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Short communication

Effects of clozapine metabolites and chronic clozapine treatment on rat brain $GABA_A$ receptors

Garry Wong [a], Mikko Kuoppamäki [b], Jarmo Hietala [c], Hartmut Lüddens [d], Erkka Syvälahti [b], Esa R. Korpi [a,b,*]

[a] *Department of Alcohol Research, National Public Health Institute, Helsinki, Finland*
[b] *Department of Pharmacology and Clinical Pharmacology, University of Turku, FIN-20014 Turku, Finland*
[c] *Department of Psychiatry, Turku University Central Hospital, Turku, Finland*
[d] *Clinical Research Group, University of Mainz, Mainz, Germany*

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Abstract

Similarly to clozapine, a clozapine metabolite, *N*-desmethylclozapine, but not clozapine *N*-oxide, antagonized brain γ-aminobutyric acid type A ($GABA_A$) receptors at high micromolar concentrations. However, daily subcutaneous injections of clozapine (10 and 25 mg/kg) and haloperidol (0.5 mg/kg) for 14 days failed to alter the modulation by GABA of rat cerebrocortical and cerebellar benzodiazepine ([^{3}H]flunitrazepam) or convulsant (*t*-[^{35}S]bicyclophosphorothionate) binding sites of the $GABA_A$ receptor. The results thus suggest that the $GABA_A$ receptor antagonism exerted by chronic in vivo clozapine treatment is weak as compared to this treatment's actions on certain monoamine receptors and is unlikely to be involved in the therapeutic actions of clozapine.

Keywords: Clozapine; Clozapine metabolite; GABA (γ-aminobutyric acid); $GABA_A$ receptor

1. Introduction

Studies to elucidate the pharmacological basis for the therapeutic actions of typical and atypical antipsychotic drugs have focused on interactions with dopamine and serotonin receptors and their associated subtypes (Meltzer et al., 1989). It has been suggested that the various clinical profiles of antipsychotic drugs are interconnected with their actions at these receptor systems (Canton et al., 1990; Kuoppamäki et al., 1993). On the other hand, several lines of evidence have implicated altered function of γ-aminobutyric acid (GABA) neurons in the differing side-effect profiles of typical and atypical antipsychotic drugs (see Scheel-Krüger, 1986). Chronic treatment with neuroleptic drugs has been shown to decrease the turnover rate of GABA in some brain regions and increase it in others (Marco et al., 1978). For instance, chronic haloperidol, but not the atypical antipsychotic drug, clozapine, has been shown to increase the density of GABA sites and the GABA sensitivity selectively in substantia nigra and globus pallidus (Gale, 1980; Huffman and Ticku, 1983; Frey et al., 1987, 1989). These findings are consistent with the idea that neuroleptics may secondarily alter GABAergic neurotransmission by primary actions on monoaminergic activity.

Clozapine treatment produces fewer extrapyramidal side effects, but is more often associated with seizures (Pacia and Devinsky, 1994). We have recently shown that clozapine acts as an antagonist directly on the $GABA_A$ receptor in a receptor-subtype-dependent manner, more efficiently in forebrain than in cerebellar receptor subtypes (Korpi et al., 1995). In the present study, we investigated the actions of chronic clozapine treatment on cerebrocortical and cerebellar $GABA_A$ receptors. We used a chronic dosing regimen already shown to decrease the density of 5-HT_{2A} and 5-HT_{2C} receptors (Kuoppamäki et al., 1995). Since the exact mechanism of the clozapine antagonism of the $GABA_A$ receptor is not known (see Korpi et al., 1995), we used two functional neurochemical tests to monitor the coupling between the transmitter site and allosteric benzodiazepine and convulsant sites. In addition, we evaluated the in vitro effects of the clozapine metabolites, clozapine

* Corresponding author at address b. Tel.: (358-21) 333-7542; Fax: (358-21) 333-7216; e-mail: esa.korpi@utu.fi

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N-oxide and *N*-desmethylclozapine, to determine how these might contribute to the actions of clozapine at $GABA_A$ receptors.

2. Materials and methods

2.1. Materials

t-[^{35}S]Butylbicyclophosphorothionate ([^{35}S]TBPS) and [^{3}H]flunitrazepam were purchased from DuPont de Nemours (NEN Division, Dreieich, Germany). Picrotoxinin and γ-aminobutyric acid (GABA) were purchased from Sigma (St. Louis, MO, USA). Ro 15-1788 was obtained from F. Hoffmann-La Roche (Basel, Switzerland). Clozapine, *N*-desmethylclozapine and clozapine *N*-oxide were donated from Sandoz Research Institute (Berne, Switzerland). Haloperidol was purchased from Orion Pharmaceutica (Espoo, Finland).

2.2. Animals and drug treatments

Adult, male Sprague-Dawley rats (240–280 g) were used. The rats were housed 3–4 per cage under a 12/12 h light/dark cycle (lights on at 6:00 a.m.) at an ambient temperature of 21°C and a relative humidity of 55 ± 5%. The rats had free access to standard pellet food and water. Saline, clozapine (10–25 mg/kg; Leponex, Sandoz Pharma, Basle, Switzerland), and haloperidol (0.5 mg/kg; Serenase, Orion Pharmaceutica, Espoo, Finland) were given s.c. once a day for 14 days (Kuoppamäki et al., 1994). The rats were decapitated 68 h after the last dose, and occipital cortical and cerebellar tissues were dissected, frozen on dry ice, and stored at −80°C until preparation of membranes for binding assays. This kind of clozapine treatment produces a dose-dependent decrease in cerebrocortical 5-HT_{2A} receptor density and in choroid plexus 5-HT_{2C} receptor density, while the haloperidol treatment induces a 30% up-regulation of striatal dopamine D_2 receptors (Kuoppamäki et al., 1993, 1994, 1995). Two other groups of rats were treated with clozapine (25 mg/kg per day, s.c.) or saline, respectively, for 14 days, killed 8 days after the last injection when the occipital cortices were dissected for binding assays.

Cerebral cortical, hippocampal and cerebellar tissues from adult male Wistar rats (Department of Laboratory Animals, University of Helsinki, Helsinki, Finland) were used in experiments with clozapine metabolites as described in detail by Korpi et al. (1995).

2.3. Ligand binding assays

For each membrane preparation, the brain tissues were homogenized with a Polytron homogenizer in 50 vols. of ice-cold 50 mM Tris-citrate buffer, pH 7.4, supplemented with 1 mM EDTA and were centrifuged at 20 000 × *g* for 20 min (Korpi et al., 1995). The pellets were resuspended in the same buffer and recentrifuged five times. The final suspension was prepared in 50 mM Tris-citrate buffer, pH 7.4, divided into aliquots, and stored frozen at −80°C.

Frozen membranes were thawed, resuspended, and centrifuged once before final resuspension in 50 mM Tris-citrate buffer to a protein concentration of 100–240 μg/ml (Bio-Rad protein assay kit), in a total volume of 0.5 ml/assay tube. After defined incubation times with dupli-

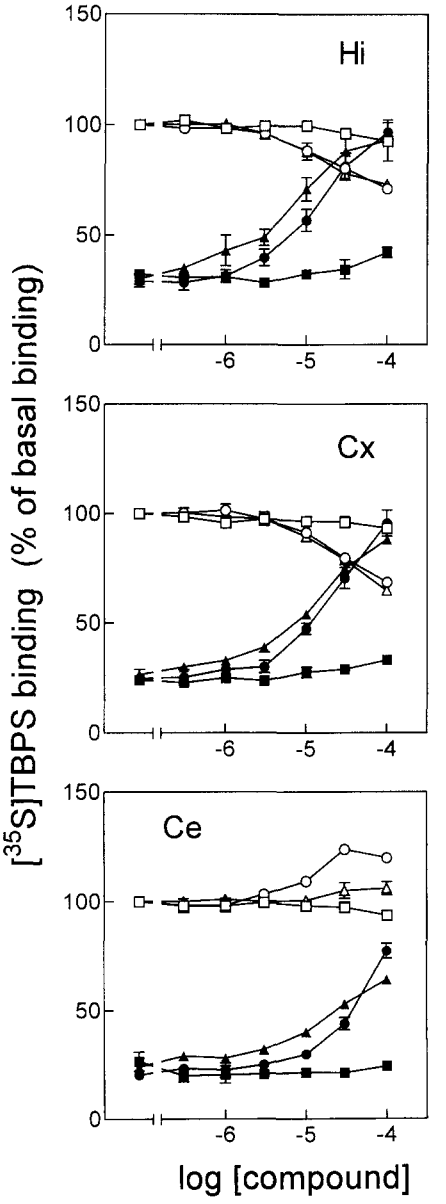

Fig. 1. Effects of clozapine (△, ▲), *N*-desmethylclozapine (○, ●) and clozapine *N*-oxide (□, ■) on picrotoxinin-sensitive [^{35}S]TBPS binding in various brain regions. [^{35}S]TBPS binding without (open symbols) and with (closed symbols) 5 μM exogenous GABA are shown in hippocampal (Hi), cerebrocortical (Cx), and cerebellar (Ce) membranes. Results are expressed as percentages (means ± S.E.M., $n = 3$) of basal binding determined in the absence of added GABA (= 100%). The values to the left of the gaps are in the absence of the drugs. The data for clozapine are from Korpi et al. (1995).

cate samples, bound and free ligands were separated by rapid filtration of the membranes onto Whatman GF/B glass fiber filters. The samples were rinsed twice with 5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. Air-dried filters were immersed in 4 ml of scintillation fluid (Optiphase HiSafe 2, Wallac, Turku, Finland), and radioactivity was determined in a Wallac 1410 scintillation counter.

[^{35}S]TBPS (2 nM) binding was determined during a 90 min incubation at 22°C in 50 mM Tris-citrate buffer supplemented with 200 mM NaCl as previously described (Korpi et al., 1995). Non-specific binding was determined with 20 μM picrotoxinin. The clozapine metabolites, *N*-desmethylclozapine and clozapine *N*-oxide, were solubilized in ethanol, diluted in assay buffer and used with or without 5 μM GABA.

[^{3}H]Flunitrazepam (1 nM) binding was determined after a 60 min incubation in an ice-water bath with 50 mM Tris-HCl buffer, pH 7.4, supplemented with 100 mM NaCl. Non-specific binding was determined with 10 μM Ro15-1788. GABA (100 nM–1 mM) was added in experiments to evaluate the coupling of GABA sites to [^{3}H]flunitrazepam binding sites.

The data were analyzed using the Prism program (GraphPad Software, San Diego, CA, USA) to calculate the best-fitting values for maximal stimulation, EC_{50}, IC_{50} and slope values for GABA-modulated [^{35}S]TBPS and [^{3}H]flunitrazepam binding.

3. Results

Micromolar concentrations of the clozapine metabolite, *N*-desmethylclozapine, inhibited picrotoxinin-sensitive [^{35}S]TBPS binding in the absence of exogenous GABA in rat hippocampal and cerebrocortical membranes, but increased it in the cerebellar membranes. Clozapine *N*-oxide was inactive (Fig. 1). The inhibition of [^{35}S]TBPS binding produced by 5 μM GABA was completely reversed by *N*-desmethylclozapine (100 μM) in the hippocampal and cerebrocortical membranes and almost completely so in the cerebellar membranes. Clozapine *N*-oxide was again inactive. Clozapine produced a GABA receptor antagonism very similar to that by its *N*-desmethyl metabolite (Fig. 1).

The coupling of GABA to [^{3}H]flunitrazepam and [^{35}S]TBPS binding sites in cerebral cortex and cerebellum was studied in rats chronically treated with saline, clozapine and haloperidol. No significant differences were observed in maximal stimulation and EC_{50} for GABA in [^{3}H]flunitrazepam binding or in maximal stimulation and

Table 1
Effects of chronic clozapine and haloperidol treatments on the coupling of GABA sites to [^{3}H]flunitrazepam and [^{35}S]TBPS binding sites

Brain region/ treatment (dose)	Basal binding (fmol/mg protein)	Maximal stimulation (% of basal binding)	EC_{50}, IC_{50} (μM)	Slope for modulation
GABA coupling to [^{3}H]flunitrazepam sites				
Occipital cortex				
Control (saline)	876 ± 15	168 ± 6	1.7 ± 0.3	1.11 ± 0.14
Clozapine (10 mg/kg)	994 ± 20	168 ± 2	2.3 ± 0.2	1.05 ± 0.07
Clozapine (25 mg/kg)	922 ± 46	171 ± 3	1.5 ± 0.1	0.94 ± 0.04
Haloperidol (0.5 mg/kg)	978 ± 51	166 ± 5	1.6 ± 0.2	1.02 ± 0.04
Cerebellum				
Control (saline)	325 ± 47	183 ± 1	1.0 ± 0.1	0.93 ± 0.03
Clozapine (10 mg/kg)	333 ± 32	178 ± 10	1.2 ± 0.3	0.92 ± 0.05
Clozapine (25 mg/kg)	292 ± 75	178 ± 12	1.0 ± 0.1	1.01 ± 0.16
Haloperidol (0.5 mg/kg)	321 ± 30	225 ± 24	0.8 ± 0.2	0.76 ± 0.15
GABA coupling to [^{35}S]TBPS sites				
Occipital cortex				
Control (saline)	219 ± 4	116 ± 2	3.8 ± 0.4	−1.7 ± 0.08
Clozapine (10 mg/kg)	237 ± 3	116 ± 1	4.3 ± 0.1	−1.6 ± 0.09
Clozapine (25 mg/kg)	253 ± 8 [a]	115 ± 1	3.8 ± 0.1	−1.7 ± 0.06
Haloperidol (0.5 mg/kg)	244 ± 7	115 ± 1	3.5 ± 0.2	−1.8 ± 0.14
Cerebellum				
Control (saline)	218 ± 10	103 ± 2	2.4 ± 0.1	−2.0 ± 0.27
Clozapine (10 mg/kg)	176 ± 5 [a]	106 ± 1	2.5 ± 0.1	−2.7 ± 0.80
Clozapine (25 mg/kg)	212 ± 5	103 ± 1	2.4 ± 0.1	−2.0 ± 0.19
Haloperidol (0.5 mg/kg)	194 ± 12	106 ± 1	2.4 ± 0.1	−2.0 ± 0.15

Values are means ± S.E.M. for three samples, each pooled from two rats. The estimates for stimulation (EC_{50}) and inhibition (IC_{50}) of [^{3}H]flunitrazepam and [^{35}S]TBPS binding, respectively, are given. Statistically significant differences between the groups (ANOVA) were only observed for the basal binding values of [^{35}S]TBPS (occipital cortex: $F(3,11) = 6.059$, $P = 0.0187$; cerebellum: $F(3,11) = 4.543$, $P = 0.0386$). [a] $P < 0.05$ for the difference from the control value (Tukey-Kramer test).

IC_{50} for GABA in [^{35}S]TBPS binding (Table 1). Also, the slopes for stimulation and inhibition were unaltered by the chronic drug treatments. The only differences from the control values regarded basal [^{35}S]TBPS binding, which was slightly elevated in the cortex and diminished in the cerebellum by high and low clozapine doses, respectively. The potency of GABA was higher in the cerebellum than in the cortex.

Since Coward et al. (1989) have suggested that supersensitivity of GABAergic mechanisms after chronic clozapine treatment might develop after a washout period, we also determined the transmitter modulation of $GABA_A$ receptors of the cerebrocortical membranes prepared from rats withdrawn for 8 days from chronic clozapine (25 mg/kg, s.c. once daily for 14 days). No differences were detected between the saline- and clozapine-treated groups for the various parameters (maximal stimulation, EC_{50} or IC_{50} for GABA, and slopes) for the [^{3}H]flunitrazepam and [^{35}S]TBPS binding assays nor in the basal binding values (data not shown).

4. Discussion

Differences between the cerebral cortex and cerebellum regarding the potency of GABA modulation of benzodiazepine and convulsant binding to the $GABA_A$ receptors are probably due to the different receptor subtypes in these regions, which are determined by their subunit composition (Korpi and Lüddens, 1993). Similar receptor subtype selectivity apparently explains why clozapine (Korpi et al., 1995) and its *N*-desmethyl metabolite were less efficient antagonists at the cerebellar than at the cerebrocortical and hippocampal $GABA_A$ receptors. Both clozapine and *N*-desmethylclozapine require high micromolar concentrations to affect $GABA_A$ receptors (Korpi et al., 1995; the present study), while they are 1000-fold more potent at certain dopamine and 5-HT receptor subtypes (Canton et al., 1990; Kuoppamäki et al., 1993). The other main clozapine metabolite in man, clozapine *N*-oxide (Gauch and Michaelis, 1971), was completely devoid of activity. Therefore, our present data support the view that the therapeutic actions of clozapine treatment are not mediated by direct antagonism of $GABA_A$ receptors.

In the present study, we observed that the coupling between GABA and its associated benzodiazepine and convulsant (chloride-ion channel) binding sites was not changed following chronic typical (haloperidol) and atypical (clozapine) antipsychotic drug treatment. Provided the GABA site coupling is an appropriate measure of $GABA_A$ receptor function and adaptable to chronic agonist and antagonist treatments (Gallager et al., 1984; Wong et al., 1994), then our results rule out any significant direct antagonism of the $GABA_A$ receptors in vivo by the antipsychotic drugs. Our results could be seen in contrast to previous reports, which have demonstrated that chronic neuroleptic treatment leads to profound changes in GABA site density (Gale, 1980; Huffman and Ticku, 1983; Frey et al., 1987, 1989). This, however, seems to depend not only upon the type of antipsychotic drug used (haloperidol active vs. clozapine inactive) but also on the brain region studied, as changes are detectable in substantia nigra reticulata and globus pallidus, but not in other regions. The alterations we now observed in basal [^{35}S]TBPS binding can be considered as minor, since no differences were observed between clozapine and haloperidol nor between the two clozapine doses.

Taken together, our results suggest that the direct $GABA_A$ receptor antagonism is unlikely to be an important aspect of the therapeutic actions of clozapine. But it still cannot be excluded that this mechanism contributes to the pathophysiology of seizures associated with clozapine treatment at high doses and rapid dose escalations (Pacia and Devinsky, 1994).

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References

Canton, H., L. Verrië and F.C. Colpaert, 1990, Binding of typical and atypical antipsychotics to 5-HT_{1C} and 5-HT_2 sites: clozapine potently interacts with 5-HT_{1C} sites, Eur. J. Pharmacol. 191, 93.

Coward, D.M., A. Imperato, S. Urwyler and T.G. White, 1989, Biochemical and behavioral properties of clozapine, Psychopharmacology 99, S6.

Frey, J.M., M.K. Ticku and R.D. Huffman, 1987, GABAergic supersensitivity within the pars reticulata of the rat substantia nigra following chronic haloperidol treatment, Brain Res. 425, 73.

Frey, J.M., M.K. Ticku, R.D. Bell and R.D. Huffman, 1989, Chronic haloperidol administration increases GABA binding and enhances neuronal responsiveness to iontophoresed GABA in rat globus pallidus, Brain Res. 491, 57.

Gale, K., 1980, Chronic blockade of dopamine receptors by antischizophrenic drugs enhances GABA binding in substantia nigra, Nature 283, 569.

Gallager, D.W., J.M. Lakoski, S.F. Gonsalves and S.L. Rauch, 1984, Chronic benzodiazepine treatment decreases postsynaptic GABA sensitivity, Nature 308, 74.

Gauch, R. and W. Michaelis, 1971, The metabolism of 8-chloro-11-(4-methyl-1-piperazinyl)5*H*-dibenzo[*b*,*e*][1,4]diazepine (clozapine) in mice, dogs and human subjects, Farmaco 26, 667.

Huffman, R.D. and M.K. Ticku, 1983, The effects of chronic haloperidol administration on GABA receptor binding, Pharmacol. Biochem. Behav. 19, 199.

Korpi, E.R. and H. Lüddens, 1993, Regional γ-aminobutyric acid sensitivity of *t*-butylbicyclo[^{35}S]phosphorothionate binding depends on γ-aminobutyric $acid_A$ receptor α subunit, Mol. Pharmacol. 44, 87.

Korpi, E.R., G. Wong and H. Lüddens, 1995, Subtype specificity of γ-aminobutyric acid type A receptor antagonism by clozapine, Naunyn-Schmiedeberg's Arch. Pharmacol. 352, 365.

Kuoppamäki, M., E. Syvälahti and J. Hietala, 1993, Clozapine and

N-desmethylchlozapine are potent 5-HT_{1C} receptor antagonists, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 245, 179.

Kuoppamäki, M., E.-P. Pälvimäki, E. Syvälahti and J. Hietala, 1994, 5-HT_{1C} receptor-mediated phosphoinositide hydrolysis in the rat choroid plexus after chronic treatment with clozapine, Eur. J. Pharmacol. 255, 91.

Kuoppamäki, M., E.-P. Pälvimäki, J. Hietala and E. Syvälahti, 1995, Differential regulation of rat 5-HT_{2A} and 5-HT_{2C} receptors after chronic treatment with clozapine, chlorpromazine and three putative atypical antipsychotic drugs, Neuropsychopharmacology 13, 139.

Marco, E., C.C. Mao, A. Revuelta, E. Peralta and E. Costa, 1978, Turnover rates of γ-aminobutyric acid in substantia nigra, N. caudatus, globus pallidus and N. accumbens of rats injected with cataleptogenic and non-cataleptogenic antipsychotics, Neuropharmacology 17, 589.

Meltzer, H.Y., S. Matsubara and J.-C. Lee, 1989, Classification of typical and atypical antipsychotic drugs on the basis of dopamine D_1, D_2 and serotonin$_2$ pK_i values, J. Pharmacol. Exp. Ther. 251, 238.

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

Scheel-Krüger, J., 1986, Dopamine-GABA interactions: evidence that GABA transmits, modulates and mediates dopaminergic functions in the basal ganglia and the limbic system, Acta. Neurol. Scand. 73 (suppl. 107).

Wong, G., T. Lyon and P. Skolnick, 1994, Chronic exposure to benzodiazepine receptor ligands uncouples the γ-aminobutyric acid type A receptor in WSS-1 cells, Mol. Pharmacol. 46, 1056.