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EVIDENCE FOR AN INHIBITORY PRESYNAPTIC COMPONENT OF NEUROLEPTIC DRUG ACTION

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1 The action of five neuroleptic drugs (haloperidol, *cis*-flupenthixol, chlorpromazine, fluphenazine and thioridazine) was studied on the synthesis and release of dopamine from rat striatal synaptosomes.

2 *In vitro* application of the drugs induced an inhibition of synthesis of [^{14}C]-dopamine from L-[U-^{14}C]-tyrosine and a decrease in the tissue content of [^{14}C]-dopamine, with IC_{50} values for the latter effect ranging from 3.6×10^{-7} to 5.9×10^{-5} M. The rank order of their potency was similar to the order of their clinical effectiveness: haloperidol>fluphenazine>*cis*-flupenthixol>chlorpromazine> thioridazine. *Trans* flupenthixol was without effect up to a concentration of 10^{-4} M.

3 The tissue level and release of GABA were not affected by concentrations of the neuroleptics up to 10^{-4} M.

4 When the neuroleptics were administered *in vivo*, changes were also detected in the synthesis and release of [^{14}C]-dopamine from subsequently prepared synaptosomes. A marked inhibition of the K^+-induced increase in [^{14}C]-dopamine synthesis was seen following a dose of 2 mg/kg *cis*-flupenthixol and haloperidol. At this concentration, haloperidol also increased the control release of [^{14}C]-dopamine and reduced the K^+-induced increase in release of [^{14}C]-dopamine.

5 *Cis*-flupenthixol at a dose of 20 mg/kg reduced the K^+-induced release of [^{14}C]-dopamine by 48% and to a lesser extent, that of γ-aminobutyric acid (GABA, 25%).

6 An inhibitory mode of action is proposed for neuroleptics mediated through a presynaptic mechanism.

Introduction

In the past few years, accounts of the mode of action of neuroleptic drugs have mainly concentrated on the ability of these agents to cause blockade of postsynaptic dopamine receptors in the striatum and substantia nigra and the linked adenylcyclase (Miller, Horn & Iversen, 1974; Aghajanian & Bunney, 1977), although interactions with presynaptic receptors or other presynaptic sites have also received attention (Farnebo & Hamberger, 1971; Kehr, Carlsson, Lindqvist, Magnusson & Atack, 1972; Christiansen & Squires, 1974a; Westfall, Besson, Giorgieff & Glowinski, 1976; Iversen, Rogawski & Miller, 1976; Dismukes and Mulder, 1977). However, the more recent advances in radioligand binding studies of dopamine receptors have shown the best correlation between clinical potency of neuroleptics as antipsychotic drugs and their ability to displace [^{3}H]-haloperidol from striatal membranes (Seeman, Chau-Wong, Tedesco & Wong, 1975; Creese, Burt & Snyder, 1976). Dopamine receptors are located at both pre- and postsynaptic sites in the striatum and although these may be distinguished to a certain extent pharmacologically (Kebabian & Calne, 1979), the aim of the present study was to concentrate on studying effects of neuroleptics on changes in dopamine synthesis and release, mediated specifically through presynaptic receptors.

Large increases in the stimulation of the turnover of striatal dopamine are seen after acute neuroleptic treatment, as judged by *increases* in: (i) production of homovanilic acid and dihydroxyphenylacetic acid, (ii) rates of synthesis of dopamine, and (iii) catabolism of dopamine (Andén, Roos & Werdinius, 1964; Nybäck & Sedvall, 1968; Hyttel, 1974a). This *stimulatory* action of neuroleptic drugs appears to be due to an increased activity of tyrosine hydroxylase resulting from a decrease in the K_m for its pteridine cofactor (Zivkovic & Guidotti, 1974). However, these changes are maximal within a few hours of administration of the drugs and return to normal 8 to 48 h later (Nybäck & Sedvall, 1968; Hyttel, 1974b; Bürki, Ruch & Asper, 1975) and are reduced during chronic drug treatment. For this reason their relevance as part of a description of the mode of action of

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these neuroleptics in man must be questioned since their therapeutic action at the low daily doses normally given (e.g. 0.1 μmol/kg *cis*-flupenthixol; (Enna, Bennett, Burt, Creese & Snyder, 1976) takes several weeks to develop.

Other actions of neuroleptics have been described which may be equally or more important in deciphering the details of drug action. These contrast with those described above which highlight a *stimulation* of metabolism since they introduce substantial *inhibition* of dopamine synthesis (e.g. 50%; Hyttel 1977) and a decrease in the size of the dopamine pool (Asper, Baggiolini, Bürki, Lauener, Ruch & Stille 1973; Kehr, Carlsson & Lindqvist, 1977). We present evidence in this paper that these inhibitory effects are due to an action of the drugs on presynaptic sites, since they can be demonstrated to occur in incubated synaptosomes from rat corpus striatum.

Whereas earlier studies (Christiansen & Squires 1974a,b) on neuroleptic action showed their ability to reverse the inhibitory action of apomorphine on tyrosine hydroxylase, the present results show a direct action of these drugs on dopamine synthesis in synaptosomes. However, these effects appear to correlate with clinical potency which was not the case for catechol formation from striatal homogenates (Iversen *et al.*, 1976). We also present evidence that neuroleptics administered *in vivo* inhibit the depolarization-induced responses of endogenous dopamine from subsequently isolated synaptosomes. These data complement the results of Seeman & Lee (1975), where an inhibition of the depolarization-induced release of preloaded dopamine from striatal slices was seen in response to the drugs administered *in vitro*, and defines the site of action.

Methods

In vivo *injection of neuroleptic drugs*

Neuroleptic drugs or control saline (0.9% w/v NaCl solution) injections were administered intraperitoneally to Sprague Dawley female rats (200 to 250 g body wt) 30 min before they were killed, in 0.5 ml volumes. Hydrochloride salts of *cis*-flupenthixol, *trans*-flupenthixol and chlorpromazine were given as solutions in saline. Haloperidol was used in solution ('Serenace') as supplied by the manufacturers (G.D. Searle & Co. Ltd.). The doses used are indicated in the legends to figures.

Preparation and incubation of synaptosomes

Synaptosomes were prepared from corpus striatum of treated or untreated rats as appropriate. The method used was a modified form of that originally described by Gray & Whittaker (1962). A 10% homogenate of tissue in 0.32 M sucrose was loaded onto a discontinuous sucrose gradient of 1.2 M/0.8 M sucrose and centrifuged at 75,000 *g* for 1 h. The synaptosome fraction occurring at the 0.8/1.2 M sucrose interface was removed and centrifuged at 65,000 *g* for 25 min. The resulting pellets were suspended (5 to 6 mg protein/ml) in Krebs-bicarbonate medium of the following composition (mM): NaCl 124, KCl 5, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.3, $CaCl_2$ 0.75, $NaHCO_3$ 26, glucose 10, L-ascorbate 1.8 and nialamide 0.54; pH 7.4, gassed with 95% O_2 and 5% CO_2. After 10 min preincubation at 37°C, L-[U-^{14}C]-tyrosine was added to give a final concentration of 4.9 μM and a final specific radioactivity of 513 mCi/mmol. Incubation was carried out for a further 30 min and where appropriate, neuroleptic drugs were included from the start of this incubation period. The hydrochloride salts of these drugs or portions of haloperidol were dissolved in Krebs-bicarbonate medium. Equivalent volumes of Krebs-bicarbonate medium were added to the controls. Potassium stimulation was initiated after 20 min of incubation by addition of 1 M KCl, made up in Krebs-bicarbonate medium, to give a final concentration of 56 mMK^+. An equivalent aliquot of Krebs-bicarbonate medium was added to the control incubations.

Extraction and analysis of [^{14}C]-*dopamine and γ-aminobutyric acid (GABA)*

At the end of incubation, synaptosomes were sedimented at room temperature in a bench ultracentrifuge, the synaptosomes and supernatant were then analysed separately using the same procedure for each. The samples were extracted with perchloric acid (0.4 M final concentration) containing sodium metabisulphite (15 mM), carrier dopamine (0.5 to 1 μmol), and internal standards of L-[U-^{14}C]-valine (15 to 37.5 nCi) and norleucine (50 to 150 nmol). The extracts were fractionated and analysed by automated cation exchange chromatography linked to fluorimetric analysis and an 'on line' liquid scintillation counter as described in detail elsewhere (de Belleroche, Dykes & Thomas, 1976). Values were related to tissue protein determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Estimates of total synthesis of [^{14}C]-dopamine were obtained by combining tissue and supernatant values of [^{14}C]-dopamine.

Results

The effects of neuroleptics on dopamine synthesis by striatal synaptosomes: in vitro *application of neuroleptic drugs*

When synaptosomes were incubated in the presence

of several neuroleptic drugs at concentrations of 10^{-7} M and above, inhibition of synthesis of [^{14}C]-dopamine from L-[U-^{14}C]-tyrosine occurred. This inhibition is seen as a reduction in the tissue content of [^{14}C]-dopamine and the total level of [^{14}C]-dopamine formed which was obtained by adding the tissue content to the amount of [^{14}C]-dopamine released during incubation. Haloperidol was the most potent agent causing inhibition of [^{14}C]-dopamine synthesis, with *cis*-flupenthixol, chlorpromazine and thioridazine following in rank order of potency. This is expressed in Table 1 as values which caused 50% inhibition of synthesis (i.e. IC_{50}) and the rank order correlates well with the clinical efficacy of these drugs as antipsychotic agents (Seeman & Lee 1975; Enna *et al.*, 1976). Fluphenazine did not show the typical pattern shown by the other agents, but displayed instead two phases of inhibition. At low concentrations (5×10^{-8} to 5×10^{-7} M), the potency (expressed as IC_{50}) was similar to that for haloperidol. Neuroleptic concentrations of 10^{-9} to 10^{-4} M were used for the study, since the inactive isomer, *trans*-flupenthixol did not cause inhibition of [^{14}C]-dopamine synthesis in this range. However, at 2×10^{-4} M and above, even *trans*-flupenthixol was inhibitory in its action. No significant change in the tissue level or release of GABA to the medium was seen in the concentration range used (below 10^{-4} M) in these experiments (Figure 1).

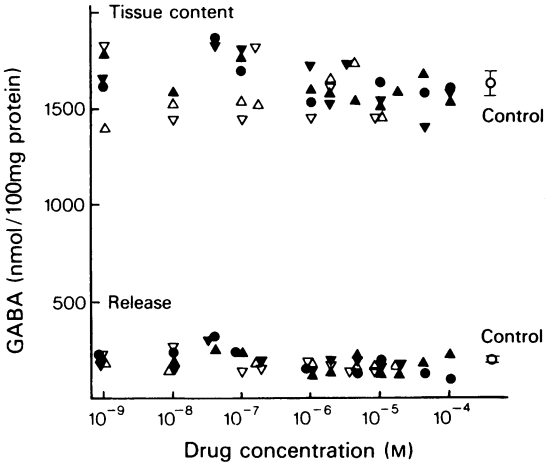

Figure 1 Effect of neuroleptics on γ-aminobutyric acid (GABA) content of striatal synaptosomes and its release. Striatal synaptosomes were incubated in the presence of neuroleptics as described in the legend of Table 1. At the end of incubation, the GABA content of the tissue and GABA released to the incubation medium were measured. These values were related to the tissue protein. Control levels of GABA (○) were obtained in the absence of drugs, the bars indicate the s.e.means (n = 20). GABA values in the presence of neuroleptics are means (n = 2 to 4), the s.e.means being approximately 10%: (▲) Thioridazine; (▼) chlorpromazine; (●) fluphenazine; (△) *cis*-flupenthixol; (▽) *trans*-flupenthixol. Data points have been shifted slightly to the left where necessary.

Table 1 Inhibition of synaptosomal [^{14}C]-dopamine synthesis from L-[U-^{14}C]-tyrosine by neuroleptics

	IC_{50} (M) *Tissue* [^{14}C]-*dopamine*	IC_{50} (M) *Total* [^{14}C]-*dopamine*
Haloperidol	3.55×10^{-7}	1.26×10^{-6}
Fluphenazine	3.72×10^{-7}	1.43×10^{-6}
cis-Flupenthixol	4.88×10^{-6}	2.33×10^{-5}
Chlorpromazine	2.75×10^{-5}	3.33×10^{-5}
Thioridazine	5.89×10^{-5}	3.98×10^{-4}

Rat striatal synaptosomes were incubated in Krebs-bicarbonate medium containing 4.9 μM L-[U-^{14}C]-tyrosine (513 mCi/mmol) and various concentrations of neuroleptics for 30 min as described in the methods. The control value for tissue incubated in the absence of drugs, tissue-[^{14}C]-dopamine was 2691.6 ± 53.8 nCi/100 mg protein (Mean value ± s.e.mean for 22 determinations). The tissue content and supernatant content of [^{14}C]-dopamine related to tissue protein were measured and combined to give total [^{14}C]-dopamine values. The control value for total [^{14}C]-dopamine was 3003.8 ± 68.9 nCi/100 mg protein (mean value ± s.e. mean for 20 determinations).

Inhibition of synthesis of [^{14}C]-dopamine caused by the neuroleptics was analysed by regression analysis of the data, to give IC_{50} values. Values for the coefficient of correlation and number of experiments (n) are given in parentheses.

Tissue [^{14}C]-dopamine: (a) haloperidol (0.931, n = 10); (b) fluphenazine (0.769, n = 6); (c) *cis*-flupenthixol (0.918 n = 11); (d) chlorpromazine (0.845, n = 15); (e) thioridazine (0.793, n = 24); (f) fluphenazine (0.887, n = 6); (g) *trans*-flupenthixol (−0.144, n = 10).

Total [^{14}C]-dopamine: (a) haloperidol (0.926, n = 9); (b) fluphenazine (0.861, n = 8); (c) *cis*-flupenthixol (0.89, n = 11); (d) chlorpromazine (0.805, n = 18); (e) thioridazine (0.795, n = 19); (f) fluphenazine (0.988, n = 8); (g) *trans*-flupenthixol (−0.54, n = 10).

Effect of neuroleptics on dopamine synthesis and release from striatal synaptosomes: in vivo *application of drugs*

Synaptosomes were prepared from the corpus striatum of rats which had been injected with haloperidol (2 mg/kg), *cis*- or *trans*-flupenthixol (2 mg/kg or 20 mg/kg) or saline. The rate of synthesis of [^{14}C]-dopamine by these synaptosomes during incubation in the presence of L-[U-^{14}C]-tyrosine was measured. *In vivo* pretreatment with *trans*-flupenthixol caused no detectable inhibition of synthesis compared to the controls of saline-treated animals. In contrast, haloperidol and *cis*-flupenthixol inhibited synthesis to a similar degree when given at a dose of 2 mg/kg. *Cis*-flupenthixol (20 mg/kg) caused a 20% inhibition of synthesis (Figure 2a). We have previously shown that in response to K^+ depolarization, the synthesis of dopamine from L-[U-^{14}C]-tyrosine by striatal synaptosomes is stimulated by approximately 60% during 10 min exposure to K^+ (de Belleroche *et al.*, 1976; de Belleroche & Bradford, 1978). This increase in synthesis is inhibited by injection of either neuroleptic drug before preparation of the synaptosomes (Figure 2b). No significant changes in the tissue levels of GABA were detected. In contrast to the similar effects produced by neuroleptics on [^{14}C]-dopamine (Figure 3), the patterns of release of [^{14}C]-dopamine from the synaptosomes differed. Thus, haloperidol pretreatment enhanced release by unstimulated synaptosomes considerably (Figure 3) whereas *cis*-flupenthixol had little or no effect. The amount of dopamine released by K^+ depolarization was reduced by the neuroleptics, e.g. 48% inhibition was produced by a dose of 20 mg/kg *cis*-flupenthixol. No significant change in the control or stimulated release of GABA was detected, except at the higher dose of *cis*-flupenthixol where a 25% decrease in release was observed in response to K^+ stimulation relative to the effect with the equivalent dose of *trans*-flupenthixol (Figure 3).

Discussion

Presynaptic site of action

The present study shows that neuroleptics of different classes are themselves able to reduce the rate of synthesis of dopamine from tyrosine by striatal synaptosomes and the tissue content of dopamine when presented to synaptosomes *in vitro* in micromolar concentrations (IC_{50} values tissue [^{14}C]-dopamine 3.6 $\times 10^{-7}$ M to 5.9 $\times 10^{-5}$ M). A comparable reduction of tissue dopamine content is known to occur *in vivo* and in the present study this was reflected in subsequently isolated synaptosomes. It was paralleled by a reduced rate of synthesis of dopamine in response to K^+ depolarization. This effect is of a quite different category from that produced by neuroleptics at lower con-

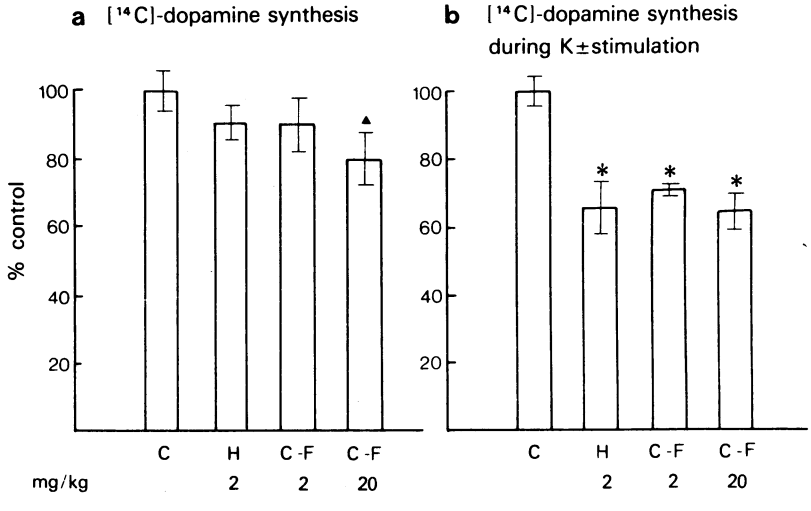

Figure 2 Effect of neuroleptics administered *in vivo* on the formation of [^{14}C]-dopamine in striatal synaptosomes. Striatal synaptosomes were prepared from rats that had been injected intraperitoneally 30 min before they were killed with: saline (C), haloperidol (H), or *cis*-flupenthixol (C-F) at the doses indicated. These were incubated in Krebs-bicarbonate medium containing 4.9 μM L-[U-^{14}C]-tyrosine (513 mCi/mmol) for 30 min. K^+-stimulation (b) was carried out during the final 10 min of incubation in the presence of 56 mM K^+. Total [^{14}C]-dopamine formed under the different conditions is expressed as a percentage of the value obtained for saline control, incubated under either control (a) or K^+-stimulated conditions (b). Values are means, with the s.e.means indicated by bars for $n = 4$ to 6. Asterisks indicate that the value is significantly reduced compared to the control; *$P<0.01$; ▲$P<0.05$.

centrations where the apomorphine-induced inhibition of dopamine synthesis has been reported to be partially *reversed* by neuroleptics (Christiansen & Squires 1974a,b; 60 to 25% reversal with drug concentrations, 1.1 to 5.4 × 10^{-7} M). The higher concentrations of drugs used *in vivo* in this study were employed to demonstrate the inhibition caused by the neuroleptic in its own right and should approximate to the tissue concentrations of drug obtained when successive smaller doses of drug are used.

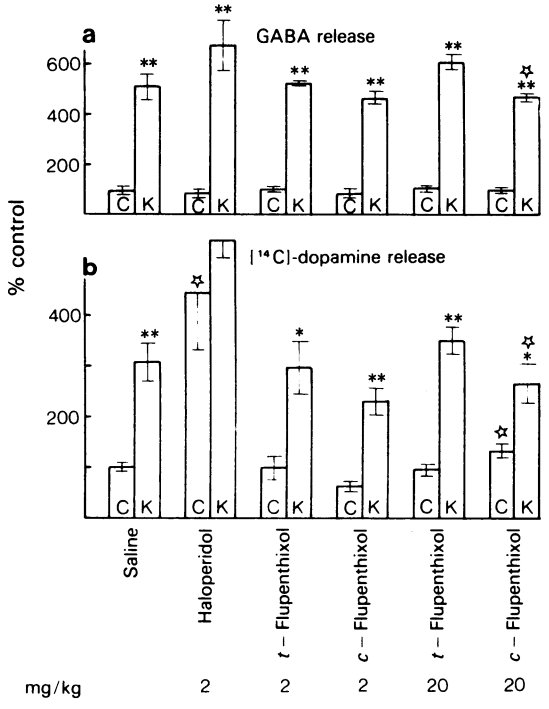

Figure 3 Effects of neuropeltics administered *in vivo* on the release is γ-aminobutyric acid (GABA) and dopamine from striatal synaptosomes. Rat striatal synaptosomes were prepared and incubated as described in the legend to Figure 2. Release of GABA (a) and [^{14}C]-dopamine (b) to the medium was measured under control (C) and K^+-stimulated conditions (K), and is expressed as a percentage of the control release obtained from the synaptosomes of saline-injected animals. Values are means, with the s.e.means indicated by bars (n = 4 to 6). Significance of differences is as follows: in (a) GABA release due to K^+ is significantly greater than the control (**$P<0.001$); release in the presence of the *cis*-isomer is significantly reduced compared to release in the presence of the *trans*-isomer (☆$P<0.025$).In (b), [^{14}C]-dopamine release due to K^+ is significantly greater than the control (**$P<0.001$; *$P<0.005$); release due to haloperidol or *cis*-flupenthixol is significantly different compared to the saline or *trans*-flupenthixol treated animals respectively, incubated under similar conditions (☆$P<0.05$).

The inhibitory effects of the neuroleptic on dopamine synthesis described here were seen both in synaptosomes isolated from pretreated animals and in synaptosomes exposed to these drugs for the first time *in vitro*. Therefore it can be concluded that these agents not only affect the performance of nerve-terminals *in situ* but must be acting directly at this presynaptic site. Any interaction which occurred between the drugs and the postsynaptic structures present on synaptosomes (i.e. postsynaptic membrane, receptors and postsynaptic thickenings) could not have led to any action on tyrosine hydroxylase activity, or on rates of dopamine release, since these postsynaptic structures have no known functional link with the presynaptic region of the synaptosome.

At present we have not investigated whether these presynaptic neuroleptic actions are produced by interaction with receptors, either dopaminergic or other types. However, presynaptic receptors which can modulate dopamine turnover and relase do exist on striatal dopaminergic terminals (Nagy, Lee, Seeman & Fibiger, 1978; de Belleroche, Luqmai & Bradford, 1979) and these may be involved in producing the effects described here.

The consequence of this inhibitory action of neuroleptics would be that less dopamine is synthesized following neuroleptic-treatment, particularly in response to nerve-terminal activation and therefore dopaminergic transmission would be reduced because of diminished transmitter output during activity in the nigrostriatal pathways. This reduction in the effectiveness of presynaptic transmitter synthesis and release would add to any direct antagonistic action of neuroleptics on the postsynaptic receptors of striatal cells and would not be counter to such a mechanism of action.

The greatly enhanced dopamine turnover seen *in vivo* in response to neuroleptics has not been demonstrated in isolated striatal tissue and may depend on the presence of the intact nigrostriatal system. This is supported by the observation that section of the nigrostriatal pathway also prevents the enhancement, and it may be that the site of action for this effect is located on nigral dopaminergic cell bodies or dendrites (Zivkovic, Guidotti & Costa, 1975). Alternatively, it may be that impulse traffic at the striatal dopaminergic nerve-terminals is essential for the effect to occur.

Clinical significance

The possible significance of the inhibitory effects described here, in understanding the basis of the clinical action of neuroleptics, relates to the similarity in the rank order of the potency of these drugs of both phenothiazine and butyrophenone classes, in the two circumstances. Also, *trans*-flupenthixol (in contrast to the *cis*-isomer) was observed to be inactive in our

system, which correlates with its clinical ineffectiveness.

The ability of a wide range of neuroleptics to displace radiolabelled haloperidol from striatal membranes (Seeman *et al.*, 1975; Creese *et al.*, 1976) shows good correlation with their potency as antipsychotic drugs. Although neuroleptics antagonize agonist action at dopamine receptors at both pre- and postsynaptic sites, the relatively weak antagonism of the postsynaptically located dopamine-sensitive adenylcyclase by the butyrophenone drugs does not correspond with their potency. Hence, it is likely that an important component of neuroleptic action occurs at other receptors either presynaptic ones, as indicated from this study or postsynaptic receptors not linked to adenylcyclase (Kebabian & Calne, 1979).

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