Possible Mechanisms By Which Repeated Clozapine Administration Differentially Affects the Activity of Two Subpopulations of Midbrain Dopamine Neurons¹

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Abstract

Extracellular single-cell recording techniques were employed to study the mechanism of action of repeated oral clozapine administration on the *in vivo* spontaneous activity of substantia nigra (A9) and ventral tegmental area (A10) dopamine (DA)-containing neurons in the rat. Clozapine was observed to affect DA neurons differentially within these two regions when compared to haloperidol. Acute treatment (1 hr) with both drugs increased the number of spontaneously firing neurons in both A9 and A10. Chronic (21 day) treatment with haloperidol decreased the number of cells encountered in both regions, whereas repeated treatment with clozapine reduced the number of DA cells per track only in A10. In all cases, the silent DA neurons were inferred to be in a state of depolarization inactivation since they could be induced to discharge normally by the microiontophoretic application of the inhibitory neurotransmitter γ -aminobutyric acid. These effects were not due to an effect of chloral hydrate anesthesia since they were also observed in gallamine-paralyzed, artificially respired animals. Chronic co-administration with haloperidol of either an anticholinergic (trihexyphenidyl) or the α_1 -norepinephrine (NE) receptor antagonist, prazosin, but not an α_2 -NE antagonist, RX781094, resulted in a differential effect on A9 and A10 DA neurons identical to that observed with repeated clozapine administration alone. Thus, chronic treatment with these combinations of drugs resulted in the depolarization inactivation of only A10 cells. These data suggest that anticholinergic and/or α_1 -NE-blocking properties of clozapine may, in part, mediate its differential effects on A9 and A10 midbrain DA neurons.

In the past few years many basic studies have examined the actions of acute and chronic dopamine (DA) receptor antagonist (i.e., antipsychotic drug (APD)) administration on DA neuronal systems. This work has been stimulated, at least in part, by the fact that APDs represent the primary pharmacological treatment for the major symptoms of psychosis. In 1978, it was shown (using single-unit extracellular recording techniques) that, whereas the acute administration of an APD increased the number of spontaneously active DA neurons within the substantia nigra (A9), chronic treatment markedly decreased the number of cells encountered (Bunney and Grace, 1978). This reduction in the number of spontaneously firing DA neurons in chronically treated animals was shown to be due to the induction of a state of tonic depolarization (i.e., depolarization inactivation; Bunney and Grace, 1978; Chido and Bunney, 1983a). Thus, these inactive DA neurons could be made to discharge in a normal fashion by the microiontophoretic application of the inhibitory neurotransmitter, γ -aminobutyric acid (GABA) but not the excitatory amino acid, glutamate. Subsequently, it was shown that typical APDs (i.e., those antipsychotic drugs associated with a high incidence of extrapyramidal side effects such as haloperidol or chlorpromazine) produce this acute increase in the number of active DA neurons observed but, when given chronically, they inactivate these cells in both A9 and the ventral tegmental area (A10) (Chido and Bunney, 1983a; White and Wang, 1983). In contrast, atypical APDs (those associated with a low incidence of side effects such as clozapine (CLZ)) produced a different pattern of response. Although both A9 and A10 had a similar effect acutely, only A10 DA cells (i.e., not A9 neurons) were inactivated after chronic treatment (Chido and Bunney, 1983a; White and Wang, 1983; Chido et al., 1984).

In the present series of experiments we have utilized this differential response of A9 and A10 neurons to typical and atypical APDs as a means to further characterize the mode of action of CLZ on midbrain DA systems. Obtaining an understanding of the mode of action of CLZ within the central nervous system is considered important for at least two reasons. First, since the use of this compound is associated with a markedly reduced incidence of extrapyramidal side effects (including tardive dyskinesia), it is hoped that elucidating the mechanism of action of CLZ upon DA neurons will aid in the development of new APDs with low side effect potential. Second, comparing the sites of action of typical and atypical APDs may allow us to find the difference between the neuronal elements involved in clinical efficacy and those involved in side effect induction.

Previous work has suggested that the anticholinergic and possibly the α -noradrenergic (NE) blocking properties of CLZ may underlie the relative inability of this drug to induce extrapyramidal reactions (Bartholini et al., 1972; Anden and Stock, 1973; Burki et al., 1974; Miller and Hiley, 1974; Snyder et al., 1974; Marco et al., 1976; McMillan and Shore, 1978; Pelham and Munsat, 1979; Souto et al., 1979; Racagni et al., 1980). For this reason, we combined the

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anticholinergic drug trihexiphenidyl (Artane) or the α_1 -NE antagonist prazosin with typical APDs in an attempt to produce an effect on the number of spontaneously firing DA cells in A9 and A10 similar to that produced by CLZ. The α_2 -NE blocker RX781094 (Idazoxan) was also tested.

Materials and Methods

Animals and drug treatment. Male Sprague-Dawley albino rats (Camm) weighing 200 to 225 gm at the start of the experiment were used. Animals were housed two per cage and maintained on a 12/12-hr light/dark cycle with free access to food and water. In the acute experiments, animals were given one of the following drug treatments via intragastric intubation: CLZ (10 mg/kg; Sandoz), haloperidol (HAL, 0.5 mg/kg; McNeal), prazosin hydrochloride (PRAZ; 0.25 mg/kg; Pfizer), RX781094 (Idazoxan (IDZ), 0.20 mg/kg; Rickett & Coleman), trihexiphenidyl hydrochloride (Artane (ART), 1.0 mg/kg; Lederle), mesoridazine besylate (MES; 2.0 mg/kg; Boehringer-Ingelheim), combined HAL and ART, combined HAL and PRAZ, or combined HAL and IDZ. In the chronic experiments additional groups of animals were given the same drugs in their drinking water for 21 days. Untreated animals were also sampled for the purpose of comparison. All chronically treated animals were weighed twice a week and the drugs were administered via light-proof water bottles. No difference in weight gain was observed. Daily water intakes were measured and the actual drug dosage was calculated from these values. All drug dosages are expressed as the weight of the salt.

Electrophysiological methods. One hour after acute drug administration or on day 21 of continuous treatment, the animals were anesthetized with the general anesthetic chloral hydrate (400 mg/kg, i.p.) or, following halothane anesthesia and a tracheotomy, were paralyzed with gallamine triethiodide (20 mg/kg, i.v.) in strict accordance with the guidelines of the American Physiological Society. As previously described (Chiodo and Bunney, 1983b), all incisions and pressure points were infiltrated with a long acting local anesthetic, mepivacaine hydrochloride. Once paralyzed, the halothane was discontinued and recordings were started 30 min later. In the paralyzed animals expired carbon dioxide was monitored with a Harvard CO₂ analyzer and maintained between 3.5 and 4.0%. No difference in the number of cells per track or in the effects of drug administration were noted between the chloral hydrate and paralyzed groups. The experimenter was always blind with respect to the treatment condition of the animal. Body temperature was monitored and maintained at 36 to 37°C. Once in the stereotaxic unit, the scalp was reflected and the skull overlying both the substantia nigra (A9, anterior 1,750 to 2,580 μ m, lateral 1,700 to 2,100 μ m; Konig and Klippel, 1974) and the ventral tegmental area (A10, anterior 1,610 to 2,420 μ m, lateral 100 to 500 μ m) was removed. Extracellular neuronal activity was monitored using a single-barrel micropipette (overall tip diameter <1 μ m) filled with 2 M NaCl containing 2% pontamine sky blue dye (*in vitro* impedance at 135 Hz, 3 to 8 megohms). Single-unit activity was recorded through a high input impedance amplifier (bandpass settings: 100 Hz to 2 kHz) the output of which was led into an analogue oscilloscope, audio monitor, and window discriminator. The amplifier input was also digitized at rates of 5 to 500 μ sec point (4,096 points, 12 bit precision) by a Nicolet Explorer III digital oscilloscope and stored on floppy discs. Spontaneously active DA neurons within both the A9 and A10 areas were counted by lowering the recording micropipette through a stereotaxically defined block of tissue (120,000 μ m²) which could be reproducibly located from animal to animal (Chiodo and Bunney, 1983a). Twelve electrode penetrations were made in each region. Each electrode track was separated from every other track by 200 μ m, and the sequence of these tracks was kept constant. Only electrophysiologically identified (see Bunney et al., 1973; Grace and Bunney, 1983) DA neurons were sampled.

Antidromic activation. In some experiments, DA neurons within the A10 region were antidromically activated from the prefrontal cortex as previously described (Chiodo et al., 1984). Concentric bipolar stimulating electrodes (SNE-100, David Kopf Instruments) were stereotaxically placed in the region of the prefrontal cortex (anterior 9,650 to 11,050 μ m, lateral 200 to 1,000 μ m; Konig and Klippel, 1974). Constant current square wave pulses (0.2 to 2.5 mA; 300 to 500 μ sec duration) were delivered by a digital pulse generator (Series 800, World Precision Instruments) through a stimulus isolation unit (model 850A, World Precision Instruments). Neurons were considered antidromically activated when the following criteria were met: (1) one antidromic spike was elicited per stimulus; (2) a fixed latency existed for the stimulus-induced action potential; (3) the antidromic spike followed stimulations up to 50 Hz; and (4) collision occurred between spontaneously occurring action potentials and stimulation-elicited spikes.

Histology. The recording sites were marked by passing a 30- μ A cathodal

current through the recording electrode for 10 min. This resulted in the ejection of a 50- μ m-diameter spot of sky blue dye. The brains were fixed in 10% buffered formalin, and then frozen sections were cut at 50- μ m intervals and stained with a formal-thionin solution (Donovick, 1974). A light microscope was used to locate the spot of ejected dye. Histological examination verified that all electrode tracks in the present experiments were located within the defined blocks of tissue corresponding to the A9 and A10 regions.

Statistics. Current electrophysiological techniques do not allow us to record from a single neuron throughout the entire period of chronic drug administration. Therefore, comparisons between populations of DA neurons sampled in both control and drug-treated (acute or chronic) animals must be made. Overall statistical significance was determined by an analysis of variance. Post hoc paired comparisons of individual group differences relative to controls were determined using a two-tailed Dunnett test.

Results

Effects of typical and atypical neuroleptics on the population response of midbrain DA-containing neurons. In control animals the mean number of DA cells per track in the A9 and A10 regions was 0.80 and 0.82, respectively (Fig. 1). The acute treatment with either CLZ, HAL, or MES significantly increased the number of cells per track in both of these regions. The mean number of cells per track for the acute HAL group was 1.76 in A9 and A10, 1.76 and 1.72 (respectively) for acute CLZ, and 1.72 and 1.79 (respectively) for acute MES animals (Fig. 1). On day 21 of continuous drug administration, there were significantly fewer cells per track in both regions in the animals receiving HAL but an increase in the number of active cells in A9 and a decrease in A10 in both the CLZ- and MES-treated groups.

Effects of anticholinergic co-administration with HAL. A dose of 1 mg/kg of the anticholinergic ART was employed in these studies. This dose was picked because oral administration of this amount of ART was observed to markedly decrease the excitatory effects of microiontophoretically applied acetylcholine on CA1 hippocampal pyramidal neurons ($N = 8$; Fig. 2). Although neither acute nor chronic treatment with ART alone altered the number of cells per track in either region (relative to control), ART did affect the response to HAL when administered concurrently. Thus, although there was an increased number of cells per track in the acute HAL + ART animals in both areas, chronic treatment with this combination of drugs induced a decrease in the number of active DA neurons within the A10 region but not the A9 area (Fig. 1).

Effect of α -NE antagonist co-administration with HAL. Acute treatment with the α_1 -NE antagonist PRAZ had no effect on the number of active DA neurons encountered in A10 but significantly increased the number of cells per track observed in A9. In contrast, chronic treatment with PRAZ did not alter the number of cells per track in either area. When PRAZ and HAL were co-administered acutely, there was once again an increase in the number of spontaneously firing DA neurons in both regions. On day 21 of repeated administration of both drugs, there was a significant decrease in the number of cells per track in A10 but an increase in the number of active DA neurons in A9 (Fig. 1). This increase was no different from that observed with acute HAL alone, CLZ alone, or PRAZ + HAL.

Acute administration of the α_2 -NE blocker IDZ alone had no effect in A10 but produced a significant elevation in the number of DA neurons observed in A9. The number of DA cells per track in chronic IDZ treatment was not different from control values. Moreover, IDZ did not alter either the increase in cells per track produced by HAL acutely or the decrease in the number of active DA neurons normally induced by chronic HAL administration in either A9 or A10. Thus, qualitatively similar results were observed between the acute HAL and HAL + IDZ groups as well as between the chronic HAL and HAL + IDZ groups.

Effects of anesthetic. No effect of chloral hydrate anesthetic was noted in this study. Approximately one-half of each of the following groups was sampled in gallamine-paralyzed animals: acute CLZ ($n = 6$ of 14), chronic CLZ (7 of 15), acute HAL (5 of 10), chronic HAL (9 of 21), and chronic MES (4 of 10). Moreover, we again have

Figure 1. The effects of both acute and repeated (21-day) oral APD treatment on the mean ($\pm$ SEM) number of spontaneously firing DA cells per track observed in both A9 and A10. The shaded areas represent the control means ($\pm$ SEM). *, $p < 0.01$ relative to untreated controls, two-tailed Dunnett test. The number below each bar indicates the number of animals studied.

examined whether or not some of the A10 DA neurons, which were still active in the 21-day HAL group, might in fact be mesoprefrontal DA cells. It has previously been suggested that these DA cells are devoid of synthesis-modulating nerve terminal, and somatodendritic autoreceptors (Chiodo et al., 1984) were demonstrated to be largely still spontaneously active in animals receiving chronic APDs (Chiodo and Bunney, 1983a; Chiodo et al., 1984). However, these latter studies were carried out in chloral hydrate-anesthetized animals. In the present set of experiments, 8 of 10 spontaneously active A10 DA neurons, which were found in animals that had received HAL for 21 consecutive days, were antidromically activated from the prefrontal cortex of gallamine-paralyzed animals ($N = 4$). These antidromically activated cells had fixed conduction latencies of 18.2 to 26.5 msec. Given a straight-line distance between the recording micropipette and the stimulating electrode tips of 9.0 to 11.0 mm, these values correspond to an estimated conduction velocity of approximately 0.5 mm/sec.

The silent DA neurons in gallamine-paralyzed animals which had been treated for 21 days with either HAL or CLZ were again shown to be in a state of depolarization inactivation. Neurons in the A10 region of both chronic HAL ($N = 8$) and chronic CLZ ($N = 7$) could be activated by the microiontophoretic application of the normally inhibitory substance, GABA (1 to 5 nA, Fig. 3). As observed in

chloral hydrate-anesthetized animals (Chiodo and Bunney, 1983a), these GABA-activated neurons first began to fire rapidly and to display relatively small amplitude action potentials. As the GABA ejection continued, the discharge rate slowed (i.e., the interspike interval increased) and the action potential amplitude increased. When GABA iontophoresis was terminated, these neurons increased their rate and their amplitude decreased until it was no longer distinguishable from the background noise. Glutamate microiontophoresis onto the same neurons had no effect.

Discussion

The present series of experiments confirms and extends earlier reports (Chiodo and Bunney, 1983a; White and Wang, 1983) which demonstrate that typical and atypical APDs have different effects on the population response of A9 and A10 DA-containing neurons in the rat. Thus, the typical APDs (e.g., HAL) produce a state of depolarization inactivation of both A9 and A10 neurons when administered repeatedly for 21 days, whereas the atypical APDs (e.g., CLZ and MES) inactivate only A10 DA neurons upon chronic administration. This response is not seen when either typical or atypical APDs are given acutely or following either the acute or chronic administration of inactive isomers of DA antagonists or antidepres-

sants (Chiodo and Bunney, 1983a; White and Wang, 1983). It has previously been suggested that the delayed onset of depolarization inactivation in A9 and A10 regions may be related to the delayed onset of both clinical efficacy (Chiodo and Bunney, 1983a; White and Wang, 1983) and extrapyramidal side effects (Bunney and Grace, 1978; Chiodo and Bunney, 1983a) in patients chronically treated with APDs. Thus, the development of a state of APD-induced depolarization inactivation in A9 may underlie the extrapyramidal side effects observed as well as contribute to development of tardive dyskinesia in humans. The induction of a state of depolarization inactivation in A9 neurons has been shown to be accompanied by a decrease in stimulation-induced release of DA within the striatum (Schenk and Bunney, 1983). While DA neurons are in this inactivated state there may be a decrease in the amount of DA in the synaptic cleft available to compete with APD molecules for striatal postsynaptic receptors. If this is the case, maximal postsynaptic DA receptor blockade would occur at this time. Over time, this effect would serve to potentiate APD-induced sensitization of postsynaptic DA receptors which is believed to be a contributing factor to the development of tardive dyskinesia (Klawans, 1976; Baldessarini and Tarsy, 1976, 1980; Jeste and Wyatt, 1982).

Given the above reasoning, it has been suggested that the inability of atypical APDs to induce depolarization inactivation of A9 would

reduce the level of functional DA receptor blockade in the striatum and, thereby, would possibly result in a lower level of extrapyramidal side effects in general, as well as a lower incidence of tardive dyskinesia. Receptor binding studies, performed after repeated CLZ treatment, tend to support this notion, since it has been reported that this treatment does not increase postsynaptic receptor binding in the striatum (Kobayashi et al., 1978; Snyder, 1980). In a similar fashion, since clinical efficacy is strongly correlated with postsynaptic receptor blockade, maximal therapeutic action (i.e., maximal postsynaptic receptor blockade in limbic and cortical regions) would be obtained only when the state of depolarization inactivation exists with respect to the A10 DA-containing neurons, a condition induced by all antipsychotic drugs studied to date.

Given the importance of developing new classes of antipsychotic drugs which are devoid of side effect potential, the present work examined whether some of the non-DA receptor-blocking actions of CLZ within the central nervous system may underlie the inability of this compound to induce depolarization inactivation of A9 neurons. Since it is known that CLZ, in addition to blocking DA receptors, exerts an anticholinergic action within the mammalian brain (Anden and Stock, 1973; Miller and Hiley, 1974; Snyder et al., 1974; Marco et al., 1976; Pelham and Munsat, 1979; Racagni et al., 1980), we examined whether the co-administration of the anticholinergic com-

Figure 2. A rate histogram demonstrating the typical effect of the anticholinergic drug ART (1 mg/kg, p.o.) on the excitatory effects of acetylcholine (ACh) applied microiontophoretically (3 nA). The cell recorded is a spontaneously active hippocampal pyramidal neuron located in region CA1. The calibration bar below the histogram represents 5 min. Horizontal bars represent the onset and duration of acetylcholine application.

Figure 3. Oscillograph tracing showing the typical response of a "silent" A10 DA neuron to microiontophoretically applied GABA (2 nA). This cell was sampled in a gallamine-paralyzed animal which had been treated with CLZ (10 mg/kg, p.o.) for 21 consecutive days. After a short delay, GABA caused the extracellular action potential of this single neuron to appear out of the background noise. Continued ejection resulted first in a decrease in activity (action potential amplitude increased and duration decreased) and then total inhibition of the cell's activity. When the GABA is turned off, the rate markedly increases (amplitude decreases and duration increases) until the action potential can no longer be discerned above the background noise. (The changes in action potential duration are not visible due to the chart speed employed.) The alterations in action potential amplitude and duration are identical to those observed in our previous studies of the effects of repeated APD administration on the activity of A9 and A10 DA cells (Bunney and Grace, 1978; Chiodo and Bunney, 1983a). Calibration bars, 400 μ V and 2 sec.

pound ART with HAL could alter the effects of HAL on A9 DA neurons. The oral administration of ART by itself, either acutely or chronically, had no effect on the number of cells per track in either A9 or A10. The acute co-administration of HAL + ART resulted in an increased number of active DA neurons in both A9 and A10 similar to that observed with either acute CLZ or acute HAL treatment. In contrast to the effects observed when HAL is administered repeatedly by itself, repeated administration of HAL and ART resulted in depolarization inactivation of only A10 DA neurons. If the pattern of response of A9 and A10 DA neurons after chronic neuroleptic treatment is an indication of both the side effect potential and the clinical efficacy of these agents in humans, then it would be expected from the above results that the co-administration of an anticholinergic drug and a neuroleptic to humans would decrease the number of extrapyramidal effects observed. In this regard, several studies have demonstrated that the co-administration of these agents does significantly reduce the incidence and severity of extrapyramidal side effects (Fann and Lake, 1976; Klein et al., 1980; Manos et al., 1981; McEvoy 1983). It is, of course, not clear where or how the anticholinergic drug is acting to produce its effect. However, given our data it may be that the prevention of depolarization block from developing in A9 cells during repeated antipsychotic drug treatment is one of its clinically relevant effects.

It has also been shown that CLZ possesses an α -NE receptor-blocking action (Burki et al., 1974; McMillan and Shore, 1978; Souto et al., 1979) which might, in part, underlie its relative inability to induce extrapyramidal side effects. To examine this issue, we treated animals both acutely and chronically with either the α_1 -NE antagonist PRAZ or the α_2 -NE receptor blocker IDZ, both alone and in combination with HAL. The acute administration of PRAZ increased the number of cells per track in only A9, whereas the chronic administration had no effect in both regions. The acute co-administration of PRAZ + HAL produced an increase in cells per track in both A9 and A10 which was similar to that observed in the HAL alone animals. The chronic administration of PRAZ + HAL produced a depolarization inactivation of only A10 DA neurons, whereas A9 neurons were increased to levels similar to that observed after acute HAL + PRAZ administration. In contrast, IDZ, when given chronically with HAL, did not alter the ability of HAL to reduce the number of spontaneously active DA neurons in A9. These findings support the hypothesis that, in addition to the anticholinergic actions of CLZ, the α_1 -NE blocking properties of this compound may also be important in its inability to induce depolarization inactivation of A9 neurons. To date, we are unaware of any clinical trials which have examined the chronic co-administration of both an α_1 -NE antagonist and an APD although a possible adrenergic influence has been suggested from subhuman primate work. Thus, it has been reported that APD-induced tardive dyskinesia observed in monkeys is successfully treated with the co-administration of clonidine (Nishikawa et al., 1983). Our findings are also in agreement with studies which have shown that CLZ possesses α_1 -NE antagonist actions while being relatively devoid of α_2 -NE blocking properties (Peroutka and Snyder, 1980; Perry et al., 1983).

In summary, we have found that combining a neuroleptic possessing a relatively high incidence of neurological side effects (i.e., HAL) with either an anticholinergic agent or an α_1 -NE blocker changes its profile of actions on midbrain DA neurons to that of an antipsychotic drug possessing a low incidence of these side effects (i.e., CLZ). One hypothesis that these findings suggest is that antipsychotic drugs which possess anticholinergic and/or α_1 -NE antagonist properties may be associated with a decreased incidence of extrapyramidal side effects.

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