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Life Sciences, Vol. 59, No. 23, pp. 2001-2008, 1996
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0024-3205/96 $15.00 + .00

PII S0024-3205(96)00551-6

TREATMENT WITH HALOPERIDOL OR CLOZAPINE CAUSES CHANGES IN DOPAMINE RECEPTORS BUT NOT ADENYLATE CYCLASE OR PROTEIN KINASE C IN THE RAT FOREBRAIN

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(Received in final form October 1, 1996)

Summary

The effect of treating rats with daily injections of haloperidol (1mg/kg/day) or clozapine (20 mg/kg/day) for four weeks on second messengers and dopamine receptors was studied. The binding of [^{3}H]forskolin to adenylate cyclase (AC), [^{3}H]phorbol 12,13-dibutyrate (PDBu) to protein kinase C (PKC), [^{3}H]SCH23390 binding to the dopamine D_1 (DA-D_1) receptor and [^{3}H]spiperone binding to the dopamine D_2 (DA-D_2) receptor were measured using quantitative autoradiography. The density of AC was greatest in the caudate-putamen, nucleus accumbens and olfactory tubercle, a distribution resembling that of DA-D_1 receptor. The distribution of PKC was relatively homogeneous in the forebrain. Neither haloperidol nor clozapine administration significantly altered the levels of AC or PKC in the caudate-putamen. By contrast treatment with haloperidol, but not clozapine, significantly increased the density of DA-D_2 receptors in the caudate-putamen without affecting the density of DA-D_1 receptors. By contrast, both haloperidol and clozapine increased the density of DA-D_1 receptors in the olfactory tubercle.

Key Words: **haloperidol, clozapine, dopamine receptors, adenylate cyclase, protein kinase C, forebrain**

The aetiology of schizophrenia, a mental disorder reported as affecting up to 1% of the population(1), is not clear. Nevertheless, it has been suggested that dopaminergic neurons in the central nervous system (CNS) play an important role in schizophrenia with the dopamine hypothesis of schizophrenia suggesting hyperactivity of certain dopaminergic pathways(2). This hypothesis is supported by the demonstration that the therapeutic efficacy of neuroleptic drugs correlates well with their *in vitro* ability to block the dopamine (DA-D_2) receptor(3).

The treatment of schizophrenia with 'typical' neuroleptics, such as haloperidol, can lead to the development of extrapyramidal side effects (EPS) or tardive dyskinesia (TD)(4). By contrast, the "atypical" neuroleptic clozapine has proven useful in treating schizophrenics who had responded poorly to typical neuroleptics and appears not to cause EPS or TD(5). Hence there has been intensive research into the mechanism(s) underlying the differing effects of 'typical' and 'atypical' neuroleptics(6). Much research has focused on the differential effects of 'typical' and 'atypical' neuroleptics on different receptors(7-9), but few studies have investigated the

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effect of these drugs on second messengers(10).

There has been accumulative evidence showing that aberrations in second messenger responses may be involved in the development of neurological disorders. As a step towards understanding the mechanisms of 'typical' and 'atypical' neuroleptics on the CNS, it is therefore of particular interest to examine whether haloperidol and clozapine can induce differential changes in neuronal second messenger status. Forskolin, a diterpene having high affinity to AC(11), is considered a valuable probe for localizing AC in the brain (12,13). Similarly, phorbol esters have been employed to localize PKC(12). We therefore used autoradiography to localize AC, PKC and the DA-D_1 and DA-D_2 receptors in the forebrain of rats after treatment with either haloperidol or clozapine.

Methods

Neuroleptic treatment

Six week-old male Spraque-Dawley rats with an initial body weight of 160-222 g (Monash University Animal Supply) received a daily intraperitoneal injection of either saline (2ml/kg/day, n=6), haloperidol (1mg/kg/day, n=6) diluted with saline to 0.5 mg/ml or clozapine (20 mg/kg/day, n=6, generously provided by Sandoz Research Institute, East Hanover, NJ) dissolved in 0.1 M HCl and the pH raised to 5.5-6.0 with 1 M NaOH prior to being diluted to 10 mg/ml with saline for 4 weeks. Animals were sacrificed by decapitation 22-24 hours after the last injection. The brains were rapidly removed, frozen in hexane/dry ice bath before storing at -70°C.

Preparation of tissue sections and protein density measurements

Frozen brains were brought to -20°C the day before sectioning. Brains were cut coronally into 5 mm tissue blocks. The forebrain, containing regions of the caudate-putamen and nucleus accumbens, were sectioned in a cryostat (-20°C). Tissue sections (15 μm) were thaw-mounted on chrom-alum/gelatin coated microscope slides and air-dried at 4°C. Each slide contained triplicate sections from a single brain.

[^{3}H]Forskolin binding studies were carried out according to the method of Gehlert et al.(13) except that 1 mM glucose was included in the binding buffer to suppress forskolin binding to glucose transporter(14). Briefly, sections were incubated with ~150 nM [^{3}H]forskolin (New England Nuclear NET-844, 26.2 Ci/mmol) for 10 minutes at ambient temperature (20-25°C) in a buffer containing 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM glucose, pH7.7 in the absence or presence of 20 μM forskolin (Sigma). Sections were then washed twice for 1 minute in 500 ml of ice-cold buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH7.7), followed by a brief rinse (1-2 seconds) in ice-cold distilled water. Sections were then dried quickly with a stream of cold air, desiccated under vacuum overnight before apposing to [^{3}H]-sensitive Hyperfilm (Amersham) for 4 weeks.

[^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) binding was carried out as described by Worley et al. (12). Briefly, frozen sections were incubated with [^{3}H]PDBu (~10 nM, New England Nuclear, NET-692, 20 Ci/mmol) for 60 minutes in the absence or presence of 1 μM PDBu (Sigma) in buffer containing 50 mM Tris-HCl, 0.1 M NaCl, 1 mM $CaCl_2$, pH 7.7. Sections were washed

twice for 10 minutes in ice-cold binding buffer, followed by a brief dip (1-2 seconds) in ice-cold distilled water. Sections were rapidly dried with a stream of cold air, desiccated under vacuum overnight before apposing to [^{3}H]-sensitive Hyperfilm for 4 weeks.

DA-D_1 receptors were labelled with the selective antagonist [^{3}H]SCH23390 ([N-methyl-^{3}H]SCH 23390, Amersham TRK.876, 72 Ci/mmol) at a concentration of ~ 2.4 nM in the absence or presence of 1 μM *cis*-flupenthixol dihydrochloride (Research Biochemicals Inc., MA)(15). DA-D_2 receptors were labelled by [^{3}H]spiperone (New England Nuclear NET-565, 17.7 Ci/mmol)(16). Total binding was obtained with ~ 2 nM [^{3}H]spiperone in the presence of 1 μM ketanserin (Janssen Pharmaceuticals, Belgium) to suppress binding to serotonin receptors. Non-specific binding was determined by displacement with 1 μM (+)-butaclamol (Research Biochemicals Inc., MA). Both [^{3}H]SCH 23390 and [^{3}H]spiperone binding were carried out at ambient temperature (20-25°C) in binding buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$. 1 mM $MgCl_2$, pH 7.4) for 60 minutes. Sections were then washed twice (2 minutes each) in ice-cold buffer, followed by a brief rinse (1-2 seconds) in ice-cold distilled water. Sections were rapidly dried under a stream of cold air and desiccated under vacuum overnight before apposing to [^{3}H]-sensitive hyperfilm for 8 weeks.

Computer-assisted image analysis and statistics

Autoradiographic films were analysed using a computerised image analysis system (MCID, Image Research Inc., St. Catherines, Ont. Canada) equipped with M1 image analysis software. Densities of receptor binding in areas of interest (caudate-putamen, nucleus accumbens, olfactory tubercle, frontal cortex) were converted from units of optical density to theoretical femtomoles of radioligand bound per mg tissue equivalent using a standard curve calibrated from optical densities generated by [^{3}H]microscales (Amersham RPA510).

Data are expressed as means ± standard errors. The results were analysed using one-way analysis of variance (ANOVA). Post-hoc comparisons of specific means were carried out using Newman-Keuls tests.

Results

The clozapine treated rats gained significantly less body weight (40-55% increase; $p<0.05$) compared to those treated with haloperidol (50-65% increase) or saline (65-78% increase).

Effects on second messenger bindings in rat forebrain

Binding of [^{3}H]PDBu (~10 nM) to rat forebrain was relatively homogeneous and was totally displaceable by 1 μM cold PDBu (Figure 1). [^{3}H]PDBu binding was measurable in the caudate-putamen (1628±65 fmol/mg tissue equivalent; n=6), nucleus accumbens (1086±66), olfactory tubercle (1789±103) and frontal cortex (1464±95) and was not affected by neuroleptic drug treatment (Table 1).

The binding of [^{3}H]forskolin (~150 nM) to the forebrain was heterogenous, with the highest density of binding sites in the caudate-putamen (910±30 fmol/mg tissue equivalent, n=6) (Figure 1). Binding was also clearly detectable in nucleus accumbens (577±69), olfactory tubercle (635±34) and the frontal cortex (204±22). In all cases, [^{3}H]forskolin binding was displaceable by 20 μM forskolin and was relatively homogeneous in the caudate-putamen although some

sections revealed slight medial to lateral density gradient. Furthermore, the levels of [^{3}H]forskolin binding in these areas of rat forebrain were not affected by haloperidol or clozapine.

Effects on DA-D1 and DA-D2 receptor bindings in rat forebrain

[^{3}H]spiperone binding was highest in the caudate-putamen (152.1±4.4 fmol/mg tissue equivalent, n=6) (Figure 1). Treatment with haloperidol caused a significant increase in [^{3}H]spiperone binding to the caudate-putamen (45%; $p < 0.001$) when compared to those animals treated with either saline ($P < 0.05$) or clozapine ($P < 0.05$)(Table 1). [^{3}H]spiperone binding in the caudate-putamen of rats treated with clozapine however did not significantly differ from those treated with saline control (Table 1).

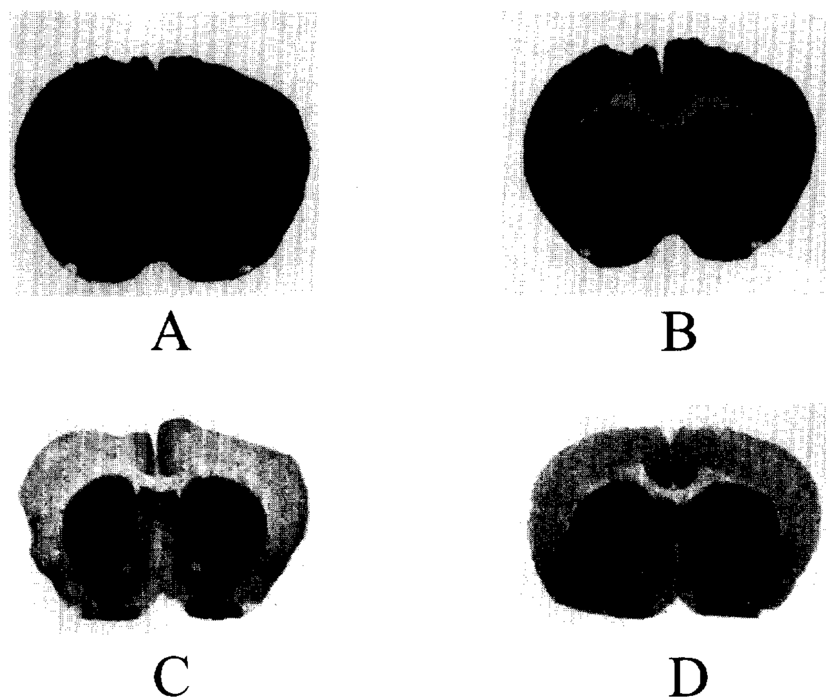

Fig. 1
Representative autoradiographs showing [^{3}H]phorbol 12,13 dibutyrate binding (A), [^{3}H]forskolin binding (B), [^{3}H]spiperone binding (C) and [^{3}H]SCH23390 binding in coronal sections of rat forebrains.

[^{3}H]SCH23390 binding was highest in the caudate-putamen (213.7± 7.9 fmol/mg tissue equivalent, n=6) but was also present in olfactory tubercle (167.1±16.7)and nucleus accumbens (127.8±20.6) (Figure 1; Table 1). Neuroleptic drug treatment did not significantly alter [^{3}H]SCH23390 binding in the caudate-putamen or in the nucleus accumbens. By contrast, there was a similar elevation of [^{3}H]SCH23390 binding in the olfactory tubercle from rats treated with either haloperidol or clozapine when compared to saline treated animals ($p < 0.05$) (Table 1).

Table 1

The Effects of Injecting Rats Daily with Haloperidol and Clozapine for Four Weeks on Second Messengers and Dopamine Receptors in the Forebrain

	[^{3}H]Forskolin Binding	[^{3}H]PDBu Binding	[^{3}H]SCH23390 Binding	[^{3}H]Spiperone Binding
	(fmol/mg)	(fmol/mg)	(fmol/mg)	(fmol/mg)
Caudate-putamen				
Saline	910±74	1628±160	213±19	125±15
Haloperidol	945±83	1723±193	228±27	183±27[a,b]
Clozapine	873±90	1556±155	228±32	136±19
Nucleus Accumbens				
Saline	577±169	1806±163	128±50	n.d
Haloperidol	606±119	1880±245	132±32	
Clozapine	718±73	1678±154	181±27	
Olfactory Tubercle				
Saline	635±84	1789±229	168±41	n.d.
Haloperidol	657±53	2023±107	218±32[a]	
Clozapine	617±45	1860±190	226±30[a]	
Frontal Cortex				
Saline	203±22	1464±232	n.d.	n.d.
Haloperidol	211±31	1670±220		
Clozapine	178±20	1557±124		

a, $p < 0.05$ compared to saline-treated animals; b, $p < 0.05$ compared to clozapine-treated animals; n.d. not determined. Data represent mean±s.e.m. (n=5 or 6) PDBu = [^{3}H]phorbol 12,13-dibutyrate

Discussion

The effect of chronically treating rats with the 'typical' neuroleptic, haloperidol, and the 'atypical' neuroleptic, clozapine, on the binding of [^{3}H]forskolin, [^{3}H]PDBu, [^{3}H]SCH23390 and [^{3}H]spiperone to the forebrain using quantitative autoradiography has been compared. Our data confirm previous studies that treatment with each type of neuroleptic drug differentially alters dopamine receptors(7,17). However, unlike previous studies we have shown that these changes occur without affecting the levels of either AC or PKC.

Previous clinical studies on human tissues have suggested a role of AC in the development of schizophrenia (18). An increase in forskolin binding in the left parahippocampal gyrus and CA1 region in post-mortem tissue from subjects with schizophrenia have been reported(18). Whilst these data implicated AC in the pathology of schizophrenia it was not clear whether the changes were due to the effects drug medication prior to death . This study, using rats, did not show a change in the levels of AC and PKC following neuroleptic drug treatment. Thus, these data would not support the hypothesis that the changes in AC in the schizophrenic brain were simply due to neuroleptic drug treatment.

The autoradiographic pattern of forskolin binding to AC in the forebrain observed in this study is similar to that reported by Appel et al.(19). The highest level of AC was in the caudate-putamen, a major target in the nigrostriatal dopaminergic pathway(20) and is closely matched with that of DA-D_1 receptor binding sites, a finding consistent with the suggestion that forskolin binds to neurons expressing predominantly DA-D_1 receptors(21). Significantly, neither haloperidol nor clozapine treatment had any significant effect on either the levels of AC or DA-D_1 receptors in these neurons. These data do not suggest that parallel changes in the density of AC and DA-D_1 receptors follow treatment with haloperidol or clozapine. However, this study does not rule out the possibility that changes in AC activity, rather than changes in the density of AC protein, might occur following neuroleptic treatment.

The binding of [^{3}H]PDBu to PKC in the caudate-putamen, nucleus accumbens, olfactory tubercle and frontal cortex was not affected by haloperidol or clozapine treatment. However, it should be noted that the levels of PKC was relatively high and therefore small changes in the binding sites affected by drug treatments may not be detected by the present approach.

The effects of neuroleptic treatment on dopamine receptor binding sites in the rat brain had previously been studied using radioligand binding to brain homogenates (17,22) or receptor autoradiography(8,9,23). Some of these studies have shown that clozapine treatment increased the densities of DA-D_1 binding sites in the caudate-putamen without having a significant effect on the DA-D_2 binding sites(8,23). We found no change in either the DA-D_1 or DA-D_2 receptors in caudate-putamen after clozapine treatment. However, like another study(7), we have shown treatment with haloperidol, but not clozapine, results in a significant increase in DA-D_2 receptor in the caudate-putamen.

One of the interesting observations resulting from this study is the up-regulation of DA-D_1 binding in the olfactory tubercle by both haloperidol and clozapine treatment. The central dopamine system consists of at least three pathways: mesostriatal, mesolimbic and mesocortical. The mesostriatal system has dopaminergic neurons from the substantia nigra projecting to the neostriatum (i.e. caudate-putamen). The mesolimbic system has its neurons in the ventral tegmental area (also known as A10) which projections to the nucleus accumbens, olfactory

tubercle and amygdala. The mesocortical system has neurons in the midbrain projecting to the prefrontal cortex and nucleus accumbens. An increase in DA-D_1 receptors in the olfactory tubercle after clozapine treatment for 28 days has been reported (9), and such action of clozapine in mesolimbic DA system has been suggested for its therapeutic efficacy(21,24). The present finding that both clozapine and haloperidol up-regulated DA-D_1 receptor in the olfactory tubercle further suggest that DA-D_1 receptor antagonism in the mesolimbic pathway plays an important role in defining the profile of atypical neuroleptics(16).

This study has attempted to determine if neuroleptic drug treatment could affect specific molecules in the rat brain. This was done to determine the likelihood of similar treatments affecting the same molecules in the human brain. Significantly, the half life of clozapine in humans has been reported as between 12 (25) and 23 (26) hours and to be very variable between individuals. By contrast, haloperidol has a half life of approximately 18 hours and this seems to be relatively constant between individuals (27). It is therefore difficult to determine appropriate treatments in rats which have different half lives for most drugs. In this study we have attempted to compensate for shorter half life by giving drugs at 5 fold higher concentrations than would be routinely given to humans (26). However, it would be equally appropriate to determine if lower doses of drug, given more frequently, could affect the density of dopamine receptors and their related second messengers in the rat brain.

In summary, the present study demonstrates that treatment with both haloperidol and clozapine caused differential changes in DA-D_1 and DA-D_2 receptors in caudate-putamen and concomitant up-regulation of DA-D_1 receptor in the olfactory tubercle. However, the same treatment did not result in any changes in the levels of AC and PKC in the rat forebrain. Importantly, this study measures the density of second messenger proteins in the rat brain, not the activity of the proteins. It therefore remains possible that neuroleptic drug treatment could alter the activity of AC and PKC, a question that now needs to be answered utilising appropriate methodology.

Acknowledgment

We thank Dr. Zinab Khalil at the North West Hospital (Parkville, Victoria) for providing animal housing facilities for this study. This work was part of the work of the NH&MRC Schizophrenia Research Unit and was also supported in part by the Rebecca L. Cooper Research Foundation, The Stanley Foundation and the Wood's Family Trust.

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