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PERSISTENT INCREASE IN STRIATAL DOPAMINE STIMULATED ADENYLATE CYCLASE ACTIVITY PERSISTS FOR MORE THAN 6 MONTHS BUT DISAPPEARS AFTER 1 YEAR FOLLOWING WITHDRAWAL FROM 18 MONTHS *CIS*-FLUPENTHIXOL INTAKE

KAMALA MURUGAIAH, SIMON FLEMINGER, ANDREAS THEODOROU, PETER JENNER and C. DAVID MARSDEN*

University Department of Neurology, Institute of Psychiatry and The Rayne Institute, King's College Hospital Medical School, Denmark Hill, London SE5, U.K.

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Abstract—Administration of *cis*-flupenthixol to rats for 18 months enhanced apomorphine-induced stereotyped behaviour, increased the number of specific [^{3}H]spiperone binding sites in striatum and potentiated striatal dopamine stimulated cyclic AMP formation, but did not alter specific [^{3}H]piflutixol binding. Following withdrawal of *cis*-flupenthixol intake, apomorphine-induced stereotypy returned to control values after 1 month and B_{max} for [^{3}H]spiperone binding returned to normal after 3 months. In contrast, the increased dopamine stimulated adenylate cyclase activity remained elevated 6 months after drug removal, but was normal 1 year after drug withdrawal.

Chronic neuroleptic treatment in man can be associated with the abnormal movements of tardive dyskinesia in up to 40% of patients [1]. The underlying cause of tardive dyskinesia is not known but present evidence suggests the involvement of cerebral dopamine receptor overactivity [2]. The movements persist following withdrawal of neuroleptic therapy in approximately 50% of those patients developing tardive dyskinesia [1]. This suggests that neuroleptics induce a change in cerebral dopamine function which endures following neuroleptic withdrawal.

Acute administration of neuroleptic drugs causes blockade of cerebral dopamine receptors but rapid tolerance develops to this effect [3–6]. Indeed, on continuous chronic administration of neuroleptic drugs to rats for 6 months or longer, the initial striatal receptor blockade is reversed [7–10]. Animals treated in this manner show enhanced stereotyped response to apomorphine and increased numbers of striatal D-2 dopamine receptor as identified by [^{3}H]spiperone. These changes are accompanied by an increase in striatal acetylcholine content as would be expected if functional dopamine receptor supersensitivity had developed [11,12]. The adaptive responses to chronic neuroleptic treatment in rats may be of relevance to the production of tardive dyskinesia in man. But do the alterations in striatal dopamine function persist following neuroleptic withdrawal?

In a previous study we showed that the increased stereotyped response and number of D-2 receptors returned to normal by 3 months following cessation of 12 months trifluoperazine treatment [10]. However, the increased dopamine stimulated cyclic AMP formation persisted throughout the 6-month withdrawal period. This persistent effect suggested altered D-1 receptor function which could be of relevance to tardive dyskinesia. In the present study we wished to discover (a) whether another neuroleptic drug, *cis*-flupenthixol, caused a similar persistent increase in striatal adenylate cyclase activity, (b) how long this effect lasted following drug withdrawal, and (c) whether changes occurred in D-1 receptors measured using [^{3}H]piflutixol binding. We administered *cis*-flupenthixol to rats for 18 months and studied striatal dopamine function in the following 12-month withdrawal period.

MATERIALS AND METHODS

Drug administration. Male Wistar rats (Olac International 218 ± 13 g at the start of the experiment) were housed in groups of eight under standard conditions of lighting (12 hr light/dark cycle) and temperature (21 ± 4°). The animals were randomly divided into two groups. One group received *cis*-flupenthixol hydrochloride (Lundbeck) at a dosage of approximately 1.5 mg/kg per day in their distilled drinking water for 18 months while the other group received distilled drinking water alone. Drug intake was adjusted at regular intervals according to water intake and body weight. The details of this experiment have been described elsewhere [13].

After 18 months of drug intake and at 1, 3, 6 and 12 months following withdrawal from *cis*-flupenthixol treatment, animals were examined for behavioural and biochemical indices of cerebral dopamine function.

Apomorphine-induced stereotyped behaviour. Stereotyped behaviour was assessed 15 min following

* To whom correspondence should be addressed.

apomorphine hydrochloride (0.5 mg/kg s.c.; MacFarlan Smith Ltd.) by observer rating as follows: 0 = animals indistinguishable from saline-treated control animals; 1 = occasional sniffing accompanied by continuous locomotor activity; 2 = continuous sniffing accompanied by discontinuous locomotor activity; 3 = occasional licking, biting or gnawing accompanied by sporadic locomotion; 4 = continuous licking, biting or gnawing with only occasional locomotor activity.

Dopamine stimulated adenylate cyclase activity. Three rats from each treatment group were killed by cervical dislocation and decapitation, and the brains rapidly removed onto ice. The paired striata from each individual animal were removed and basal and dopamine (1–150 μM) stimulated adenylate cyclase activity was measured in individual tissue homogenates according to the method of Miller *et al.* (14). Cyclic AMP formation was measured in duplicate at each dopamine concentration. The linear increase in cyclic AMP produced by dopamine was subjected to linear regression analysis and the increase in cyclic AMP formation caused by 50 μM dopamine determined.

Specific striatal [H^3]*spiperone binding.* Animals were killed by cervical dislocation and decapitation, and the brains removed and placed on ice. The paired striata were dissected out and placed in ice-cold 50 mM Tris–HCl buffer (pH 7.7). Tissue from seven animals from each group was pooled and specific [^{3}H]spiperone (26 Ci/mmole; 0.125–4.0 nM; Amersham International) binding was determined in triplicate according to the technique of Leysen *et al.* [15]. Specific binding was defined by the incorporation of 10^{-4} M dopamine (Sigma Chemical Co.). Data were analysed using Scatchard analysis and linear regression analysis of the mean individual values at each ligand concentration to determine the number of binding sites (B_{max}; pmole/g tissue) and the dissociation constant (K_D; nM).

Specific striatal [^{3}H]*piflutixol binding.* Rats were killed by cervical dislocation and decapitation, and brains were rapidly removed and placed on ice. Paired striata were dissected out and placed in ice-cold 50 mM Tris–HCl buffer (pH 7.7). Striatal tissue from three animals was pooled and specific [^{3}H]piflutixol (10.5 Ci/mmole; 0.25–4.0 nM; Lundbeck) binding determined in triplicate on two separate tissue pools according to Hyttel [16]. Total specific binding of [^{3}H]piflutixol to both D-1 and D-2 receptors was defined by incorporation of 10^{-6} M *cis*-flupenthixol (Lundbeck). Specific binding to D-1 receptors alone was defined by 10^{-6} M *cis*-flupenthixol in the presence of 3×10^{-5} M sulpiride (Delagrange). Data were analysed by Scatchard analysis and linear regression analysis of the mean individual values at each ligand concentration to determine B_{max} and K_D.

Statistical analysis. Differences in stereotyped behaviour produced by apomorphine in control and drug-treated animals were compared using Mann–Whitney U test for non-parametric data. Dopamine stimulation of cyclic AMP formation was analysed by Student's *t*-test of the 50 μM values. Differences in ligand binding were made by comparison of the intercept and slope of the regression line for each mean individual ligand concentration using the standard error of the regression line. All results represent mean values ± 1 S.E.M.

RESULTS

Drug dose and animal weights

Over the 18-month period of drug administration, intake of *cis*-flupenthixol was between 1.3 and 1.6 mg/kg per day. At the cessation of drug intake the body weight of animals receiving *cis*-flupenthixol was slightly lower than that of control animals (control animals 478 ± 10 g; *cis*-flupenthixol-treated rats 449 ± 11 g; $P < 0.05$).

Apomorphine-induced stereotyped behaviour

Administration of apomorphine hydrochloride (0.5 mg/kg s.c. 15 min previously) to the control animals produced a similar stereotyped response

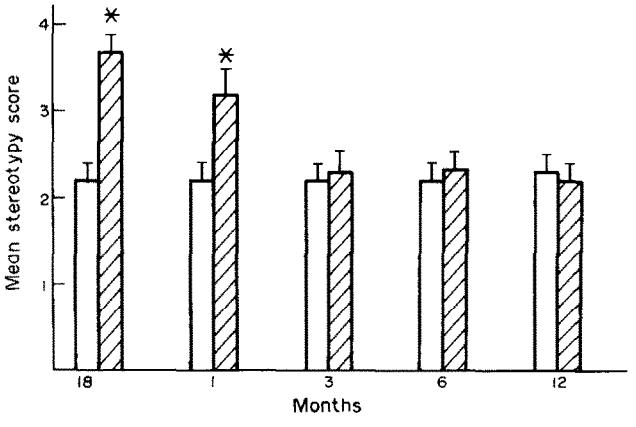

Fig. 1. Apomorphine (0.5 mg/kg s.c. 15 min previously)-induced stereotyped behaviour in rats receiving *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months and then withdrawn for up to 12 months (▨) compared to age-matched control animals (□). Values are the mean scores (±1 S.E.M.) for six individual animals from each treatment group. The effects of drug treatment were assessed using Mann–Whitney U-test $n = 6$. *$P < 0.05$ compared to control animals.

throughout the period of study, consisting of continuous sniffing, occasional licking, gnawing or biting accompanied by some exploratory activity (mean score 2.2 ± 0.2) (Fig. 1).

Following 18 months *cis*-flupenthixol (1.3–1.6 mg/kg per day) intake, apomorphine-induced stereotyped behaviour was enhanced. The enhancement was due to an increase in the number of animals exhibiting licking, gnawing or biting. After 1 months drug withdrawal, apomorphine-induced stereotypy remained enhanced, but by 3 months and thereafter this increase had disappeared (Fig. 1).

Striatal dopamine sensitive adenylate cyclase activity

Basal striatal dopamine stimulated cyclic AMP formation ranged between 15.7 and 36.6 pmole/2 mg tissue per 2.5 min in control rats and between 17.1 and 44.1 pmole/2 mg tissue per 2.5 min in *cis*-flupenthixol-treated animals. This variation was not age-dependent and was due to inherent day-to-day variation.

Administration of *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months caused an increase in striatal cyclic AMP formation in response to dopamine (1–150 μM) (Table 1;). Increased cyclic AMP formation was apparent also at 1, 3 and 6 months following cessation of *cis*-flupenthixol administration. However, after 12 months drug withdrawal, cyclic AMP formation was not different from that found in striatal tissue from age-matched control animals.

Specific striatal [^{3}H]*spiperone binding*

Continuous administration of *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months enhanced the number of striatal specific [^{3}H]spiperone binding sites (Fig. 2; Table 2). The increase in B_{max} was apparent also 1 and 3 months following drug withdrawal but returned to normal values by 6 months and thereafter. The dissociation constant for striatal [^{3}H]spiperone binding was elevated after 18 months *cis*-flupenthixol intake. This increase was exaggerated 1 month after drug withdrawal but K_D had returned to control values 3 months after drug withdrawal. However, 6 and 12 months after cessation of drug intake, K_D fell below control values although this was a significant difference only at 6 months.

Specific [^{3}H]*piflutixol binding*

Specific striatal binding of [^{3}H]piflutixol, as defined using 10^{-6} M *cis*-flupenthixol alone or in the presence of 3×10^{-5} M sulpiride, was not different in animals receiving *cis*-flupenthixol for 18 months compared to age-matched control animals (Fig. 3). Similarly, 6 months following drug withdrawal, B_{max} and K_D for specific [^{3}H]piflutixol were not different from control values (Fig. 3).

DISCUSSION

Continuous chronic administration of *cis*-flupenthixol for 18 months resulted in the appearance of striatal dopamine receptor super-sensitivity as judged by enhanced apomorphine-induced stereotypy and increased numbers of [^{3}H]spiperone binding sites. This confirms the previous finding of ourselves

Table 1. Formation of cyclic AMP produced by dopamine (50 μM) in striatal preparations from animals treated with *cis*-flupenthixol (1.3–1.6 mg/kg per day for 18 months and then withdrawn for up to 12 months compared to age-matched control animals

Length of drug treatment	Cyclic AMP formed (pmoles/2 mg tissue/2.5 min)	
	Control	*cis*-flupenthixol
18 months	7.9 ± 0.3	30.4 ± 0.8*
Drug withdrawal		
1 month	19.7 ± 0.6	31.6 ± 1.2*
3	20.0 ± 1.2	45.4 ± 6.0*
6	15.7 ± 1.3	26.8 ± 4.0*
12	18.7 ± 8.7	14.8 ± 3.3

Formation of cyclic AMP over basal levels was determined using range of dopamine (1–150 μM) concentrations. Using regression analysis the amount of cyclic AMP formed by 50 μM dopamine was calculated. Differences between control and drug-treated animals were determined using a two-tailed Student's *t*-test. The values shown represent the mean (±1 S.E.M.) for three individual animals examined in duplicate at each dopamine concentration. n =6.
*P <0.05 compared to control animals.

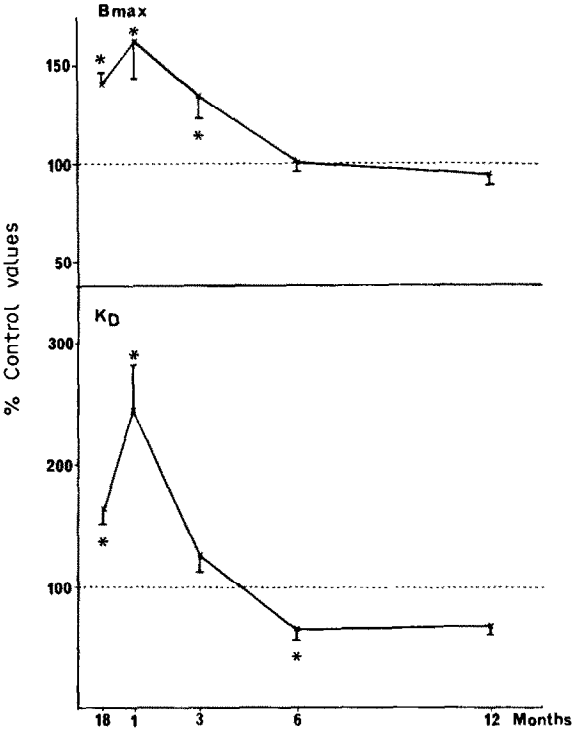

Fig. 2. Alterations in the number of binding sites (B_{max}) and the dissociation constant (K_D) for specific [^{3}H]spiperone binding to striatal preparations in animals receiving *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months and then withdrawn for up to 12 months expressed as a per cent of values found in age-matched control animals. n = 7. *P < 0.05.

Table 2. The number of binding sites (B_{max}; pmole/g tissue) and dissociation constant (K_D; nM) for specific [^{3}H]spiperone (0.125–4.0 nM) binding to striatal membrane preparations from animals receiving *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months and then withdrawn for up to 12 months compared to age-matched control animals

Length of drug treatment	[^{3}H]spiperone			
	B_{max} (pmoles/g tissue)		K_D (nM)	
	Control	*cis*-flupenthixol	Control	*cis*-flupenthixol
18 months	10.2 ± 0.8	14.4 ± 0.6*	0.14 ± 0.60	0.23 ± 0.02*
Drug withdrawal				
1 month	9.5 ± 0.9	15.5 ± 1.5*	0.20 ± 0.05	0.49 ± 0.09*
3	13.3 ± 0.6	17.9 ± 1.1*	0.36 ± 0.03	0.46 ± 0.05
6	14.3 ± 1.2	14.5 ± 0.9	0.28 ± 0.04	0.17 ± 0.02*
12	11.4 ± 1.4	10.8 ± 0.5	0.40 ± 0.10	0.27 ± 0.03

The results are expressed as the values (±1 S.E.M.) obtained for the intercept and slope of the regression analysis carried out on the individual values obtained for each ligand concentration. Differences between control and drug-treated animals were assessed using the standard error of the whole regression in each case. Assays were carried out on the pooled tissue from seven animals, examining each ligand concentration in triplicate. $n = 7$.

*P <0.05 compared to control animals.

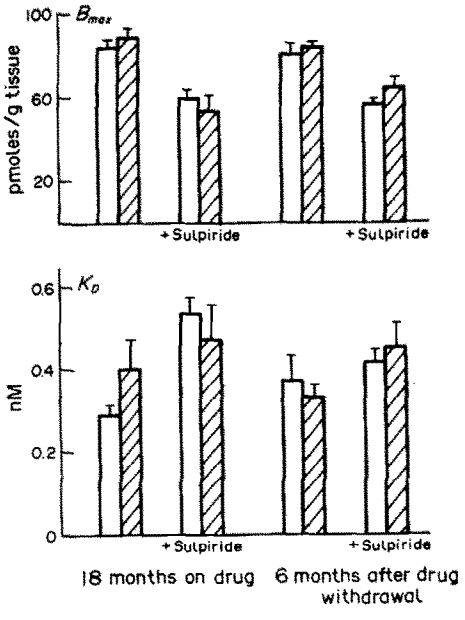

Fig. 3. The number of binding sites (B_{max}; pmole/g tissue) and the dissociation constant (K_D; nM) for (A) total specific [^{3}H]piflutixol binding (as defined using 10^{-6}M *cis*-flupenthixol) and (B) specific [^{3}H]piflutixol binding in the presence of 3×10^{-5} M sulpiride to striatal preparations from rats receiving *cis*-flupenthixol (1.3–1.6 mg/kg per day) for 18 months (▨) and then withdrawn for 6 months compared to age-matched control animals (□). The results are expressed as the values obtained from the intercept and slope of the regression analysis carried out on the individual points obtained for each ligand concentration. Differences between control and drug-treated animals were assessed using the standard error of the whole regression in each case. Assays were carried out on the pooled tissue from three animals on two separate occasions, examining each ligand concentration in triplicate. $n = 6$ on each occasion. *P <0.05 compared to control animals.

and others that a variety of neuroleptic drugs induce this effect [7–10, 17, 18].

Cerebral dopamine receptors are thought to exist in multiple forms [19]. A common division is into those receptors linked to the enzyme adenylate cyclase (D-1) and those which act independently of this enzyme (D-2) [20]. It is currently considered that all functional effects of dopamine receptor occupation are mediated via D-2 receptors [19,21]. There is no known physiological role for cerebral D-1 receptors.

The increase in striatal [^{3}H]spiperone binding sites suggests increased numbers of adenylate cyclase independent dopamine receptors for, in the concentrations employed, this ligand is selective for D-2 receptors. The enhanced apomorphine-induced stereotyped behaviour is considered to be mediated via these receptors. The increase in stereotypy was apparent only 1 month after drug withdrawal and then disappeared. However, alteration of striatal D-2 function, as judged by [^{3}H]spiperone binding, persisted for up to 3 months following drug withdrawal. This sequence of events is similar to that observed following termination of 12 months administration of trifluoperazine [10].

Chronic *cis*-flupenthixol administration for 18 months also caused an increase in striatal dopamine stimulated adenylate cyclase suggesting altered D-1 receptor function. However, at this time we were unable to observe any increase in D-1 receptor numbers as judged by [^{3}H]piflutixol binding. Specific [^{3}H]piflutixol binding identifies both D-1 and D-2 receptors but by incorporating a high concentration of a selective D-2 antagonist sulpiride, it is possible to examine D-1 receptors alone ([22]; unpublished observation). Total specific [^{3}H]piflutixol binding to both D-1 and D-2 receptors also did not reflect the increase in D-2 sites observed with [^{3}H]spiperone. However, the actual increase in number of D-2 sites

is small in comparison with the total number labelled by [^{3}H]piflutixol, so perhaps this is not so surprising.

The increase in dopamine stimulated adenylate cyclase occurs, therefore, in the absence of any change in the number of dopamine D-1 recognition sites. It is possible that the enhanced response is caused by a change in D-1 receptor occupancy for total occupation may not be necessary to elicit a maximal pharmacological response. The alternative explanation is that neuroleptic treatment enhances cyclic AMP formation evoking some change beyond the D-1 recognition site. A number of possible sites exist for such an action, but one has been shown to be affected by neuroleptic treatment. Repeated administration of neuroleptic drugs causes alteration in membrane-bound calmodulin and protein phosphorylation [23]. It may be that the altered adenylate cyclase response is evoked by long-term neuroleptic treatment acting at this level.

The increase in striatal adenylate cyclase activity persisted for between 6 and 12 months following cessation of *cis*-flupenthixol intake. Again, the increase was apparent after 6 months withdrawal in the absence of altered D-1 receptor sites as labelled by [^{3}H]piflutixol or of D-2 sites as labelled by [^{3}H]spiperone. Why this effect should be present for such prolonged periods remains to be determined, but it is unlikely to be due to residual drug; all other parameters had returned to normal. However, eventually even striatal adenylate cyclase activity returns to normal after 1 year of drug withdrawal.

In conclusion, continuous chronic neuroleptic treatment caused a persistent increase in striatal adenylate cyclase activity which occurred independent of alteration in neuronal receptors. If such a change is responsible for persistent tardive dyskinesia it may be the absence of accompanying receptor changes which makes this such a difficult therapeutic problem.

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