol, UK). AMPA and bicuculline were purchased from Sigma-Aldrich (St. Louis, MO), and GABA was from Wako (Japan). Clozapine, NDMC and CNQX were dissolved in DMSO as stock solutions.

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References

Arvanov, V. L., Liang, X., Schwartz, J., Grossman, S., Wang, R. Y., 1997. Clozapine and haloperidol modulate N-methyl-D-aspartate- and non-N-methyl-D-aspartate receptor-mediated neurotransmission in rat prefrontal cortical neurons in vitro. J Pharmacol Exp Ther 283, 226-234.

Baskys, A., Wang, S., Remington, G., Wojtowicz, J. M., 1993. Haloperidol and loxapine but not clozapine increase synaptic responses in the hippocampus. Eur J Pharmacol 235, 305-307.

Bishara, D., Taylor, D., 2008. Upcoming agents for the treatment of schizophrenia:

mechanism of action, efficacy and tolerability. Drugs 68, 2269-2292.

Choi, K. H., Rhim, H., 2010. Inhibition of recombinant Ca(v)3.1 (alpha(1G)) T-type calcium channels by the antipsychotic drug clozapine. Eur J Pharmacol 626, 123-130.

Correll, C. U., 2010. From receptor pharmacology to improved outcomes: individualising the selection, dosing, and switching of antipsychotics. Eur Psychiatry 25 Suppl 2, S12-21.

Couchman, L., Morgan, P. E., Spencer, E. P., Flanagan, R. J., 2010. Plasma clozapine, norclozapine, and the clozapine:norclozapine ratio in relation to prescribed dose and other factors: data from a therapeutic drug monitoring service, 1993-2007. Ther Drug Monit 32, 438-447.

Devinsky, O., Pacia, S. V., 1994. Seizures during clozapine therapy. J Clin Psychiatry 55 Suppl B, 153-156.

Enyeart, J. J., Biagi, B. A., Mlinar, B., 1992. Preferential block of T-type calcium channels by neuroleptics in neural crest-derived rat and human C cell lines. Mol Pharmacol 42, 364-372.

Gemperle, A. Y., Enz, A., Pozza, M. F., Luthi, A., Olpe, H. R., 2003. Effects of clozapine, haloperidol and iloperidone on neurotransmission and synaptic plasticity in prefrontal cortex and their accumulation in brain tissue: an in vitro study. Neuroscience 117, 681-695.

Gershkovich, P., Sivak, O., Sharma, A., Barr, A. M., Procyshyn, R., Wasan, K. M., 2010. Effect of hypertriglyceridemia on the pharmacokinetics and blood-brain barrier

penetration of clozapine and norclozapine following administration to rats. Eur Neuropsychopharmacol 20, 545-552.

Haddad, P. M., Dursun, S. M., 2008. Neurological complications of psychiatric drugs: clinical features and management. Hum Psychopharmacol 23 Suppl 1, 15-26.

Kane, J., Honigfeld, G., Singer, J., Meltzer, H., 1988. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 45, 789-796.

Korpi, E. R., Wong, G., Luddens, H., 1995. Subtype specificity of gamma-aminobutyric acid type A receptor antagonism by clozapine. Naunyn Schmiedebergs Arch Pharmacol 352, 365-373.

Lameh, J., Burstein, E. S., Taylor, E., Weiner, D. M., Vanover, K. E., Bonhaus, D. W., 2007. Pharmacology of N-desmethylclozapine. Pharmacol Ther 115, 223-231.

Leucht, S., Wahlbeck, K., Hamann, J., Kissling, W., 2003. New generation antipsychotics versus low-potency conventional antipsychotics: a systematic review and meta-analysis. Lancet 361, 1581-1589.

Mendoza, M. C., Lindenmayer, J. P., 2009. N-desmethylclozapine: is there evidence for its antipsychotic potential? Clin Neuropharmacol 32, 154-157.

Michel, F. J., Trudeau, L. E., 2000. Clozapine inhibits synaptic transmission at GABAergic synapses established by ventral tegmental area neurones in culture. Neuropharmacology 39, 1536-1543.

Ninan, I., Wang, R. Y., 2003. Modulation of the ability of clozapine to facilitate NMDA- and electrically evoked responses in pyramidal cells of the rat medial prefrontal

cortex by dopamine: pharmacological evidence. Eur J Neurosci 17, 1306-1312.

Ohno-Shosaku, T., Maejima, T., Kano, M., 2001. Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. Neuron 29, 729-738.

Pacia, S. V., Devinsky, O., 1994. Clozapine-related seizures: experience with 5,629 patients. Neurology 44, 2247-2249.

Park, T., Bae, S., Choi, S., Kang, B., Kim, K., 2001. Inhibition of nicotinic acetylcholine receptors and calcium channels by clozapine in bovine adrenal chromaffin cells. Biochem Pharmacol 61, 1011-1019.

Squires, R. F., Saederup, E., 1998. Clozapine and several other antipsychotic/antidepressant drugs preferentially block the same 'core' fraction of GABA(A) receptors. Neurochem Res 23, 1283-1290.

Weigmann, H., Hartter, S., Fischer, V., Dahmen, N., Hiemke, C., 1999. Distribution of clozapine and desmethylclozapine between blood and brain in rats. Eur Neuropsychopharmacol 9, 253-256.

Weiner, D. M., Meltzer, H. Y., Veinbergs, I., Donohue, E. M., Spalding, T. A., Smith, T. T., Mohell, N., Harvey, S. C., Lameh, J., Nash, N., Vanover, K. E., Olsson, R., Jayathilake, K., Lee, M., Levey, A. I., Hacksell, U., Burstein, E. S., Davis, R. E., Brann, M. R., 2004. The role of M1 muscarinic receptor agonism of N-desmethylclozapine in the unique clinical effects of clozapine. Psychopharmacology (Berl) 177, 207-216.

Wong, G., Kuoppamaki, M., Hietala, J., Luddens, H., Syvalahti, E., Korpi, E. R., 1996.

Effects of clozapine metabolites and chronic clozapine treatment on rat brain GABAA receptors. Eur J Pharmacol 314, 319-323.

Wong, J., Delva, N., 2007. Clozapine-induced seizures: recognition and treatment. Can J Psychiatry 52, 457-463.

Yang, T. T., Wang, S. J., 2005. Effects of haloperidol and clozapine on glutamate release from nerve terminals isolated from rat prefrontal cortex. Synapse 56, 12-20.

Figure legends

Figure 1

Recordings of IPSCs and EPSCs. (A) Schematic drawing showing measurements of evoked IPSCs or EPSCs from a pair of neurons having a synaptic connection. IPSCs and EPSCs show different decay kinetics. (B) IPSCs are consistently blocked by the $GABA_A$ antagonist bicuculline (10 μM). (C) EPSCs are consistently blocked by 25 μM CNQX, an AMPA-type glutamate receptor antagonist.

Figure 2

Both clozapine and NDMC depress IPSCs in a dose-dependent and reversible manner. (A, D) The time courses of changes in IPSC amplitudes and IPSC traces (top) from representative experiments with 30 μM clozapine (A) and NDMC (D). (B, E) Relative amplitudes of IPSCs acquired before (C), during (T) and after (R) application of 1, 3 or 30 μM clozapine (B) and NDMC (E). They are normalized to the values before drug application. (C, F) Individual (open symbols) and averaged (closed circles) data for the

IPSC amplitude in the presence of 1, 3 or 30 μM clozapine (C) or NDMC (F). The data are normalized to the means of the IPSC amplitudes obtained before and after drug application. Open squares, diamonds, and circles are the data from excitatory, inhibitory, and unidentified postsynaptic neurons. The number of experiments is indicated in parentheses for this and subsequent figures.

Figure 3

EPSCs are less sensitive to clozapine and NDMC than IPSCs. (A, D) Representative examples of the experiments showing effects of 30 μM clozapine (A) and NDMC (D). (B, E) Relative amplitudes of EPSCs acquired before, during and after application of 3 or 30 μM clozapine (B) and NDMC (E). (C, F) Individual (open symbols) and averaged (closed circles) data for the EPSC amplitude in the presence of 3 or 30 μM clozapine (C) or NDMC (F). Open squares, diamonds, and circles are the data from excitatory, inhibitory, and unidentified postsynaptic neurons.

Figure 4

Both clozapine and NDMC reduce the GABA-induced currents in a reversible manner. (A) Schematic drawing showing local application of 10 μM GABA to the tested neuron. The GABA-induced currents are blocked by 10 μM bicuculline. (B, F) Representative examples of the experiments showing effects of 30 μM clozapine (B) and NDMC (F) on GABA-induced currents. (C, G) Relative amplitudes of GABA-induced currents acquired before, during and after application of 1, 3 or 30 μM clozapine (C) and NDMC

(G). (D, H) Individual (open circles) and averaged (closed circles) data for the amplitude of GABA-induced currents in the presence of 1, 3 or 30 μM clozapine (D) or NDMC (H). (E, I) Summary bar graphs showing effects of 1, 3 or 30 μM clozapine (E) and NDMC (I) on IPSCs and GABA-induced currents. Averaged values shown in Fig. 2C, 2F, 4D and 4H are assembled in this figure for comparison.

Figure 5

Slight potentiation of the AMPA-induced currents by clozapine and NDMC. (A) The currents induced by local application of 50 μM AMPA are blocked by 25 μM CNQX. (B, D) Representative examples of the experiments showing effects of 30 μM clozapine (B) and NDMC (D) on AMPA-induced currents. (C, E) Left: Relative amplitudes of AMPA-induced currents acquired before, during and after application of 30 μM clozapine (C) and NDMC (E). Right: Individual (open circles) and averaged (closed circles) data for the amplitude of AMPA-induced currents in the presence of 30 μM clozapine (C) or NDMC (E).

Figure 6

Effects of clozapine and NDMC on the paired-pulse ratio of IPSCs and EPSCs. (A) An example showing an increase in the paired-pulse ratio during presynaptic suppression induced by the $GABA_B$ agonist baclofen. Traces acquired before and during application of baclofen are superimposed. The traces scaled to the first IPSCs are shown on the right. (B) A representative experiment showing an increase in the paired-pulse ratio of IPSCs in

the presence of 30 μM clozapine. (C, D) Summary data for the paired-pulse ratio (top) and the amplitude of IPSCs (C) or EPSCs (D) obtained before, during and after application of 30 μM clozapine or NDMC.

Figure 7

Effects of clozapine and NDMC on voltage-gated Ca^{2+} channels. (A) Depolarizing voltage pulses from -80 to -10 mV for 50 ms induce inward currents (left), which are subtracted by the Cd^{2+}-resistant component (middle) to yield Ca^{2+} currents (right). (B, E) Representative examples of the experiments showing effects of 30 μM clozapine (B) and NDMC (E) on Ca^{2+} currents. (C, F) Relative amplitudes of Ca^{2+} currents acquired before, during and after application of 3 or 30 μM clozapine (C) and NDMC (F). (D, G) Individual (open circles) and averaged (closed circles) data for the amplitude of Ca^{2+} currents in the presence of 3 or 30 μM clozapine (D) or NDMC (G).

Figure 8

Effects of clozapine and NDMC on voltage-gated Na^{+} channels. (A, C) Representative examples showing effects of 30 μM clozapine (A) and NDMC (C) on inward Na^{+} currents. (B, D) Left: Relative amplitudes of Na^{+} currents acquired before, during and after application of 30 μM clozapine (B) and NDMC (D). Right: Individual (open circles) and averaged (closed circles) data for the amplitude of Na^{+} currents in the presence of 30 μM clozapine (B) or NDMC (D).

Figure 9

Effects of clozapine and NDMC on voltage-gated K^+ channels. (A, C) Representative examples showing effects of 30 μM clozapine (A) and NDMC (C) on outward K^+ currents. (B, D) Left: Relative amplitudes of K^+ currents acquired before, during and after application of 30 μM clozapine (B) and NDMC (D). Right: Individual (open circles) and averaged (closed circles) data for the amplitude of K^+ currents in the presence of 30 μM clozapine (B) or NDMC (D).

Table 1. Series resistance, holding current and input resistance during application of clozapine and NDMC

Drug		Series resistance (MΩ)			Holding current at -80 mV (pA)		Input resistance (MΩ)	
		Before	Drug	After	Control	Drug	Control	Drug
Clozapine	3 μM (n=6)	12.9 ± 1.9	13.2 ± 2.0	14.0 ± 2.4	-236 ± 50	-234 ± 50	131 ± 25	131 ± 25
	30 μM (n=9)	13.0 ± 1.4	13.4 ± 1.6	13.6 ± 1.7	-236 ± 42	-211 ± 44**	130 ± 17	139 ± 21
NDMC	3 μM (n=7)	12.1 ± 1.2	13.0 ± 1.8	13.6 ± 2.1	-239 ± 47	-236 ± 48	133 ± 20	132 ± 19
	30 μM (n=9)	12.3 ± 1.1	12.8 ± 1.3	13.1 ± 1.5	-240 ±40	-206 ± 45**	126 ± 14	148 ± 23*

* $P<0.05$, ** $P<0.01$.

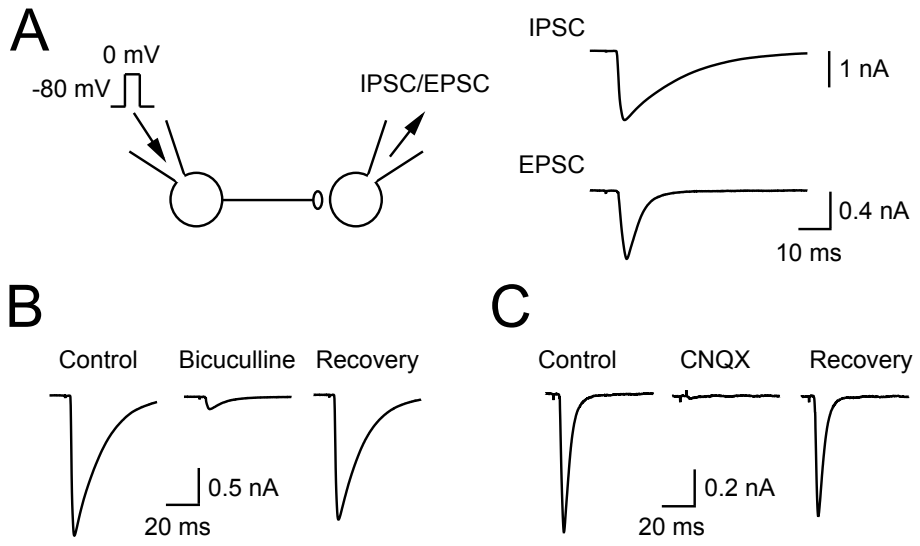


Figure 1 (T. Ohno-Shosaku)

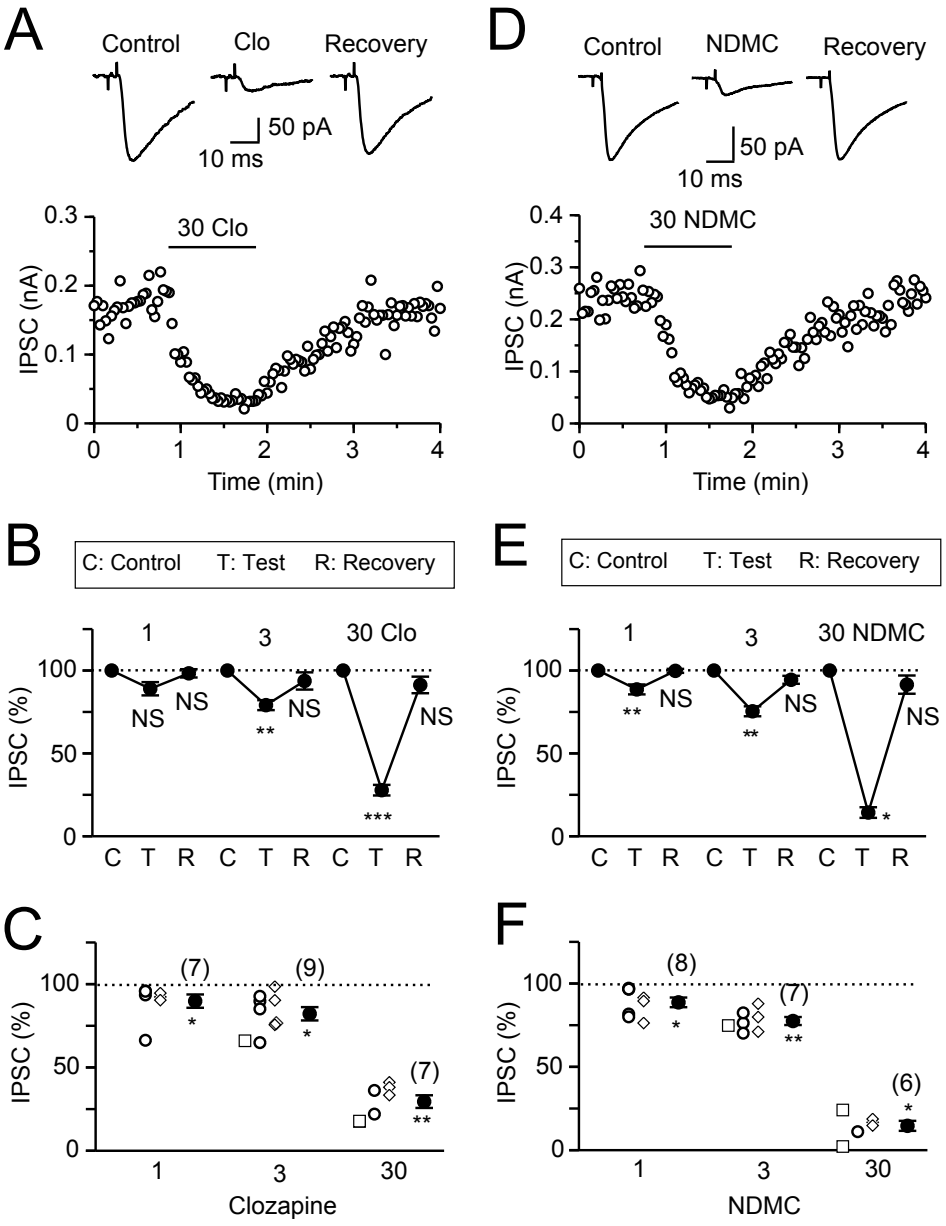
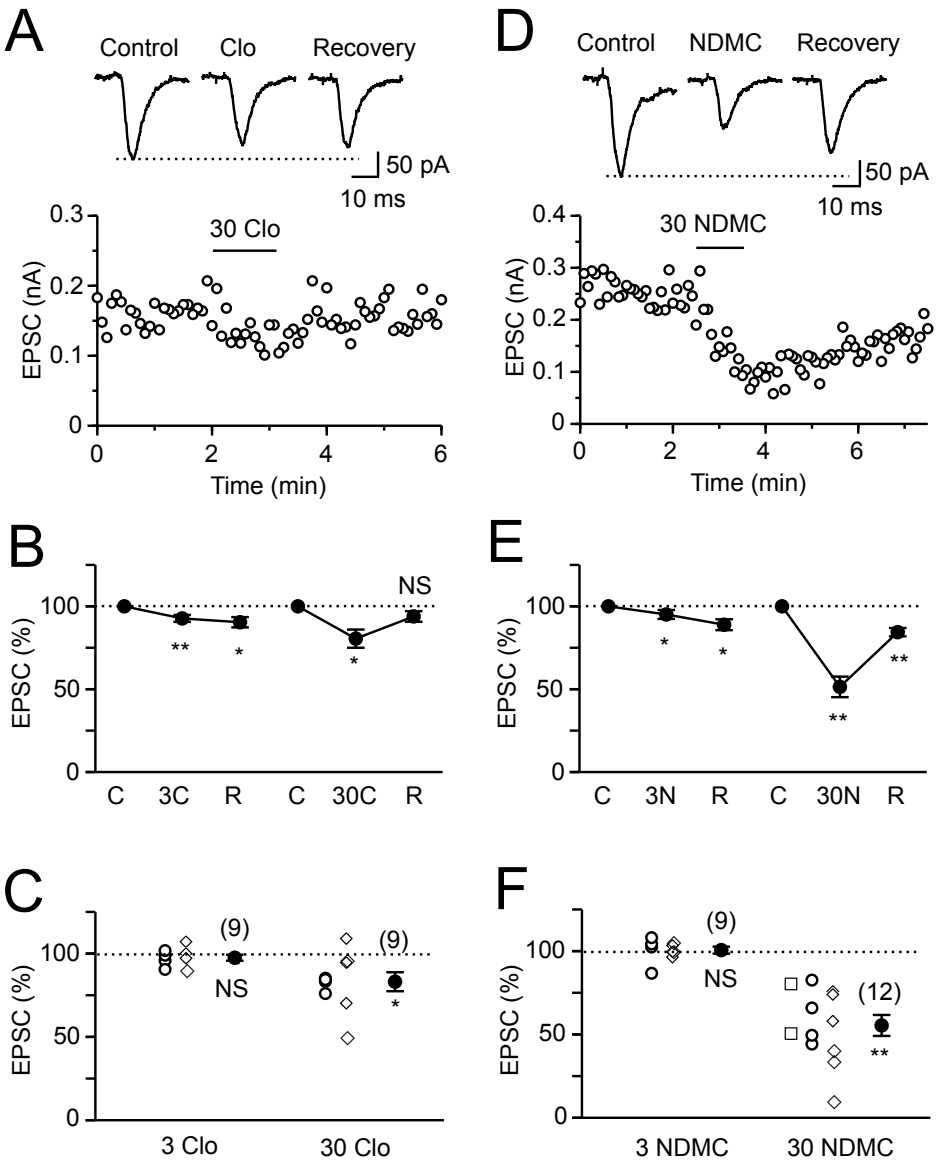


Figure 2 (T. Ohno-Shosaku)



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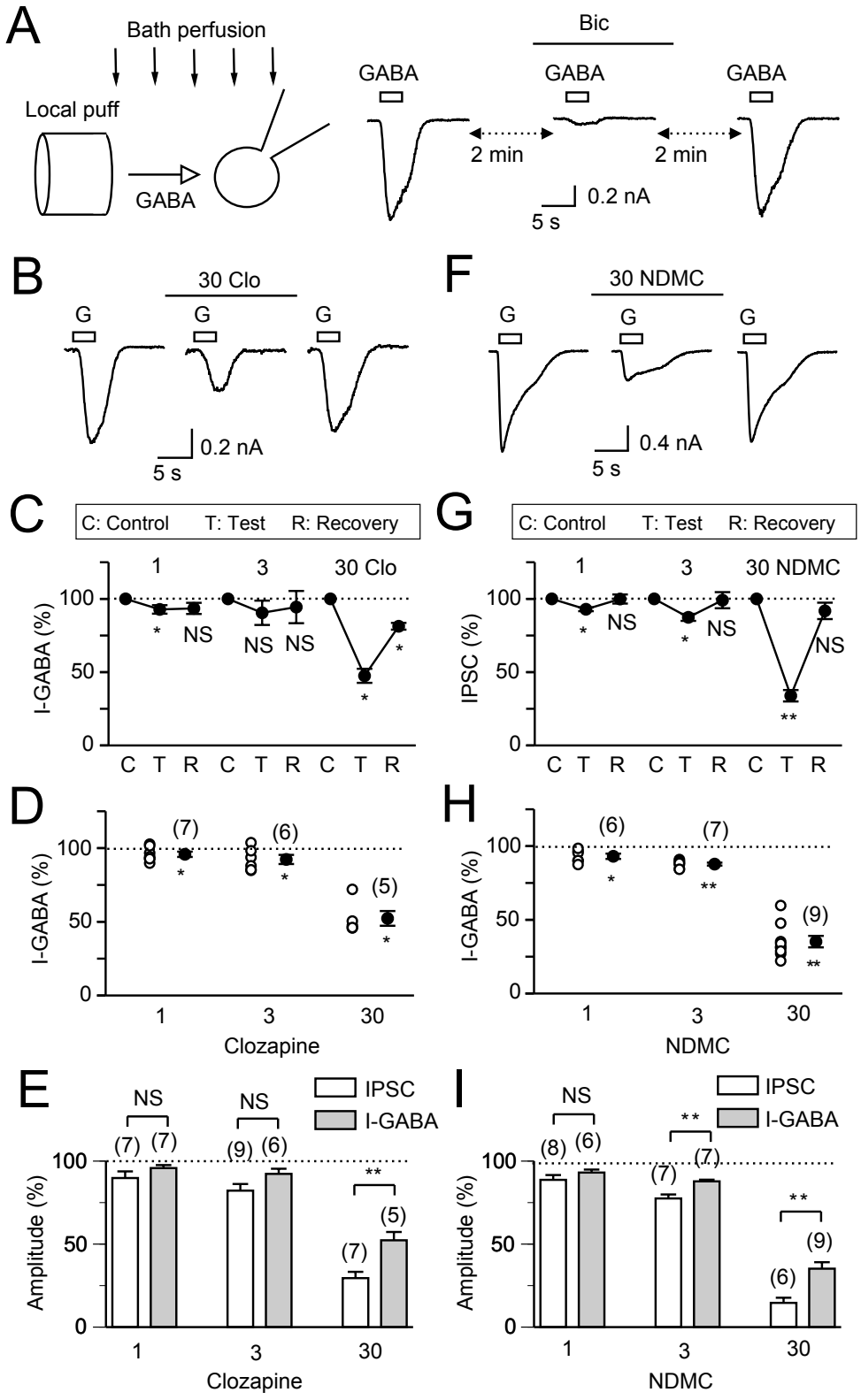

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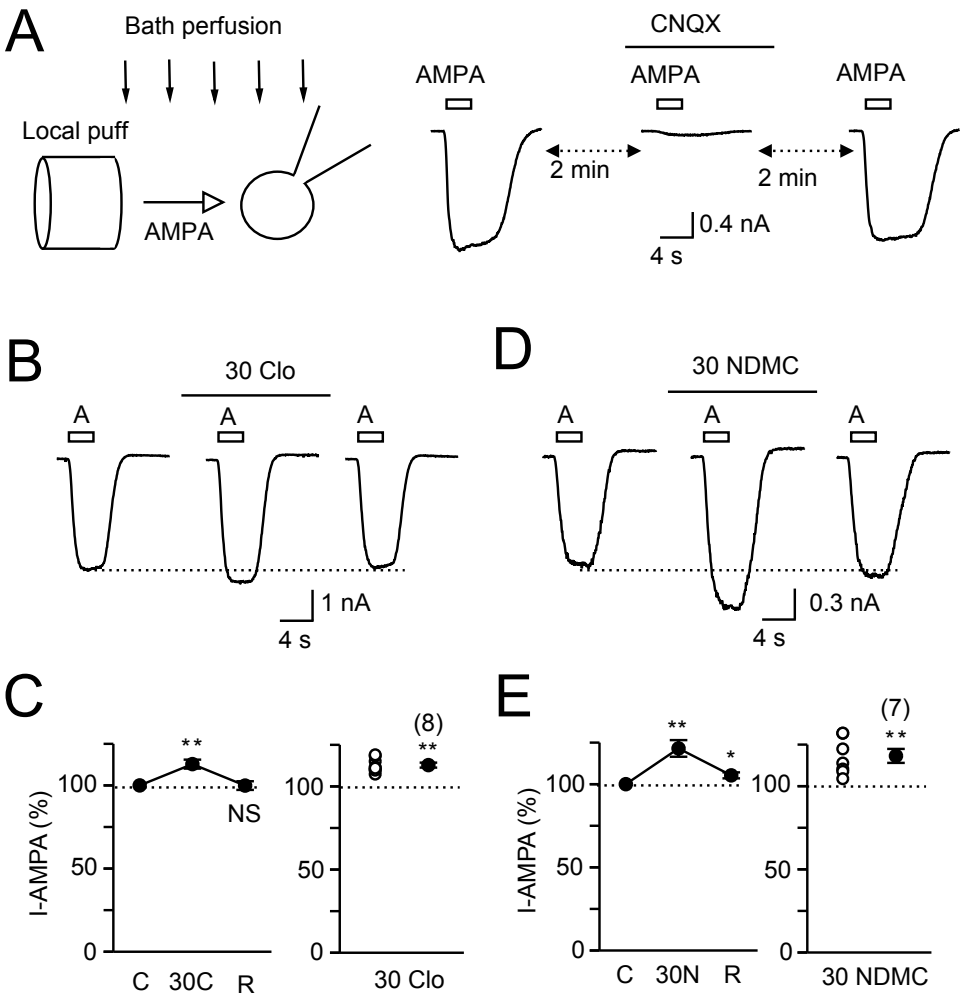

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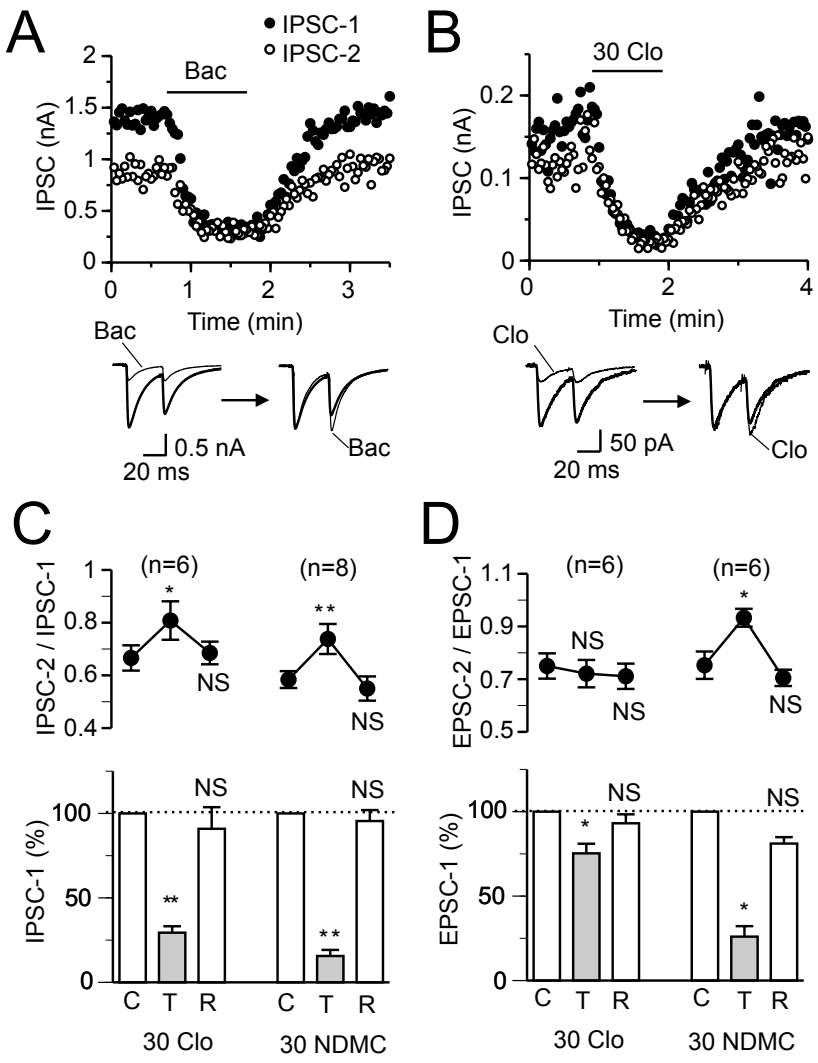

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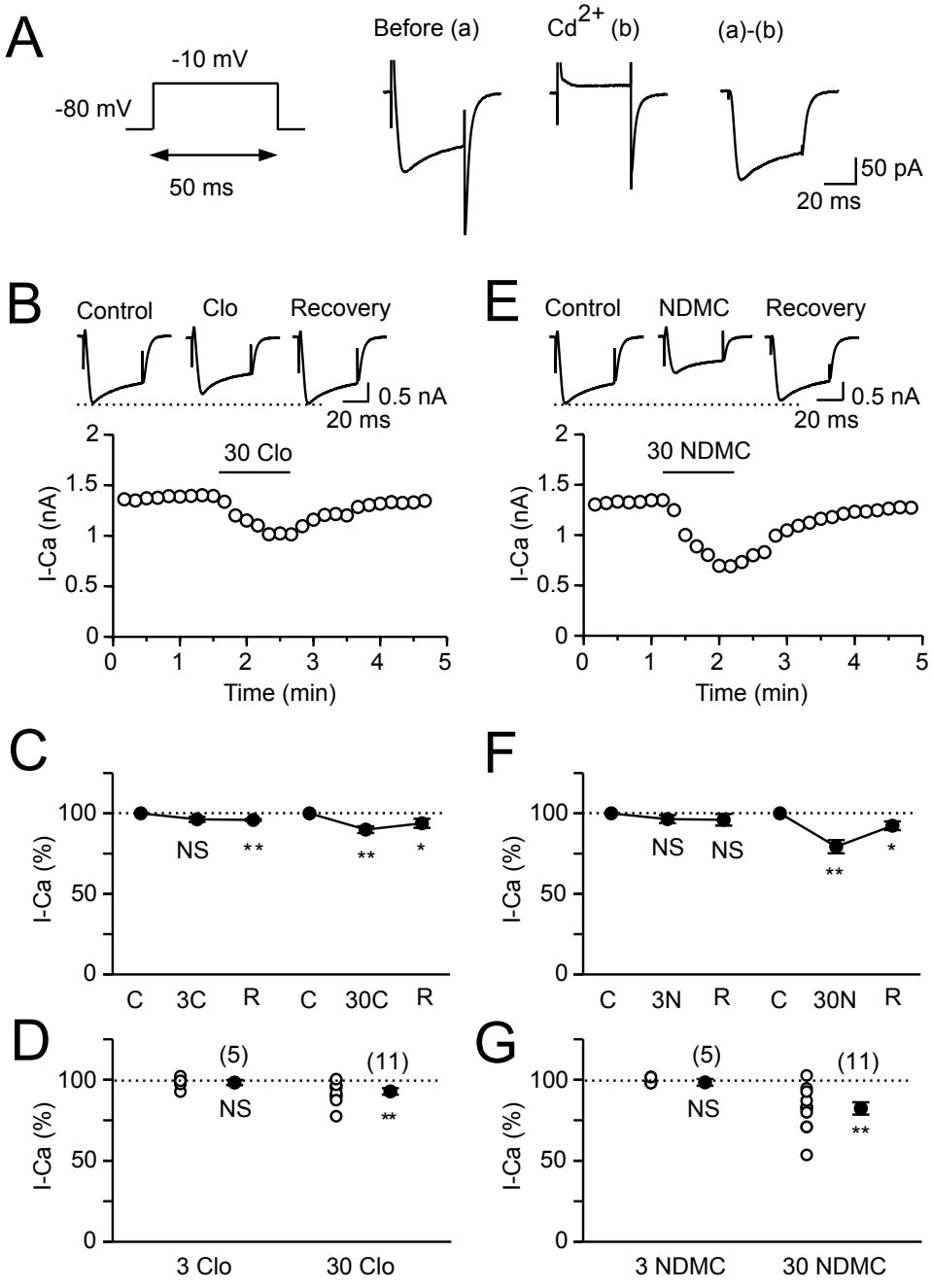

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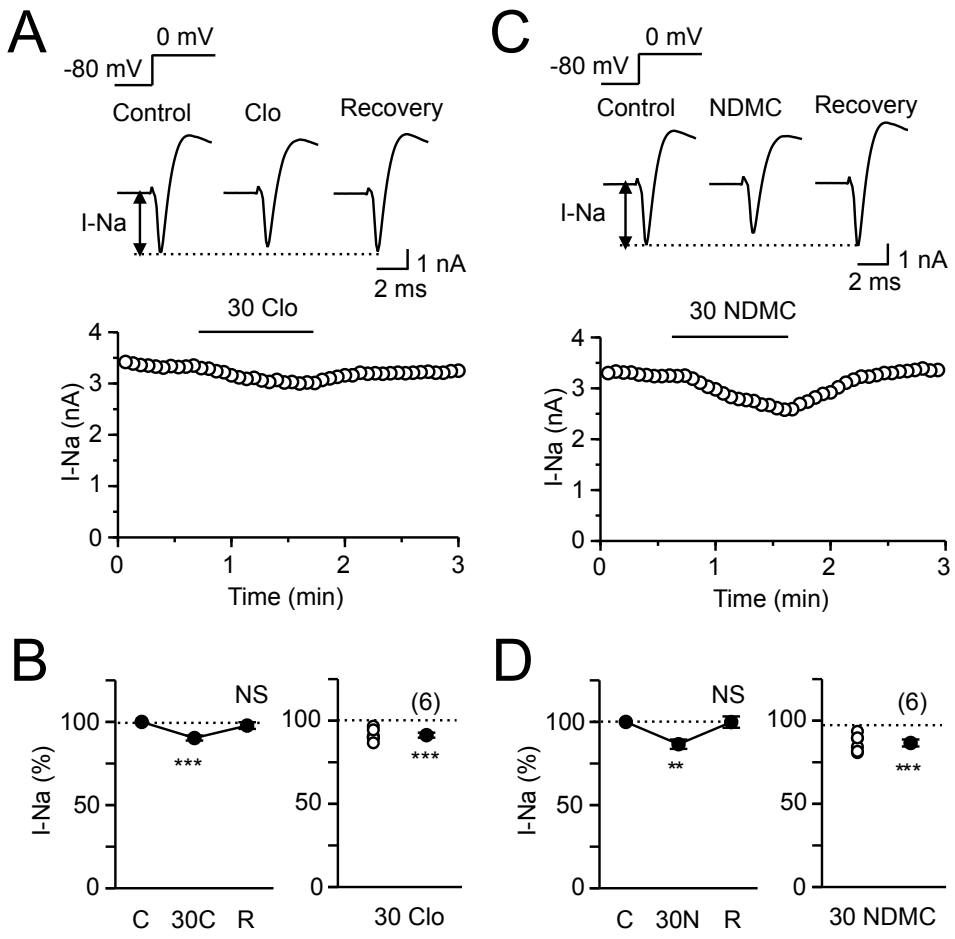

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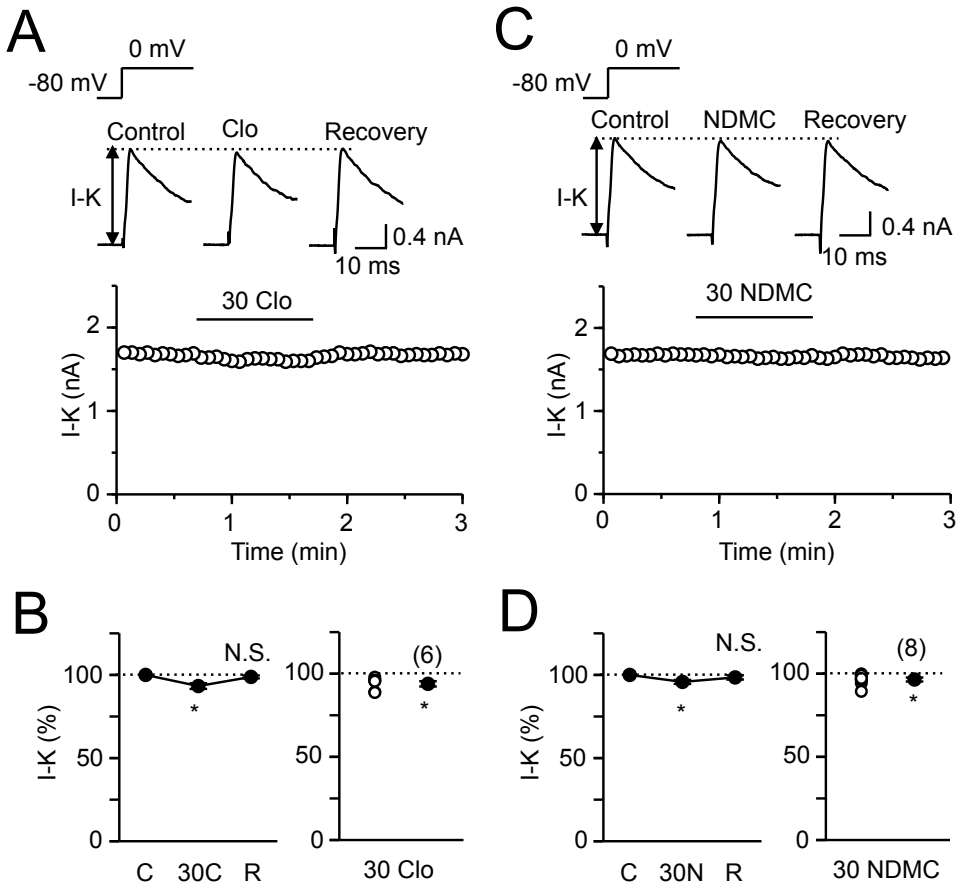

Figure 9 (T. Ohno-Shosaku)