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DEVELOPMENT OF SUPERSENSITIVITY OF APOMORPHINE-INDUCED INCREASES IN ACETYLCHOLINE LEVELS AND STEREOTYPY AFTER CHRONIC FLUPHENAZINE TREATMENT*

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Summary—Dopamine agonists administered systemically produce an increase in striatal levels of acetylcholine (ACh). Possible development of postsynaptic dopamine receptor supersensitivity after neuroleptic treatment was studied by measurement of apomorphine (APO)-induced increase in ACh levels in the striatum and olfactory tubercle. Apomorphine-induced stereotypic behaviour was also measured. Rats received a single subcutaneous injection of either sesame seed oil vehicle or fluphenazine (FLU) decanoate (10 mg kg^{-1}), a long-acting neuroleptic preparation. After 14 days, rats received APO intraperitoneally, in various doses (0.03–1.0 mg kg^{-1}). Fifteen minutes later, brain tissue was rapidly fixed by microwave irradiation, dissected, and ACh levels determined by means of gas chromatography. Acetylcholine levels were 75 nmol g^{-1} in olfactory tubercle and 70 nmol g^{-1} in striatum. Apomorphine treatment resulted in dose-dependent increases of ACh level in both regions. Apomorphine-induced increases were greater in rats pretreated with FLU than in controls. Using 0.1 mg kg^{-1} APO, the higher striatal ACh-elevating effect found 14 days after FLU treatment was also present 21 days, but not 27 days after FLU treatment. At 21 days after subcutaneous injection, 0.25 mg kg^{-1} APO induced significantly greater stereotypic behaviour in FLU-treated rats than in controls. Thus, FLU treatment led to an apparent temporary supersensitivity of APO-induced increases in ACh levels and stereotypic behaviour.

There have been numerous reports in which chronic blockade of synaptic transmission, by lesion of the presynaptic neurones or by pharmacological antagonism of the transmitter substance, resulted in an enhancement of the observed effects associated with synaptic transmission. This has been interpreted as evidence for the development of postsynaptic receptor supersensitivity. Recent studies also indicate the occurrence of this phenomenon in dopaminergic neuronal systems in the CNS. After chronic lesion of the dopaminergic nigro-neostriatal pathway, there is an increase in the stereotypic response to the direct-acting dopaminergic agonist, apomorphine (APO) (Price and Fibiger, 1974). Chronic treatment with neuroleptic agents, which have dopaminergic antagonist properties, also result in increased stereotypies induced by APO given after withdrawal of the neuroleptic agent (Klawans and Rubovits, 1972; Tarsy and Baldessarini, 1974; Sayers, Bürki, Ruch and Asper, 1975; Smith and Davis, 1975, 1976).

In addition to stereotypic movements, striatal acetylcholine (ACh) concentration is another parameter of dopaminergic nigro-neostriatal transmission that can be studied. Numerous studies indicate that there seems to be an inverse relationship between ACh levels and cholinergic neuronal activity, and that the dopaminergic nigro-neostriatal neurones act to inhibit cholinergic interneurones in the striatum (for review, see Roth and Bunney, 1976). Thus, APO treatment results in an increase in striatal ACh levels (Consolo, Ladinsky and Garattini, 1974; McGeer, Grewaal and McGeer, 1974; Sethy and Van Woert, 1974) and a decrease in ACh turnover (Trabucchi, Cheney, Racagni and Costa, 1975).

Using this paradigm, Fibiger and Grewaal (1974) demonstrated that 2 months after unilateral lesion of the dopaminergic nigro-neostriatal pathway, APO treatment resulted in a greater increase in striatal ACh level on the lesioned side as compared to the intact side. Their study provided neurochemical evidence for denervation supersensitivity.

This paper reports on the effect of chronic treatment with the neuroleptic, fluphenazine (FLU), on the APO-induced increase in striatal and olfactory tubercle ACh levels and in stereotypic behaviour. It indicates the development of dopaminergic supersensitivity after withdrawal from chronic neuroleptic treatment.

METHODS

Subjects

Male Sprague–Dawley rats weighing 150–200 g (Charles River, Inc., Wilmington, MA) were used.

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Key words: Acetylcholine, apomorphine, fluphenazine, olfactory tubercle, stereotypy, striatum, supersensitivity.

They were kept in metal cages and offered food and water *ad libitum*. The cages were kept under diurnal lighting cycle and controlled temperature and humidity.

Drug treatments

The rats were given a subcutaneous injection of either 0.08 ml fluphenazine decanoate, 25 mg ml^{-1} (Prolixin decanoate®) or 0.08 ml sesame seed oil as control. A previous study has shown that the single subcutaneous injection of FLU decanoate was equivalent to a chronic administration of the drug (Nowycky and Roth, 1977). Indirect measurement of the presence of pharmacologically active concentrations of FLU in the brain was done by examining the antagonism of the APO- or amphetamine-induced inhibition of firing rate in substantia nigra dopaminergic neurons (Bunney, Aghajanian and Roth, 1973). Two days after a single injection of FLU decanoate, inhibition of firing rate was completely blocked, indicating the presence of effective levels of FLU. Inhibition of firing rate gradually returned to normal after 8 days. Thus, this route of administration of FLU subjects the rats to effective levels of FLU for about one week. On either 14, 21 or 28 days after the initial injection, rats were given an intraperitoneal injection of either apomorphine hydrochloride or saline. The doses of APO ranged from 0.03 to 1.0 mg kg^{-1}. The rats were sacrificed 15 min later.

Method of sacrifice

Each rat was killed by microwave irradiation directed to the head. Duration of microwave exposure (sec) was approximately 1/70 of body weight (g). The microwave device used was the Model LMM of Medical Engineering Consultants (Lexington, MA). The microwave output is 1.3 kW at a frequency of 2.45 GHz.

Dissection and estimation of ACh concentration

The fixed brain was dissected and the olfactory tubercles and striata were weighed, then homogenized in 2 ml ice-cold 15% 1 *N* formic acid/85% acetone, to which 0.1 ml 50 μM propionylcholine bromide in 0.1 M sodium acetate buffer (pH 4.5) was added as internal standard. The homogenates were processed essentially as described by Freeman, Choi and Jenden (1975), except that the samples did not receive silver tosylate in acetonitrile and propionyl chloride. Control samples containing equimolar quantities of ACh and propionylcholine were processed to allow calculation of the quantity of ACh in tissue samples.

Gas-chromatographic analysis was performed with a Barber-Colman Model 5000 with a flame ionization detector. The column was a 3.5 mm × 6 ft silanized glass column packed with Pennwalt 223 amine packing. Instrument temperatures (°C) were as follows: column oven, 185; injector, 200; detector, 250. Carrier gas (N_2) flow rate was 60 ml min^{-1}

Test for stereotypy

Nine FLU-treated and nine control rats were used 21 days after injection. They each received 0.25 mg kg^{-1} APO intraperitoneally, and were placed in uncovered plastic cages containing wood shavings. Stereotypic behaviour was rated according to the scale of Creese and Iversen (1975). Each rat was observed for one min every 10 min and a stereotypy score was recorded. The rater had no knowledge of which rats were FLU-treated, and which were control. After the experiment, the scores for each group at a given time point were averaged.

RESULTS

Effect of APO treatment on striatal ACh levels

The effect of intraperitoneal injections of APO on striatal ACh levels after 15 min is shown in Table 1. Striatal ACh levels in control rats (receiving sesame seed oil and saline) were 68.9 nmol g^{-1}. Similar values for striatal ACh level in rats killed by microwave irradiation have been reported by Stavinoha, Weintraub and Modak (1974), Haubrich, Wang, Clody and Wedeking (1975) and Sethy (1976). Apomorphine pretreatment for 15 min in sesame seed oil control rats resulted in a dose-dependent increase in striatal ACh level. The percentage increase was 26% after 1 mg kg^{-1}, the highest dose tested.

Effect of FLU pretreatment on the APO-induced increase in striatal ACh level

Pretreatment with FLU 14 days previously affected the response of striatal ACh levels to APO treatment

Table 1. Effect of apomorphine on striatal acetylcholine levels in control and fluphenazine-treated rats

	Striatal acetylcholine concentration (nmol g^{-1})				
	Dose of apomorphine (mg kg^{-1})				
Pretreatment	Saline	0.03	0.1	0.3	1.0
Sesame seed oil control	68.9 ± 2.0 $N = 20$	71.9 ± 3.3 $N = 13$	74.7 ± 2.2 $N = 15$	79.7 ± 2.3 $N = 11$	87.1 ± 4.8 $N = 6$
Fluphenazine decanoate	70.7 ± 4.1 $N = 6$	76.6 ± 1.9 $N = 12$	83.9* ± 3.2 $N = 14$	82.0 ± 2.6 $N = 9$	84.8 ± 4.5 $N = 4$

* Significantly greater than control (*t*-test, $p < 0.012$).

Data presented as mean ± S.E.M., N = number of observations. Fluphenazine decanoate (2 mg) or sesame seed oil administered subcutaneously 14 days previously. Apomorphine or saline administered intraperitoneally 15 min before microwave irradiation.

Table 2. Effect of apomorphine on olfactory tubercle acetylcholine levels in control and fluphenazine-treated rats

	Olfactory tubercle acetylcholine concentration (nmol g^{-1})				
	Dose of apomorphine (mg kg^{-1})				
Pretreated	Saline	0.03	0.1	0.3	1.0
Sesame seed oil control	74.3 ± 3.0 $N = 11$	77.5 ± 3.0 $N = 5$	82.3 ± 1.0 $N = 6$	88.8 ± 4.0 $N = 6$	90.0 ± 5.5 $N = 3$
Fluphenazine decanoate	81.1 ± 1.5 $N = 3$	87.7* ± 2.6 $N = 6$	89.6 ± 4.2 $N = 7$	90.4 ± 4.5 $N = 4$	88.3 ± 0.4 $N = 2$

* Significantly greater than control (*t*-test, $p < 0.015$).
Data presented as mean ± S.E.M., N = number of observations. Fluphenazine decanoate (2 mg) or sesame seed oil administered subcutaneously 14 days previously. Apomorphine or saline administered intraperitoneally 15 min before microwave irradiation.

as shown in Table 1. The striatal ACh levels were higher in FLU pretreated rats than in sesame seed oil controls in the groups which received APO in doses of 0.03, 0.1 and 0.3 mg kg^{-1}. The difference was statistically significant (*t*-test) after the dose of 0.1 mg kg^{-1}. This suggests the development of supersensitivity of the effect of APO in elevating striatal ACh levels.

Effect of APO treatment on ACh concentrations in the olfactory tubercle

The effect of APO given intraperitoneally 15 min previously on ACh concentrations in the olfactory tubercle are shown in Table 2. Olfactory tubercle ACh levels in control rats (receiving sesame seed oil and saline) were 74.3 nmol g^{-1}. This level is quite high relative to other brain regions, and indicates dense cholinergic innervation in the olfactory tubercles. Apomorphine treatment for 15 min in sesame seed oil controls resulted in a dose-dependent increase in olfactory tubercle ACh level, as was found in the striatum. The 1 mg kg^{-1} dose resulted in a 21% increase in olfactory tubercle ACh level.

Effect of FLU pretreatment on the APO-induced increase in olfactory tubercle ACh level

Pretreatment with FLU 14 days previously affected the response of olfactory tubercle ACh levels to APO treatment as shown in Table 2. The effect was similar to that seen in the striatum. The ACh levels were higher in FLU pretreated rats than in sesame seed oil controls in the groups which received APO in doses of 0.03, 0.1 and 0.3 mg kg^{-1}. The difference was statistically significant at a dose of 0.03 mg kg^{-1}. This suggests the development of supersensitivity of the effect of APO in elevating ACh levels in the olfactory tubercles, as well as the striatum.

Time course of FLU effect on APO-induced elevation of striatal ACh level

Additional rats which received FLU were given APO (0.1 mg kg^{-1}, i.p.) 21 or 27 days after FLU. The rats were killed 15 min after APO injection. Table 3 shows the resultant percentage increase in striatal ACh level induced by 0.1 mg kg^{-1} APO as a function of time after FLU pretreatment. The enhanced APO-induced increase in striatal ACh seen at 14 days after FLU injection is also seen at 21 days after FLU. At 27 days after FLU, however, the APO-induced elevation in the ACh level is the same as that seen in sesame seed oil controls. This indicates that the supersensitivity of the APO-induced elevation in striatal ACh that develops is a temporary phenomenon, and eventually disappears.

Effect of FLU pretreatment on APO-induced stereotypy

The effect of FLU pretreatment on APO-induced stereotypic behaviour 21 days after FLU injection is shown in Fig. 1. Significantly higher stereotypy scores were recorded for FLU-treated rats than for controls. This difference occurred throughout the one-hour duration of the experiment. The behaviour of the FLU-treated rats consisted mainly of sniffing, with occasional grooming, licking and gnawing. Most control rats did not show this behaviour.

Table 3. Time course of effect of fluphenazine treatment on apomorphine-induced elevation of striatal acetylcholine level

Time after fluphenazine injection (days)	N	% Increase in striatal acetylcholine level after 0.1 mg kg^{-1} apomorphine
Sesame seed oil	15	8.5 ± 3.2
14	14	18.7* ± 4.5
21	7	19.2* ± 6.5
27	5	7.1 ± 2.0

* Significantly greater than sesame seed oil ($p < 0.05$, *t*-test).
Data presented as mean ± S.E.M., N = number of observations.

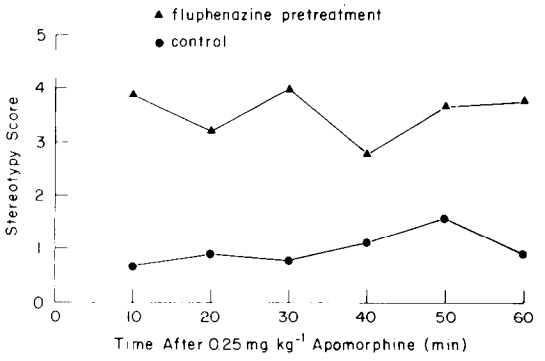

Fig. 1. Effect of fluphenazine pretreatment on apomorphine-induced stereotypic behaviour.

Mean stereotypy scores are plotted as a function of time after intraperitoneal injection of 0.25 mg kg^{-1} apomorphine. Rats received fluphenazine decanoate (2 mg) or sesame seed oil, subcutaneously, 21 days previously. Mean score was significantly ($p < 0.05$) higher for fluphenazine-treated rats than for controls at each time point (two-sample rank test, one tail). $N = 9$ in each group.

DISCUSSION

This study shows that after chronic treatment with the potent neuroleptic agent, FLU, there is an enhanced response to the dopaminergic agonist, APO, as measured by elevation in ACh concentration and induction of stereotyped behaviour. The results constitute neurochemical and behavioural evidence in support of the hypothesis that dopaminergic receptors become supersensitive during withdrawal from chronic exposure to receptor blocking agents. This hypothesis was formulated based on findings of enhanced behavioural effects of dopaminergic drugs after chronic treatment with neuroleptics (Klawans and Rubovits, 1972; Tarsy and Baldessarini, 1974; Fjalland and Møller Nielsen, 1974).

Additional evidence on the molecular level has recently been reported. Burt, Creese and Snyder (1977) found increased dopamine receptor ([^{3}H] haloperidol) binding in striatal homogenates of rats one week after chronic neuroleptic treatment for 3 weeks. Thus, chronic neuroleptic treatment has been reported to enhance (i) striatal dopamine receptor binding; (ii) APO-induced elevation of ACh levels in cholinergic neurones, which are postulated to receive dopaminergic innervation; and (iii) APO-induced stereotypic behaviour, also postulated to be modulated by striatal dopaminergic innervation.

The supersensitivity observed after chronic neuroleptic treatment may develop as a result of the blockade of receptor stimulation. Consistent with this hypothesis is the observation that after chronic lesion of the nigro-neostriatal pathway, another treatment which would result in long-term lack of receptor stimulation, supersensitivity of APO-induced stereotypy and increase in striatal ACh level are also observed (Price and Fibiger, 1974; Fibiger and Grewaal, 1974).

One other biochemical parameter used to assess postsynaptic dopamine receptor sensitivity is dopamine-sensitive adenylate cyclase activity. This measure, however, has been reported not to be enhanced after chronic neuroleptic treatment (Rotrosen, Friedman and Gershon, 1975; Von Voigtlander, Losey and Triezenberg, 1975). Since several laboratories have reported an increase in dopamine-sensitive adenylate cyclase after chronic destruction of the nigro-neostriatal pathway (Mishra, Gardner, Katzman and Makman, 1974; Krueger, Forn, Walters, Roth and Greengard, 1976), the negative results obtained following chronic neuroleptic treatment are somewhat puzzling. However, it is likely that they may be explained, at least in part, by the fact that only several time points were selected for the duration of drug treatment and withdrawal.

In the present study, the apparent supersensitivity that develops is a temporary phenomenon. It was present at 14 and 21 days after FLU injection, but after 27 days the response had returned to the normal magnitude. It is interesting that increased dopamine receptor binding (Burt *et al.*, 1977) was seen 5 and 12 days after withdrawal of neuroleptic treatment, but not at 17 days afterward. Considering the differences in mode of neuroleptic administration (single depot injection lasting about one week in the present study, daily injection in Burt *et al.*, 1977) the time courses for disappearance of supersensitivity after neuroleptic levels subside are similar.

The experiment involving APO-induced stereotypic behaviour confirms previous reports that chronic neuroleptic administration results in supersensitivity to the stereotypic effects of dopaminergic drugs (Klawans and Rubovits, 1972; Tarsy and Baldessarini, 1974; Fjalland and Møller Nielsen, 1974; Sayers *et al.*, 1975; Smith and Davis, 1975, 1976). It also shows that the method of single subcutaneous depot injection of FLU decanoate gives results similar to daily injections of neuroleptics followed by withdrawal. The behavioural supersensitivity was observed 21 days after FLU injection, a time when APO-induced elevation of striatal ACh levels was also enhanced.

The striatum and olfactory tubercle were the brain regions examined in this study because both receive major innervation from the midbrain dopaminergic neurones (Ungerstedt, 1971). Many studies have demonstrated an effect of dopaminergic input on striatal cholinergic neurones (see Roth and Bunney, 1976). Dopaminergic agonists have been found to cause an increase in ACh levels in this region (Sethy and Van Woert, 1974; Consolo *et al.*, 1974; McGeer *et al.*, 1974). This study, which used rapid microwave fixation of the brain tissue, confirms the previous ones and shows that this effect is a dose-dependent one.

The results of this study also indicate that the olfactory tubercles may contain cholinergic neurones which are affected by the dopaminergic input. This is indicated by the relatively high concentration of ACh found in the olfactory tubercles, the dose-dependent increase in these levels observed after APO administration and the development of supersensitivity of the

APO effect after chronic FLU treatment. The value of 75 nmol g^{-1} reported here for olfactory tubercle ACh level is much higher than a value of about 20 nmol g^{-1} reported by Cheney, Le Fevre and Racagni (1975). The difference in results could be due to the difference in dissection procedure. Cheney *et al.* (1975) made 400 μm sections of frozen brain, then punched out areas to be studied with stainless-steel tubing. In our study, the brain regions were dissected free-hand. Further studies are required to characterize the location and function of postulated cholinergic neurones in the olfactory tubercle and the influence of the dopaminergic neurons on them.

In humans, chronic neuroleptic treatment can bring about tardive dyskinesia, a syndrome characterized by abnormal, involuntary movements or orofacial muscles and extremities (Kobayashi, 1977). Paradoxically, the symptoms are reduced when the dosage of the neuroleptic agent is increased, while lowering the dosage enhances the dyskinesias. The observation that the dyskinesias resemble the stereotypic movements induced in rats by APO or amphetamine led Klawans and Rubovits (1972) to propose development of dopamine receptor supersensitivity as the cause of tardive dyskinesia, when they found that after chronic chlorpromazine treatment, rats were more sensitive to the stereotypic effect of APO and amphetamine. This explanation is consistent with the paradoxical response of the syndrome to changes in dosage of neuroleptics.

Anticholinergic drugs worsen or even induce tardive dyskinesia (Klawans, 1973). This led to the hypothesis that cholinergic neurones play a role in the expression of tardive dyskinesia. The results of this study, namely that supersensitivity of APO-induced increase in ACh level develops after chronic FLU, support this hypothesis. Since dopaminergic drugs act to reduce striatal ACh turnover (Trabucchi *et al.*, 1975), supersensitivity of dopamine receptors probably manifests itself as reduced cholinergic neuronal activity. Several attempts have been made to treat tardive dyskinesia by attempting to promote cholinergic neurotransmission with administration of dimethylaminoethanol (Casey and Denney, 1974; Miller, 1974) or choline (Davis, Hollister, Barchas and Berger, 1976).

The evidence that supersensitivity of dopamine receptors is responsible for the enhanced APO-induced increase in striatal ACh level further supports the hypothesis that nigro-neostriatal dopaminergic afferents act on striatal cholinergic neurones. Thus, analysis of changes in striatal cholinergic function is a valuable method for studying the effects of treatments which modify nigro-neostriatal dopaminergic transmission, since it reveals effects on the functioning of the postulated postsynaptic neurones. Since ACh turnover rate is a more direct and meaningful measure of cholinergic neuronal function than ACh level, future studies should include the investigation of this parameter. In view of this, further studies should be done on the decrease in striatal ACh turnover rate induced by apomorphine (Trabucchi *et al.*, 1975) to determine whether chronic neuroleptic treatment results in development of supersensitivity to this effect also.

Other experiments which would be interesting and important to do would be to determine if antipsychotic agents which are associated with a lower incidence of extrapyramidal side effects (e.g. clozapine, thioridazine) result in development of supersensitivity after chronic administration. If not, this may predict a lower incidence of tardive dyskinesia upon long-term treatment with these drugs.

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