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Effects of acute and chronic clozapine on dopamine release and metabolism in the striatum and nucleus accumbens of conscious rats

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1 The effect of single and repeated (once daily for 23 days) oral doses of 20 and 60 mg kg^{-1} clozapine on dopamine release and metabolism were studied by intracerebral dialysis in the striatum and nucelus accumbens of conscious rats.

2 The basal output of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum and nucleus accumbens of rats given clozapine 20 or 60 mg kg^{-1} chronically, measured one day after the last drug dose, was not significantly different from that of vehicle-treated animals.

3 Challenge doses of 20 or 60 mg kg^{-1} clozapine produced similar increases in dopamine levels in the striatum and nucleus accumbens of animals which had received vehicle or clozapine 20 or 60 mg kg^{-1} once daily for 23 days, except that 1 h after administration 60 mg kg^{-1} clozapine had a greater effect in the nucleus accumbens.

4 In animals treated chronically with clozapine 20 and 60 mg kg^{-1} or vehicle, DOPAC levels in the striatum and nucleus accumbens were increased to the same extent by challenge doses of clozapine (20 or 60 mg kg^{-1}). In animals treated chronically with clozapine, a challenge dose of 60 mg kg^{-1} had significantly greater effect on HVA only in the nucleus accumbens.

5 When DOPAC and HVA were measured post mortem in the striatum and nucleus accumbens 2 h after various oral doses of clozapine, it was found that 10 mg kg^{-1} significantly increased dopamine metabolites only in the nucleus accumbens whereas 100 mg kg^{-1} had this effect in both regions. Clozapine, 30 mg kg^{-1} significantly raised DOPAC levels in both regions but HVA was elevated only in the nucleus accumbens.

6 There appeared to be no appreciable changes in dopamine release and metabolism nor any reduction in the effect of clozapine in the nucleus accumbens after chronic drug treatment. In fact the effect was greater in chronically treated rats, particularly in the nucleus accumbens of animals given 60 mg kg^{-1} clozapine.

7 It was confirmed that measurement of dopamine metabolites in post mortem tissue provides no valuable information on changes in the availability of synaptic dopamine.

Introduction

In 1983 Chiodo & Bunney reported that 21 days' treatment with various neuroleptics markedly reduced the number of active dopamine cells in A9 and A10, except for clozapine which affected only the A10 cells. White & Wang obtained similar results (1983). These findings were particularly interesting in view of the delayed onset of antipsychotic and extrapyramidal effects in patients treated chronically with neuroleptics (Cotes *et al.*, 1978; Beckman *et al.*, 1979) and because clozapine induces only limited extrapyramidal effects (Gerlach *et al.*, 1975), commonly attributed to changes in the nigrostriatal A9 dopamine cells (Baldessarini & Tarsy, 1976).

In the same study Chiodo & Bunney found that dopaminergic neurones in animals chronically treated with neuroleptics were in a state of tonic depolarization inactivation, a finding later confirmed by *in vivo* extracellular recording (Grace & Bunney, 1986); interestingly, dopaminergic neurones of the mesolimbic, but not the mesocortical system were affected, at least at the doses used.

On the basis of these findings it was suggested that, besides blockade of postsynaptic dopamine receptors in terminal regions, a drop in the synaptic availability of dopamine might be associated with the inactivation state and these combined effects could be responsible for the antipsychotic and extrapyramidal effects of chronic neuroleptics. Thus, one would expect chronically treated animals to have reduced dopamine release and/or to develop tolerance to the acute effects of clozapine in the nucleus accumbens, but not in the striatum, terminal regions respectively of A10 and A9 dopamine cells. Compatible with this hypothesis, a reduction of basal dopamine metabolism and development of tolerance to the acute effects of haloperidol, but not to clozapine, have been found in the striatum of chronically treated rats (Ladinsky *et al.*, 1980). Similar results were found with haloperidol on measuring dopamine release and metabolism by intracerebral dialysis (Bettini *et al.*, 1987).

A recent *in vivo* voltammetry study found no development of tolerance to the acute effects of clozapine on extracellular dihydroxyphenylacetic acid (DOPAC) in the striatum and nucleus accumbens of rats treated subcutaneously with 50 mg kg^{-1} for 21 days (Maidment & Marsden, 1987). *In vivo* dialysis studies have shown that changes in extracellular DOPAC do not necessarily reflect changes in dopamine release (Imperato & Di Chiara, 1984; 1985). Thus Maidment & Marsden's findings do not definitely exclude the possibility of changes in dopamine release in the nucleus accumbens resulting from depolarization inactivation of A10 cells in animals chronically treated with clozapine.

To gain information on this issue, we employed intracerebral dialysis to investigate the effects of acute and chronic treatment with clozapine at two doses on dopamine release and metabolism in the striatum and nucleus accumbens of rats.

A preferential effect of clozapine on A10 dopamine cells after acute treatment could not account for the chronic effect since a single dose (20 mg kg^{-1}) increased the number of spontaneously firing cells in A9 and A10 (Chiodo & Bunney, 1983). There is some disagreement on whether acute treatment with clozapine preferentially increases dopamine metabolism in limbic regions (Westerink & Korf, 1975; Waldmeier & Maitre

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1976a), although initial studies seemed to suggest this (Anden & Stock, 1973; Bartholini, 1976). Surprisingly, our preliminary experiments with 20 mg kg^{-1} clozapine indicated that the compound was more effective on striatal dopamine release, so in an additional experiment we measured the dose-response effect of clozapine on dopamine metabolism in post-mortem tissues to look for differences between the two measures.

Methods

Male Sprague-Dawley rats (CD-COBS Charles River, Italy) weighing 200–300 g (75–100 g for dialysis experiments at the beginning of chronic treatment) were used. The animals were housed at constant temperature (21 ± 1°C) and relative humidity (60%) under a regular light/dark schedule (light 07 h 00 min–19 h 00 min). Food and water were freely available.

Drug treatment

For microdialysis experiments, rats were given clozapine (Sandoz Basel, Switzerland) or vehicle orally once daily for 23 days. Clozapine was dissolved in 0.8% acetic acid and the pH of the solution was adjusted to 5 with 1 M NaOH. On day 23 a dialysis fibre was inserted, as described below, through the striatum and nucleus accumbens of rats and approximately 24 h after the last drug injection, once basal output of dopamine, homovanillic acid (HVA) and DOPAC had become constant, the animals were given 20 or 60 mg kg^{-1} clozapine orally and samples were collected every 20 min for 3 h.

In another experiment the rats were given 10, 30 and 100 mg kg^{-1} clozapine orally and were killed 2 h later by microwave irradiation focussed on the head (1.3 kW at 2.45 GHz for 5 s) from a commercial microwave unit adapted by Medical Engineering Consultants (Lexington, MA, U.S.A.).

Dialysis procedure

Rats were anaesthetized with 3 ml kg^{-1} Equithesin and placed on a stereotaxic apparatus (David Kopf Instruments) so dialysis fibres could be positioned in the striatum and nucleus accumbens. Stereotaxic coordinates were as follows: striatum: AP = +7890; H = +1.4 from zero plane; nucleus accumbens: AP = +8920; H = −1.2 from zero plane according to the Konig & Klippel atlas (1963). A copolymer of acrylonitrile-sodium methallyl sulphonate dialysis fibre (AN 69 Hospal SpA; 0.3 mm outer diameter, with more than 44,000 Daltons cut-off) was used. A short piece of the dialysis tube was covered with epoxy glue except for a zone in the middle (5 and 10 mm wide for the nucleus accumbens and striatum respectively).

The rat's skull was exposed and two holes were made on the lateral surface at the level of the head of the nucleus caudatus or nucleus accumbens. The dialysis fibre, held straight by a tungsten wire inside, was inserted transversely into the brain so that the middle glue-free zone was exactly in the target area. The tungsten wire was withdrawn and two stainless steel cannulae (22 gauge inner diameter, about 15 mm long) were glued to the ends of the fibre. These ends were bent up and fixed vertically to the skull with dental cement and modified Eppendorf tips. Finally, the skin was sutured and the rats were allowed to recover from anaesthesia for 24 h before the dopamine release study.

On the day of the experiment the rat was placed in a cage and one of the two steel cannulae was connected by polyethylene tubing to a 1 ml Hamilton syringe containing Ringer solution (composition (mM): NaCl 147, KCl 4, $CaCl_2$ 3.4 in distilled water, pH 6.1). The fibre was perfused at a constant flow rate of 2 μl min^{-1} with a CMA/100 microinjection pump (Carnegie Medicin, Stockholm, Sweden). Every 20 min, samples of perfusate containing dopamine and metabolites were collected in mini-vials containing 10 μl of 1 M perchloric acid and directly assayed by high performance liquid chromatography with electrochemical detection.

Placement of the dialysis fibres in the striatum and nucleus accumbens was verified by histological examination of fibre tracts.

Biochemical assays

Extracellular dopamine, DOPAC and HVA were separated through a 200 × 3 mm glass column (Chrompack, Middelburg, NL) with 5 μm reverse phase packing (Nucleosil, Mackerey, Nagel & Co.) using a mobile phase consisting of 0.1 M sodium acetate, 15% methanol, 50 mg l^{-1} sodium octyl sulphate, 0.1 mM disodium EDTA adjusted to pH 4.8 with concentrated acetic acid. A flow rate of 0.5 ml min^{-1} was maintained by a Gilson pump mod. 302.

Dopamine and its metabolites were measured by an electrochemical detector (Coulochem 5100 A, ESA, Bedford, MA) equipped with two in-series electrodes (mod. 5011, ESA, Bedford, MA). The first electrode was used to oxidize dopamine and its metabolites at +0.35 V, the second to reduce them at −0.15 V. DOPAC and HVA were read as first electrode output signal, dopamine as second electrode output signal. The method was sufficiently sensitive to detect about 25 fmol of dopamine (S/N = 2).

To measure HVA and DOPAC in brain tissues, the striatum and nucleus accumbens were dissected out according to Glowinski & Iversen (1966), frozen on dry-ice and stored at −20°C until assay. The tissues were homogenized by sonication in 500 μl of 0.1 M perchloric acid and centrifuged at 15,000 *g* for 20 min; 20 μl supernatant were directly injected into the high-performance liquid chromatograph equipped with an electrochemical detector (BAS, West Lafayette, IN, U.S.A.). HVA and DOPAC were separated by use of a mobile phase containing 0.1 M sodium acetate, 200 mg l^{-1} sodium hexyl sulphate, 11.5% methanol and 0.1 mM disodium EDTA. The pH of the solution was adjusted to 4.3 with concentrated acetic acid.

Statistics

Basal values of dopamine and its metabolites (mean of four 20 min samples of stable output before drug treatment) in animals repeatedly given clozapine or vehicle were compared by Student's *t* test for unpaired data. Basal values and values obtained at each time after clozapine were compared by one-way ANOVA (randomized-block design) followed by Dunnett's *t* test. A two-factor mixed design was used to compare the effects of acute and repeated clozapine treatment. The remaining data were anlaysed by one-way ANOVA and post-hoc comparisons with vehicle were made by Dunnett's *t* test.

Results

Effects of chronic clozapine on basal dopamine release and metabolism in the striatum and nucleus accumbens

Table 1 shows the basal output of dopamine, DOPAC and HVA expressed as the mean (pmol 20 min^{-1}) of four 20 min samples of stable output before drug treatment. The levels of dopamine and its metabolites in the striatum and nucleus accumbens of rats treated chronically with clozapine 20 or 60 mg kg^{-1} were not significantly different from those of vehicle-treated animals.

Effects of acute clozapine challenge on extracellular dopamine levels in chronically treated rats

As shown in Figure 1, administration of 20 mg kg^{-1} clozapine to animals treated chronically with the vehicle raised dopamine levels in the striatum and nucleus accumbens with peak

Table 1 Extracellular levels of dopamine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the striatum and nucleus accumbens of rats chronically treated with clozapine

	Dose ($mg\,kg^{-1}$)	*Dopamine*	*DOPAC* ($pmol\,20\,min^{-1}$)	*HVA*
Striatum				
Vehicle		0.643 ± 0.088	72.8 ± 14.7	64.1 ± 10.7
Clozapine	20.0	0.622 ± 0.062	83.5 ± 11.0	69.8 ± 4.9
Vehicle		0.652 ± 0.105	69.8 ± 4.9	51.4 ± 4.8
Clozapine	60.0	0.736 ± 0.120	85.7 ± 5.9	54.9 ± 5.1
Nucleus accumbens				
Vehicle		0.272 ± 0.054	86.2 ± 15.2	50.7 ± 10.8
Clozapine	20.0	0.210 ± 0.033	73.8 ± 8.6	35.5 ± 4.1
Vehicle		0.204 ± 0.020	72.8 ± 3.2	42.7 ± 21.6
Clozapine	60.0	0.230 ± 0.011	63.0 ± 10.4	38.3 ± 6.8

Rats received vehicle, 20 or $60\,mg\,kg^{-1}$ clozapine orally once daily for 23 days. Samples were collected 24 h after the last dose. Each value is the mean ± s.e.mean of 4–8 animals.

effect 1 h after injection, by 52% and 29% respectively. Sixty $mg\,kg^{-1}$ clozapine did not further elevate dopamine concentrations in the striatum whereas the increase in the nucleus accumbens was dose-dependent.

Dopamine levels in the striatum and nucleus accumbens of rats chronically treated with 20 or $60\,mg\,kg^{-1}$ clozapine were similar to those in vehicle-treated animals, except that $60\,mg\,kg^{-1}$ had a greater effect in the nucleus accumbens 1 h after administration.

Effects of acute clozapine challenge on extracellular DOPAC and HVA levels in chronically treated rats

The effects of clozapine on extracellular DOPAC and HVA concentrations are shown in Figures 2 and 3. Doses of 20 and $60\,mg\,kg^{-1}$ clozapine in vehicle-treated animals dose-dependently raised extracellular DOPAC and HVA in the nucleus accumbens, whereas only $60\,mg\,kg^{-1}$ produced a statistically significant increase of dopamine metabolites in the striatum. DOPAC levels in the striatum and nucleus accumbens of rats treated chronically with 20 and $60\,mg\,kg^{-1}$ clozapine were not different from vehicle-treated animals. In animals chronically treated with clozapine the $20\,mg\,kg^{-1}$

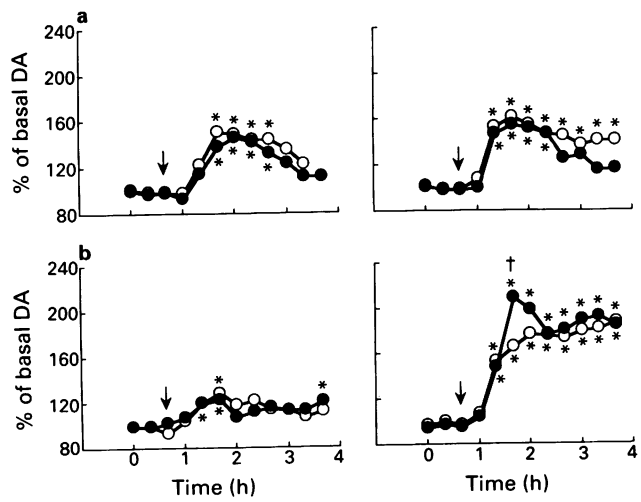

Figure 1 Effect of chronic oral doses (once daily for 23 days) of 20 (left) and 60 (right) $mg\,kg^{-1}$ clozapine on dopamine (DA) release in the striatum (a) and nucleus accumbens (b) of conscious rats. Approximately 24 h after the last dose, another oral dose of 20 or $60\,mg\,kg^{-1}$ clozapine was given and samples were collected for the next 3 h. Each value is the mean of 4–8 rats. Variability between samples did not exceed 29%. (○) Chronic vehicle and (●) clozapine. The arrow indicates the time at which the drug was administered. Statistical analysis was done on actual data. * $P < 0.05$ vs. basal values; † $P < 0.05$ chronic vehicle vs. chronic clozapine.

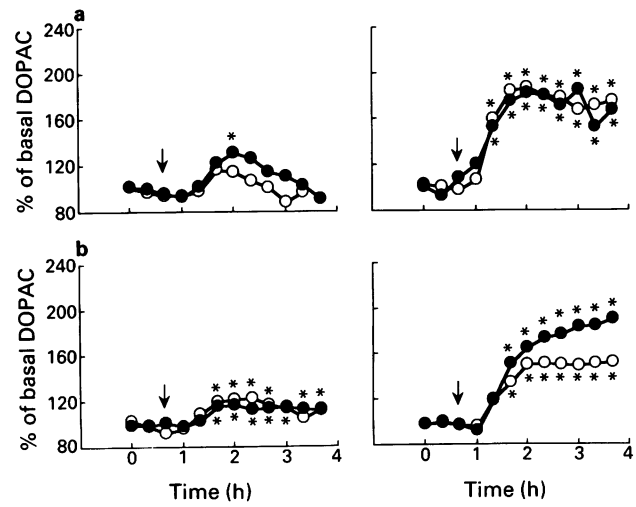

Figure 2 Effect of chronic oral doses (once daily for 23 days) of 20 (left) and 60 (right) $mg\,kg^{-1}$ clozapine on dihydroxyphenylacetic acid (DOPAC) levels in the striatum (a) and nucleus accumbens (b) of conscious rats. See Figure 1 for other details. * $P < 0.05$ vs. basal values.

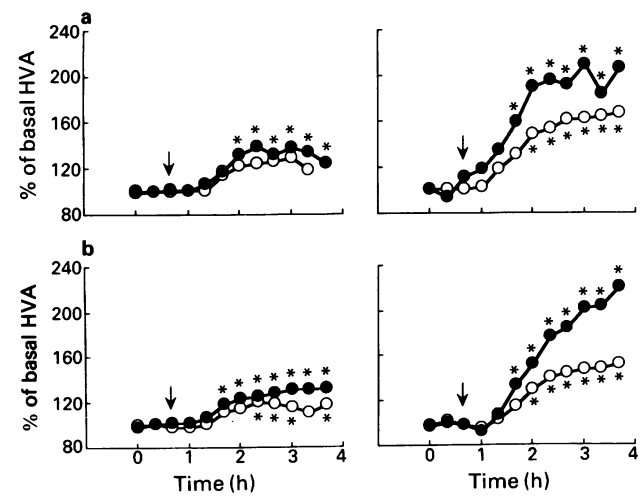

Figure 3 Effect of chronic oral doses (once daily for 23 days) of 20 (left) and 60 (right) $mg\,kg^{-1}$ clozapine on homovanillic acid (HVA) levels in the striatum (a) and nucleus accumbens (b) of conscious rats. See Figure 1 for other details. $F\ (9,54) = 4.8$, $P < 0.01$ (Split-plot design) for the effect of clozapine on HVA levels in the nucleus accumbens of chronic clozapine vs. vehicle treated animals. * $P < 0.05$ vs. basal values.

Table 2 Effect of various doses of clozapine on the levels of homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) in the striatum and nucleus accumbens of rats

	Dose ($mg\,kg^{-1}$)	HVA ($ng\,g^{-1}$)	DOPAC ($ng\,g^{-1}$)
Striatum			
Control		400 ± 32	418 ± 17
Clozapine	10	388 ± 25	444 ± 27
Clozapine	30	492 ± 27	606 ± 36*
Clozapine	100	940 ± 59**	1216 ± 93**
Nucleus accumbens			
Control		339 ± 26	551 ± 46
Clozapine	10	465 ± 30*	749 ± 34**
Clozapine	30	682 ± 87**	1168 ± 163**
Clozapine	100	1053 ± 105**	1414 ± 210**

Rats received various doses of clozapine orally and were killed 2 h later. Values are mean ± s.e.mean. * $P < 0.05$; ** $P < 0.01$ vs. control, Dunnett's t test.

dose significantly raised striatal HVA from 80 to 180 min after administration. The $60\,mg\,kg^{-1}$ dose had a greater effect on HVA in chronically treated animals, although statistical significance was reached only in the nucleus accumbens.

Effects of acute clozapine on dopamine metabolism in post mortem tissues

Table 2 shows the effects of various doses of clozapine on DOPAC and HVA in the whole striatum and nucleus accumbens, measured in post mortem tissues. A dose-dependent increase of dopamine metabolites was found in the nucleus accumbens, the lowest effective dose being $10\,mg\,kg^{-1}$. This dose did not affect the levels of dopamine metabolites in the striatum and $30\,mg\,kg^{-1}$ clozapine significantly raised only striatal DOPAC. The $100\,mg\,kg^{-1}$ dose significantly raised both dopamine metabolites in the striatum.

Discussion

The main aim of the present study was to examine whether in conditions similar to those described by Chiodo & Bunney (1983) and White & Wang (1983) for depolarization inactivation of A10 dopamine cells, there was preferential reduction of dopamine release and development of tolerance to the acute effects of clozapine in the nucleus accumbens of chronically treated rats. Single oral doses of 20 and $60\,mg\,kg^{-1}$ clozapine significantly increased dopamine release in both striatum and nucleus accumbens, with peak effect about 1 h after injection. The two terminal regions appeared to respond differently to the two clozapine doses since the lowest dose had more effect on dopamine release in the striatum while the higher one had similar activity (in terms of percentage increase) in both regions. Recently a preferential effect of $40\,mg\,kg^{-1}$ clozapine on dopamine release in the dorsolateral compared with limbic striatum has been reported (O'Connor *et al.*, 1989). We found no significant differences in basal dopamine release in either region of animals treated chronically with 20 to $60\,mg\,kg^{-1}$ clozapine. There was also no reduction in clozapine's effect in the nucleus accumbens (or in the striatum) after chronic treatment. In the nucleus accumbens of chronically treated animals, $60\,mg\,kg^{-1}$ clozapine actually tended to have more effect on dopamine release and metabolism, suggesting a sensitization to its effect in this brain region.

These data therefore clearly indicate that depolarization inactivation of A10 cells induced by chronic treatment with clozapine does not result in any appreciable reduction of basal dopamine release or any loss of effect in one terminal region, as assessed by intracerebral dialysis. This cannot be attributed to inadequate dosage or length of treatment since, besides using $20\,mg\,kg^{-1}$ clozapine as in the electrophysiological studies, we also studied the effect of $60\,mg\,kg^{-1}$, and the length of treatment was similar. Even with $100\,mg\,kg^{-1}$ clozapine once daily for 28 days no tolerance was seen though a greater effect on dopamine release and metabolism was seen in the striatum (unpublished results).

Since the A10 region is the site of origin of dopaminergic neurones of the mesolimbic and mesocortical systems it could be argued that terminal regions of the latter system, such as the prefrontal cortex, may better reveal the association between the electrical and biochemical phenomena. However this does not seem to be the case since Chiodo & Bunney (1983) reported that mesocortical dopaminergic neurones projecting to the prefrontal cortex do not go into depolarization inactivation as a result of chronic neuroleptic treatment.

Studies in which dopamine metabolism was measured in post mortem tissues showed that tolerance to the effect of clozapine developed in limbic regions but not in the striatum (Burki *et al.*, 1974; Waldmeier & Maitre, 1976b; Racagni *et al.*, 1980). These data suggest that changes in dopamine metabolites may provide a better picture of the changes in dopamine cell firing after chronic treatment with neuroleptics.

This hypothesis is not borne out by the present study in which no significant differences in extracellular DOPAC and HVA levels were found in the striatum and nucleus accumbens of rats treated acutely or chronically with 20 or $60\,mg\,kg^{-1}$ clozapine. It should be noted that changes in extracellular dopamine metabolites, particularly DOPAC, are not necessarily associated with changes in dopamine release and may better reflect changes in dopamine synthesis and consequently spill-over of intraneuronal dopamine (Imperato & Di Chiara, 1984).

It is interesting that in one study of the effects of various acute doses of clozapine on dopamine metabolism in post mortem tissue, $10\,mg\,kg^{-1}$ caused an increase only in the nucleus accumbens and $30\,mg\,kg^{-1}$ also had a preferential effect. This appears to agree with acute effects seen in experiments in which intracerebral dialysis was used since $20\,mg\,kg^{-1}$ clozapine significantly increased dopamine metabolites in the nucleus accumbens but not in the striatum, although the percentage increase was similar in both regions. Doses higher than $30\,mg\,kg^{-1}$ show no selectivity and may even preferentially affect dopamine metabolism in the dorsomedial striatum (O'Connor *et al.*, 1989).

The present results show that single, relatively low doses of clozapine preferentially increase the synthesis and metabolism of dopamine in the nucleus accumbens but this was not reflected by changes in dopamine release. In fact, the lowest dose had more effect on striatal dopamine release. The dissociation between changes in dopamine release in the nucleus accumbens and firing of A10 dopamine cells in animals chronically treated with clozapine is not necessarily surprising in view of the fact that different mechanisms may control these two processes. Clozapine possesses potent antimuscarinic and α-adrenoceptor blocking properties that are involved in its preferential effect on depolarization inactivation of A10 cells (Chiodo & Bunney, 1985). How these properties influence its effects on dopamine release in the striatum and nucleus accumbens is not clear.

Thioridazine, which also has high affinity for muscarinic and α-adrenoceptors (Leysen, 1984), has been reported to have effects similar to those of clozapine on A10 dopamine cells (White & Wang, 1983). Recently, development of tolerance to the ability of thioridazine to raise DOPAC levels in the nucleus accumbens was reported in one study in which *in vivo* voltammetry was used (Maidment & Marsden, 1987) but, as discussed above, the relation between changes in dopamine metabolism and in dopamine synaptic availability is not clear. Changes in A10 cell firing and dopamine release in the nucleus accumbens with this drug may possibly be more closely associated.

In conclusion, no appreciable changes in dopamine release and metabolism and no reduction in the effect of clozapine could be detected in one terminal region in conditions similar

to those in which chronic treatment with clozapine caused depolarization inactivation of A10 dopamine cells. These findings therefore apparently disprove the suggestion (Chiodo & Bunney, 1983) that, in addition to postsynaptic dopamine receptors in the nucleus accumbens being blocked, less synaptic dopamine is available in animals chronically treated with clozapine; in fact, the effect tended to be greater in the nucleus accumbens of animals treated chronically with 60 mg kg^{-1} clozapine. The extent to which the relationship between changes in dopamine cell firing and dopamine release in terminal regions may be closer with other neuroleptic agents remains to be clarified.

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