Clozapine inhibits synaptic transmission at GABAergic synapses established by ventral tegmental area neurones in culture

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Abstract

Elucidation of the mechanism of action of the atypical antipsychotic clozapine is complicated by the finding that this molecule interacts with multiple targets including dopaminergic and serotonergic receptors. Binding studies have suggested that clozapine also antagonises $GABA_A$ receptors, but physiological evidence for such a block at functional synapses is lacking. In this study, we explored this antagonism by using electrophysiological techniques on GABAergic neurones of the ventral tegmental area in culture. Inhibitory post-synaptic currents (IPSCs) evoked in isolated GABAergic neurones were found to be dose-dependently inhibited by clozapine. Compatible with a post-synaptic mechanism, we found that membrane currents evoked by exogenous applications of GABA were similarly dose-dependently inhibited by clozapine. An analysis of miniature inhibitory post-synaptic currents (mIPSCs) showed that clozapine reduced the amplitude of quantal events in a way similar to SR-95531, a specific $GABA_A$ receptor antagonist. Both drugs caused a similar leftward shift of the cumulative probability distribution of mIPSC amplitudes. This suggests that clozapine acts on both synaptic and extrasynaptic $GABA_A$ receptors. In conclusion, our work demonstrates that clozapine produces a functional antagonism of $GABA_A$ receptors at synapses. Because this effect occurs at concentrations that could be found in the brain of patients treated with clozapine, a reduction in GABAergic synaptic transmission could be implicated in the therapeutic actions and/or side-effects of clozapine. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Although atypical antipsychotic drugs such as clozapine are widely used in the treatment of schizophrenia (Andersson et al., 1998; Brunello et al., 1995), their exact mechanism of action remains ill-defined. The discovery that classical antipsychotics are potent antagonists of dopamine (DA) receptors has been instrumental in the elaboration of a "dopamine hypothesis" for the pathophysiology of schizophrenia (Bacopoulos et al., 1979; Davis et al., 1991; Grace, 1991; Joyce, 1993; Reynolds, 1983; Seeman, 1992). Although a number of variants of this hypothesis have been proposed, most of these suggest that, one way or another, some aspect of the functioning of the central dopaminergic pathways is perturbed in schizophrenia and that most antipsychotics act by interacting with dopamine receptors.

Despite a large number of studies on clozapine, our understanding of its mechanism of action remains fragmentary. This drug interacts not only with all DA receptors [the D4 subtype showing the highest affinity (~9 nM) (Van Tol et al., 1991)], but also with other metabotropic receptors such as those for serotonin (5HT1 and 5HT2) (Canton et al., 1990), norepinephrine (Coward, 1992), acetylcholine (Snyder et al., 1974) and histamine (Coward, 1992) (for review see Brunello et al., 1995; Coward, 1992). It has also been shown that clozapine can interact with some ionotropic receptors such as the NMDA-subtype glutamate receptor (Arvanov et al., 1997; Ossowska et al., 1999) and the $GABA_A$ receptor (Squires and Saederup, 1991).

The possibility that GABAergic receptors could be a relevant target for clozapine can be considered interesting in part because dopaminergic neurones such as those in the ventral tegmental area (VTA) are known to be

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regulated by the activity of GABAergic neurones both of local origin and from areas such as the nucleus accumbens (Ikemoto et al., 1997; Johnson and North, 1992; Steffensen et al., 1998). Most evidence in favour of an interaction between clozapine and GABA receptors has originated from binding studies. Squires and Saederup (1991) demonstrated that clozapine in the micromolar range can reverse the inhibitory effect of GABA on the binding of [^{35}S]TBPS (^{35}S-t-butylbicyclophosphorothionate), a competitive GABA$_A$ receptor antagonist, to rat brain membranes. A clozapine metabolite, N-desmethylclozapine, seems to have the same effect (Wong et al., 1996). The same authors have suggested recently that in addition to clozapine, several other antipsychotic drugs may also act as antagonists at GABA$_A$ receptors (Squires and Saederup 1997, 1998). These observations have been confirmed by Korpi et al. (1995) who found that clozapine at 10 µM significantly reduces GABA-mediated Cl$^-$ influx in brain vesicles and that at 100 µM it regionally antagonises GABA inhibition of [^{35}S]TBPS binding in brain sections. Finally, using a system allowing the expression of various combinations of recombinant GABA$_A$ receptor subunits, this same group has demonstrated that clozapine interacts preferentially with GABA$_A$ receptors containing specific combinations of α and β subunits (Korpi et al., 1995). Although physiological evidence is lacking, taken together these experiments support the idea that clozapine can act to reduce GABA$_A$ receptor activation.

In this report we have sought to determine whether the reported antagonism of GABA$_A$ receptors by clozapine could be of physiological importance at the synapse. Using GABAergic neurones from the VTA in primary culture we have tested whether clozapine acts to regulate the synaptic activation of GABA$_A$ receptors.

2. Methods

2.1. Cell culture

Primary cell cultures were prepared using methods derived from Cardozo (1993). Briefly, neonatal (P$_0$ or P$_1$) Sprague–Dawley rats were anaesthetised with Halothane and their brains were removed and transferred to ice-cold medium. A 1.5 mm slice was cut at the level of the midbrain flexure. Using a custom tissue punch, the VTA was isolated and digested with papaïn for 30 min before being mechanically triturated. Cells were then centrifuged and re-suspended in Basal Medium Eagle containing 5% FBS (GIBCO) and Mito+ serum extender (VWR Canlab). Cells were plated at a density of 200 000 cells per ml on poly(L-lysine)/collagen-coated coverslips (standard cultures) or at 100 000 cells per ml on agarose-covered coverslips sprayed with poly(D-lysine)/collagen micro-dots ("micro-dot" cultures). The latter permitted

the establishment of single neurone cultures in which the formation of autaptic synaptic contacts facilitated electrophysiological recordings of GABA-mediated synaptic currents (Bekkers and Stevens, 1991). Experiments were performed on neurones between 10 and 30 days after plating.

2.2. Electrophysiological recordings

Cells were perfused with extracellular medium that contained (in mM): NaCl 140, KCl 5, MgCl$_2$ 2, CaCl$_2$ 2, HEPES 10, glucose 10, sucrose 6 (pH 7.35). Whole-cell recordings were performed at room temperature using a WARNER PC-505 patch-clamp amplifier (Warner Instrument Corp, Hamden, CT). Signals were digitised at 5 kHz and recorded and analysed with Pclamp 6.0.4 software (Axon Instruments, Foster City, CA). Autaptic currents and inhibitory post-synaptic currents (IPSCs) were recorded with a potassium gluconate intrapipette solution containing (in mM): K$^+$ gluconate 140, EGTA 10, HEPES 10, ATP (Mg^{2+} salt) 4, GTP (Tris salt) 0.2 (pH 7.35). Miniature IPSCs (mIPSCs) were recorded with a caesium gluconate intrapipette solution which contained (in mM): Cs$^+$ gluconate 117.5, NaCl 10, MgCl$_2$ 4, HEPES 15, EGTA 5, ATP (Mg^{2+} salt) 2, GTP (Tris salt) 0.2 (pH 7.35). When recording mIPSCs, 0.5 µM tetrodotoxin (TTX) and 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were added to the extracellular medium in order to block respectively fast sodium spikes and glutamatergic non-NMDA EPSCs. All drugs were purchased from RBI/Sigma (St Louis, MO) except TTX (Alomone Labs, Jerusalem, Israel). mIPSCs were analysed with Mini Analysis software by Synaptosoft Inc. (Leonia, NJ). Data are expressed as mean±standard error of the mean (SEM). Significance levels are expressed as: $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

3. Results

3.1. Clozapine inhibits GABAergic autaptic IPSCs

To determine whether clozapine affects GABAergic synaptic transmission in VTA interneurones, autaptic synaptic responses were recorded in single neurone micro-dot cultures. GABAergic autaptic responses were triggered by a 1.5 ms depolarising step to +30 mV from a holding potential between −50 and −40 mV. This elicited a fast inward sodium action current followed by a slow outward GABA$_A$ receptor-mediated IPSC [Fig. 1(A)]. These IPSCs were GABAergic because they were abolished by SR-95531 (2 µM), a specific GABA$_A$ receptor antagonist [Fig. 1(A)]. Clozapine was found to have a dose-dependent inhibitory action on IPSCs starting from a threshold concentration of 1 µM (11.8±2.1%
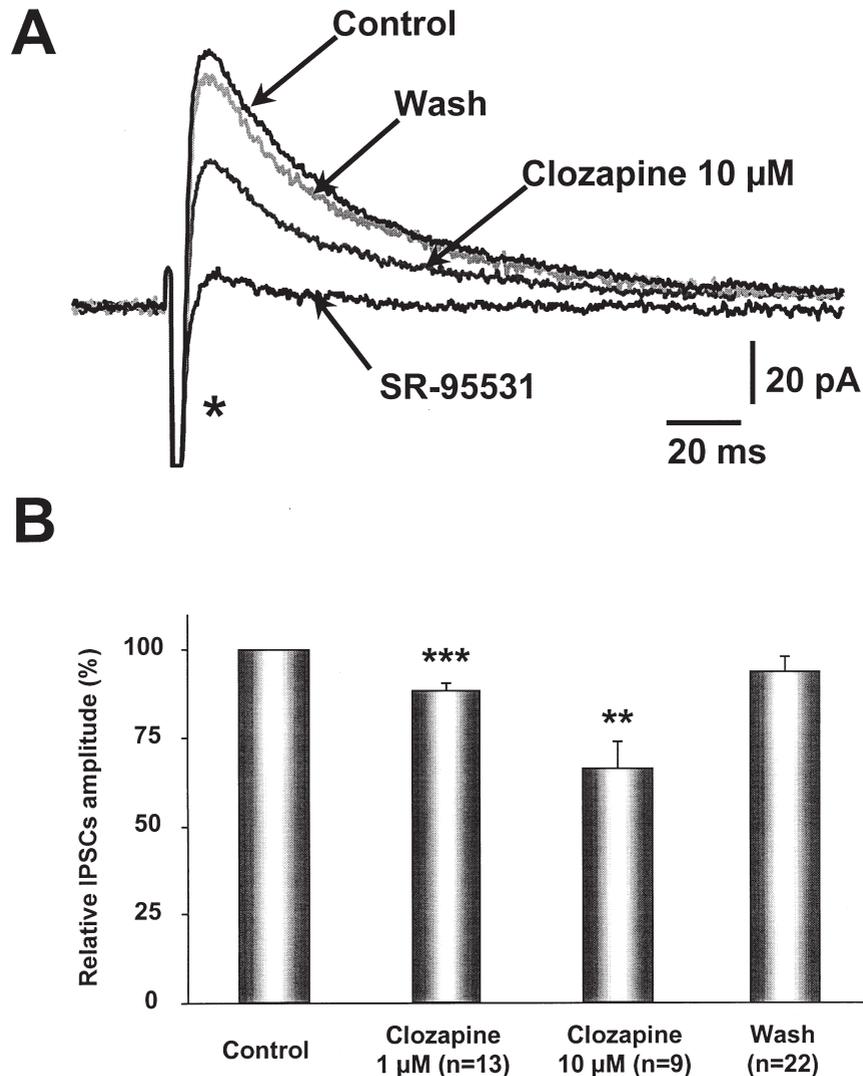
Fig. 1. Reduction of GABA-mediated synaptic currents by clozapine. (A) Recording of an autaptic GABA-mediated synaptic current evoked by a step depolarisation to +30 mV for 1.5 ms. The neurone was voltage-clamped at −40 mV. Clozapine (10 μM) caused a clear and reversible (*Wash*) decrease in IPSC amplitude. The outward current was almost totally abolished by 2 μM SR-95531, a specific GABA$_A$ receptor antagonist. Note that the initial inward current (*) is a truncated sodium action current. The recordings shown are five-trace averages. (B) Summary data showing the reduction of autaptic IPSC amplitudes by 1 and 10 μM clozapine and the reversal of these effects by wash. The number of experiments is indicated in parentheses for each column. Bars represent the mean±SEM (paired *t*-tests were used for statistical comparisons).

inhibition; $n=13$; $P<0.001$). At 10 μM, IPSCs were decreased by 33.9±7.6% ($n=9$; $P<0.01$) [Fig. 1(A) and (B)]. The effect was reversible by wash [Fig. 1(A) and (B)]. At a concentration of 50 μM and higher we found that clozapine appeared to interfere with action potential generation, which could suggest a depolarising action or an interaction with voltage-dependent Na$^+$ channels (results not shown).

3.2. Clozapine inhibits GABAergic receptors

The inhibition of IPSCs by clozapine could result from a number of possible mechanisms, either pre- or post-synaptic, or both. To determine whether, as predicted by binding studies, a direct block of GABA$_A$

receptors is involved, we tested the effect of clozapine on membrane currents activated by local applications of exogenous GABA on the cell bodies of VTA neurones. GABA was pressure-ejected at 500 μM on to patch-clamped neurones. A clear antagonism of functional GABA responses was detected [Fig. 2(A)]. At a concentration of 10 μM, clozapine reversibly decreased GABA responses by 28.4±6% ($n=18$; $P<0.01$) [individual means were compared using a Bonfferoni post hoc test after a one-way analysis of variance (ANOVA) ($F(4,56)=13.9$; $P<0.001$)] [Fig. 2(A) and (B)]. A maximal effect was reached at 50 μM with a 48.8±7.3% inhibition [Fig. 2(B)]. The IC$_{50}$ was found to be 8.2 μM [Fig. 2(B)]. Because the inhibition of the autaptic IPSCs and the membrane currents evoked by direct applications of
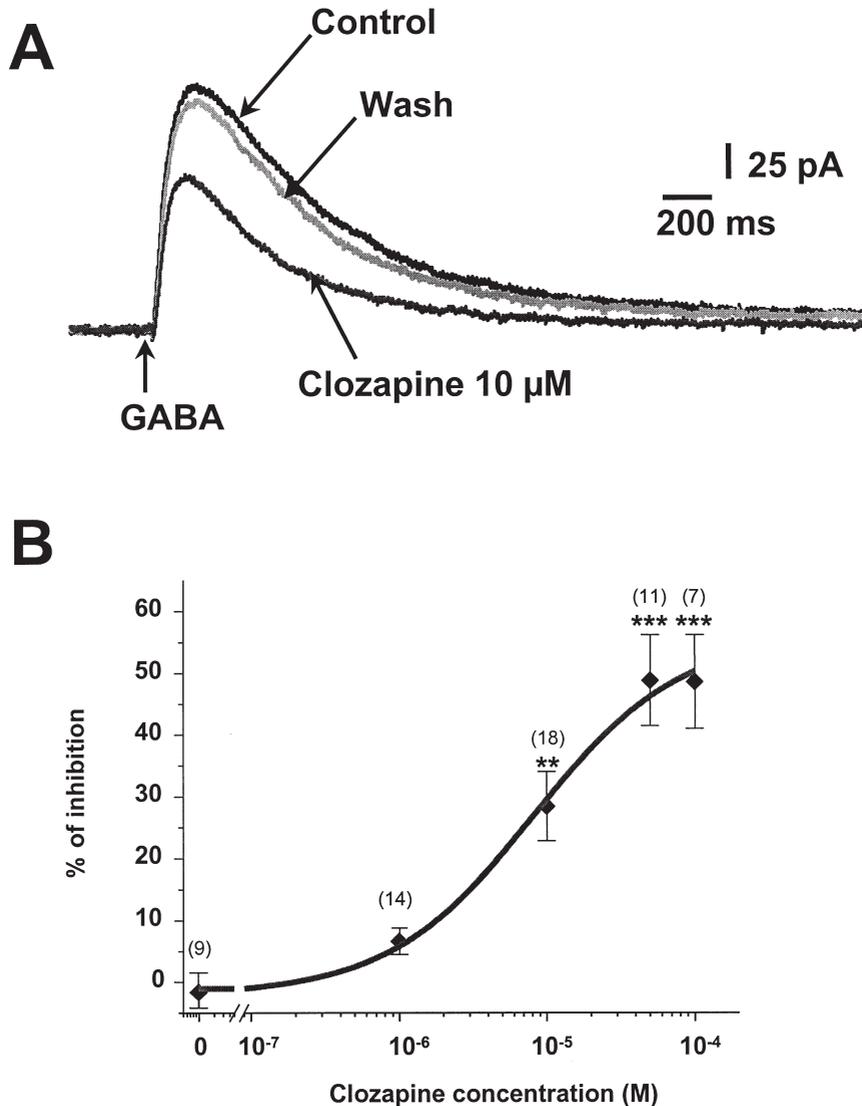
Fig. 2. Clozapine reduces exogenous GABA-mediated currents. (A) Outward currents evoked by direct application of exogenous GABA (500 µM) by pressure ejection from a local pipette (3.5 ms pulse). The cell was voltage-clamped at -40 mV. In this cell, the outward GABA current was inhibited by 33% by clozapine (10 µM). This effect was completely reversible. Traces are averages of five. (B) Dose–response curve of the antagonism of exogenous GABA-mediated currents by clozapine. The IC_{50} was 8.2 µM and corresponds to an inhibition of 27.5%. The number of experiments at each dose is indicated in parentheses. Bars represent mean±SEM.

GABA were quantitatively similar, these results are compatible with the idea that clozapine acts at GABAergic synapses through a post-synaptic mechanism.

3.3. Clozapine acts on mIPSCs similarly to SR-95531

Because somatic and synaptic $GABA_A$ receptors could possibly have different pharmacological properties (Brickley et al., 1999), we tested the effect of clozapine on the amplitude and frequency of miniature inhibitory post-synaptic currents (mIPSCs) in standard VTA cultures. This allowed us to determine more directly whether $GABA_A$ receptors located at the synapse can be blocked by clozapine and to exclude a pre-synaptic site of action. These events were recorded in the presence of

TTX in order to block sodium action potentials and isolate quantal events. We predicted that if clozapine acts through a post-synaptic mechanism, it should decrease the amplitude of mIPSCs. Such experiments are complicated by the fact that as events decrease in amplitude they will have a tendency to fall below the detection threshold, therefore giving the appearance of a decrease in mIPSC frequency and leading to an underestimation of the decrease in event amplitudes. In order to control for such a problem we have plotted cumulative probability distributions of mIPSC amplitudes before, during and after application of clozapine. This method allows one to compare synaptic event amplitude distributions directly, irrespective of the absolute change in event frequency. In addition, we compared the effect of clozapine

with that of SR-95531, a specific GABA$_A$ receptor antagonist. The latter was used at a concentration (100 nM) sufficient to produce a block of 51.1±9.6% of autaptic IPSC amplitudes [Fig. 3(C)]. We found that, at 10 µM, clozapine caused a significant and reversible decrease in mIPSC amplitudes in two out of six neurones tested. The decrease in mIPSC amplitude was observed directly as a shift to the left of the cumulative probability distribution of mIPSCs for individual experiments (Kolmogorov–Smirnov two-sample test; $P<0.01$) [Fig. 3(A)]. At 50 µM a clear reduction in mIPSC amplitude was detected in seven out of seven neurones tested [Fig. 3(B)] ($P<0.001$; Kolmogorov–Smirnov two-sample test). However, in two neurones the effect was not fully reversible. In these same neurones, a quantitatively similar decrease in mIPSC amplitudes was caused by the GABA$_A$ receptor antagonist SR-95531 (100 nM) [Fig. 3(B)] ($P<0.001$; Kolmogorov–Smirnov two-sample test). These observations confirm that clozapine acts to antagonise not only somatic but also synaptic GABA$_A$ receptors in our preparation.
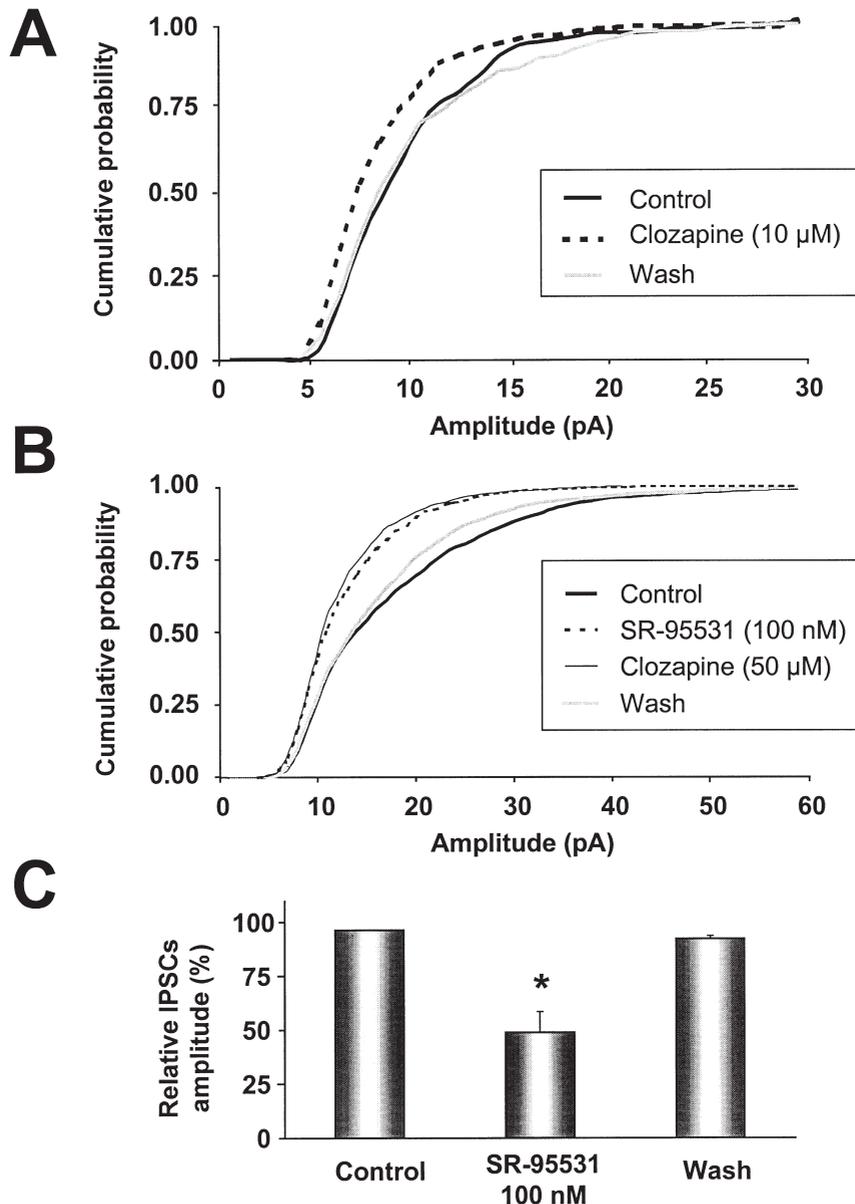
Fig. 3. Clozapine acts similarly to a GABA$_A$ receptor antagonist on mIPSCs. (A) Miniature GABAergic IPSCs (mIPSC) were recorded from standard VTA cultures in the presence of TTX (0.5 µM) and CNQX (10 µM). Clozapine (10 µM) caused a clear and reversible shift to the left of the cumulative probability distribution of mIPSC amplitudes recorded from a VTA neurone, thus revealing a reduction in mIPSC amplitudes. The experimental distribution (*Clozapine*) was significantly different from that of the control ($P<0.02$; Kolmogorov–Smirnov test). (B) Clozapine (50 µM) and the GABA$_A$ receptor antagonist SR-95531 (100 nM) caused a quantitatively similar shift to the left of the mIPSC amplitude distribution. Data are from one representative neurone. Both experimental distributions (*Clozapine* and *SR-95531*) were significantly different from that of the control ($P<0.0001$; Kolmogorov–Smirnov test). (C) At a concentration of 100 nM, SR-95531 caused a 51.1±9.6% decrease in the amplitude of autaptic GABAergic IPSCs ($n=5$). Bars represent mean±SEM.

4. Discussion

The results presented herein provide direct physiological evidence for an action of clozapine at $GABA_A$ receptors located on VTA neurones. An inhibitory effect of clozapine on GABAergic synaptic currents (Fig. 1) and membrane currents evoked by direct applications of GABA (Fig. 2) was detectable from a threshold concentration of 1 μM and was maximal at concentrations reaching 50 μM. An analysis of miniature synaptic currents also suggests that the action of clozapine closely resembles that of SR-95531, a specific $GABA_A$ receptor antagonist (Fig. 3). Taken together, our results suggest that clozapine can act as an antagonist at both synaptic and extrasynaptic $GABA_A$ receptors on VTA neurones.

At face value, our observation that clozapine appears to be less effective at reducing the amplitude of mIPSCs (two out of six neurones) than GABA-mediated membrane currents might be taken as evidence for a heterogeneity in the sensitivity of synaptic and extrasynaptic $GABA_A$ receptors for clozapine. This is not impossible theoretically, because synaptic and extrasynaptic $GABA_A$ receptors have been shown to display certain differences (Brickley et al., 1999) and a previous binding study has suggested that the affinity of clozapine for the $GABA_A$ receptor is dependent on the subunit composition of the receptor (Korpi et al., 1995). However, in the present situation, it is likely that this difference simply results from the considerable variability in mIPSC amplitudes (from 4 to 50 pA). Distributions established from very large numbers of mIPSCs would be required to reliably detect changes in mIPSC amplitudes in the range of 20 to 30%. In favour of the idea that populations of synaptic and extrasynaptic $GABA_A$ receptors on VTA neurones display similar sensitivities towards clozapine, we find that IPSCs (Fig. 1) and GABA-mediated membrane currents (Fig. 2) are decreased to approximately the same extent by 10 μM clozapine.

In order to be relevant for the therapeutic effects of clozapine, such an antagonism of GABA receptors would need to occur at concentrations that are found in the brains of patients during treatment with this drug. Steady-state plasma concentrations of clozapine in humans have been reported to be between 200 ng/ml and 450 ng/ml (Olesen, 1998). This corresponds to between 0.8 μM and 1.8 μM. In rodents, it has been reported that brain concentrations of clozapine are 16 to 24 times higher than in plasma (Baldessarini et al., 1993; Weigmann et al., 1999). Taking into account various pharmacodynamics parameters, Weigmann and colleagues (1999) have compared human and rodent data on clozapine. From their work, it seems likely that clozapine concentrations in the human brain should also be much higher than in plasma, perhaps between 10 to 15 times. Similar conclusions have been reached for other psychoactive drugs such as antidepressants or the classical antipsychotic haloperidol (Altamura et al., 1987; Kornhuber et al., 1999). Concentrations of clozapine in the brain could thus reach between 8 and 27 μM. At such concentrations our data suggest that clozapine might produce a 25 to 35% decrease in GABAergic synaptic transmission in some brain areas. Nonetheless, it should be considered that the studies mentioned above provided determinations of total brain concentrations and not aqueous concentrations. It is thus possible that the concentration of clozapine available to interact with $GABA_A$ receptors will be somewhat lower than the total brain concentration.

Although our experiments do not allow us to conclude that $GABA_A$ receptors located on VTA neurones are especially sensitive to clozapine in comparison to GABAergic neurones elsewhere in the brain, our data nonetheless lead to the prediction that under conditions where clozapine reaches concentrations approaching 10 μM in the brain, DA neurones of the VTA could be partially disinhibited because of a reduction in the efficacy of GABAergic synaptic inhibition. Clozapine has previously been shown to lead to an enhancement of the firing of VTA DA neurones, but this has usually been interpreted as resulting solely from the block of D2-type somatodendritic DA receptors on DA neurones (Hand et al., 1987). A partial contribution of $GABA_A$ receptor blockade to this effect might need to be considered.

Although our results do not allow us to predict directly the extent of antagonism of $GABA_A$ receptors by clozapine in patients receiving standard therapeutic doses, it remains a possibility that such an action on $GABA_A$ receptors could be implicated in the generation of seizures that have been reported in a small subset of patients after treatment at high doses or during rapid dose escalation (Hyde and Weinberger, 1997; Pacia and Devinsky, 1994). If a significant block of $GABA_A$ receptors also occurs in patients at standard therapeutic concentrations, the possibility that a reduction of GABA transmission by clozapine could be involved in its therapeutic actions should also be considered. Interestingly, it has been previously suggested that clozapine could potentially act through a "kindling" mechanism: it might fail to cause enhanced cellular excitability following initial dosages but, in analogy with kindling models using electrical stimulation, an initially sub-threshold dosage of clozapine might eventually come to cause "micro-seizures" after repeated administration (Stevens et al., 1997; see also George and Kulkarni, 1998; Minabe et al., 1998), possibly leading to long-term changes in neuronal pathways involved in schizophrenia. Recent work has shown that an acute application of clozapine to medial prefrontal cortex slices produced a marked facilitation of NMDA receptor-evoked responses (Arvanov et al., 1997). Additionally, Ossowska et al. (1999) demonstrated that chronic treatment with clozapine produces a long-term increase in NMDA receptor

expression. An interesting possibility is that opposite actions of clozapine at GABA$_A$ (antagonism) and NMDA receptors (facilitation) might synergistically influence the activity of various neuronal networks.

Acknowledgements

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