ALTERATION IN DOPAMINERGIC AND MUSCARINIC CHOLINERGIC RECEPTORS AFTER SUBCHRONIC TREATMENT WITH HALOPERIDOL IN THE DEVELOPING RAT BRAIN

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Influence of subchronic treatment with haloperidol (0.5 to 5.0 mg/kg for 10 days, twice a day) on sensitivity of both central dopaminergic (DA) and muscarinic cholinergic (ACh) receptors was behaviorally and neurochemically investigated 4 days after withdrawal stage of the drug in the developing rat aged 28 days. An *i.p.* injection of apomorphine 0.2 mg/kg induced locomotor stimulation more effectively in haloperidol-treated animals than in controls. Scatchard analysis of the specific [³H]spiperone binding to the striatal membranes showed that haloperidol treatment produced an increase in the maximal number of binding sites ($B_{\max}$) without alteration in a dissociation constant (K_D). Pilocarpine (100 and 150 mg/kg) was less effective in inducing catalepsy in the treated animals than in controls, although the treatment did not induce any alteration in saturation parameters of specific [³H]quinuclidinyl benzilate ([³H]QNB) binding to the homogenates of striatum, mesolimbic area and hippocampus. It is suggested that a decrease in $B_{\max}$ in the striatal [³H]spiperone binding by chronic haloperidol treatment underlies DA receptor hypersensitivity in developing rats. Behavioral muscarinic hyposensitivity caused by chronic haloperidol may not to be due to alteration in the striatal [³H]QNB binding.

Keywords — haloperidol; dopamine; acetylcholine; ligand binding; locomotion; catalepsy; young rat

INTRODUCTION

Several studies indicate that, when animals are previously exposed to haloperidol, followed by an injection of a dopamine (DA) agonist, they display enhanced locomotion^{1–4)} and stereotypy.^{5–6)} An increased sensitivity of DA receptors by long-term treatment with haloperidol has been shown by enhanced inhibition of the striatal DA turnover after an injection of apomorphine in adult rats²⁾ and in developing rats.⁷⁾ In addition, chronic haloperidol treatment significantly increases the number of DA receptor binding sites in the striatum.^{8–11)} On the other hand, a close interrelationship between the DA and acetylcholine (ACh) neurons in the CNS^{12–14)} prompts that long-term haloperidol treatment affects sen-

sitivity of ACh receptors. In fact, repeated treatment with haloperidol retards the reduction in locomotor activity produced by pilocarpine.³⁾ Chronic haloperidol administration reduces the specific binding of [³H]quinuclidinyl benzilate ([³H]QNB) binding in hippocampus 14 days after withdrawal of the neuroleptic.¹⁰⁾

The rat is responsive to DA agents immediately after birth or at the early stage of postnatal life, subsequently whose responsiveness to atropine reaches functional maturity between 10 and 20 days after birth.^{15–17)} Little is known, however, with regard to influence of long-term haloperidol treatment on rapidly developing DA and ACh systems in the brain. A biochemical and behavioral experimentation was designed to study

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alterations in DA and ACh receptors sensitivity by subchronic haloperidol treatment in 28-day-old rats.

MATERIALS AND METHODS

Wistar rats of both sexes were used. After birth all litters were culled to 10–12 pups/mother. Pups were maintained with their mother at 23° at normal conditions, and weaned at 22 days.

One to 10 mg of haloperidol was dissolved in 1 ml of propyleneglycol : ethylalcohol : 0.1 N HCl (32 : 8 : 20), and the mixture made up with saline to 10 ml. Freshly prepared haloperidol solution or its vehicle was administered by a s.c. injection to the 15-day-old rat twice daily (8:00 A.M. and 8:00 P.M.) beginning with a dose of 0.5 mg/kg. The dosage was increased by 0.5 mg/kg every time until a final dose of 5 mg/kg was reached (10 days). The 28-day-old animal was used for experiments 4 days after cessation of haloperidol treatment.

Measurement of Apomorphine-induced Locomotor Stimulation — Apomorphine-induced locomotor stimulation was measured by the method previously described.¹⁶⁾ Animals at each stage were placed singly into a plastic cage, 27 × 17 cm, and their locomotor activity was measured with an ANIMEX activity meter (Type S, LKB Instrument, sensitivity, 40 μ A). Before an injection of apomorphine, general behavior and locomotor activity of the test animal were observed for 10 min in the same cage as used for further observation. The recording began at 10 min after an *i.p.* injection of apomorphine (0.05, 0.2, 0.5, 1.0 and 2.0 mg/kg) dissolved in normal saline solution containing 0.01 N acetic acid. Measurements were made for 5 min periods with a 10 min intermission between recordings. The measurements were accumulated for 3 consecutive observation periods during a 35 min session.

Measurement of Pilocarpine-induced Catalepsy — The test for catalepsy was carried out by the method of Nomura and Segawa.¹⁷⁾ The 28-day-old rat of both control and haloperidol treated groups were received a s. c. injection of 5 mg/kg atropine methyl nitrate, dissolved in nor-

mal saline solution, 30 min before a pilocarpine injection in order to minimize peripheral cholinceptive side effects. Pilocarpine, dissolved in normal saline solution, was injected *i. p.* The catalepsy test was performed at intervals of 15, 30, 45, 60, 75, 90 and 120 min after pilocarpine administration. The rat was gently placed with forelimbs resting on a rubber can 4 cm from the floor and the latency to correct the abnormal position was greater than 30 sec. The righting reflex was also examined at each test time after the drug injection.

[³H]Sipiperone Binding Assay — After cervical decapitation, the brain was rapidly removed and dissected on ice. The striatum was dissected out by the method of Nomura *et al.*¹⁸⁾ The [³H]siperone binding was done using homogenates of the striatum by the method of Seeman *et al.*¹⁹⁾ The fresh tissues were suspended in 15 vol. of 15 mM Tris-HCl buffer (pH 7.4) containing 5 mM Na₂-EDTA, 1.1 mM ascorbate and 13.3 μ M pheniprazine, crudely homogenized with a glass-teflon homogenizer, preincubated at 37° for 60 min. The sample was centrifuged at 39000 × *g* for 15 min, resuspended in 14 ml of buffer and finally homogenized with a Polytron homogenizer (setting, 6) for 20 sec. A 0.8 ml portion of homogenates (0.25–0.4 mg protein) was transferred into polyethylene tubes. 0.1 ml of buffer or (+)-butaclamol (final concentration, 1 μ M) and 0.1 ml of [³H]siperone (final concentration, 0.0954 nM to 0.899 nM). After 30 min incubation at 23°, an aliquot of 0.5 ml was filtered through a Whatman GF/B filter. The filter washed with 10 ml of buffer and then placed into a liquid scintillation vial, 10 ml of scintillator (ACS II, Amersham) was added and radioactivity was measured. Specific binding of [³H]siperone was defined as that displaceable by 1 μ M (+)-butaclamol.

[³H]Quinuclidinyl Benzilate Binding Assay — The striatum, mesolimbic area and hippocampus were dissected by the method previously described.^{18,20)} The [³H]QNB binding assay was carried out by the method of Nomura *et al.*¹⁸⁾

Estimation of protein was carried out by the

Folin reagent method of Lowry *et al.*²¹⁾

Differences between experimental values were analyzed by Student's *t*-test.

Chemicals — [³H]Spiperone (23 Ci/mmol) was purchased from Radiochemical Center, Amersham. [³H]Quinuclidinyl benzilate (29.4 Ci/mmol) was obtained from New England Nuclear. (+)-Butaclamol hydrochloride was donated by Dr. D. J. Marshall (Ayerst Res. Lab., Canada). Haloperidol (Dainippon Pharmaceutical Co., Ltd.); apomorphine hydrochloride (Takeda Chemical Industries, Ltd.); atropine sulfate (Merck); atropine methyl nitrate and pilocarpine hydrochloride (Nakarai Chemicals Ltd.).

RESULTS

1. Apomorphine-induced Locomotor Hyperactivity

Apomorphine-induced locomotor stimulation in the 28-day-old rat, subchronically treated with haloperidol for 10 days, was examined 4 days after cessation of the drug administration. Apomorphine 0.05 to 2.0 mg/kg produced locomotor hyperactivity in controls and treated groups (Fig. 1). Apomorphine dose-response curve of the haloperidol-treated animals tended to shift away from the control (Fig. 1). In addition, the treated animals were significantly ($p < 0.01$) more responsive to apomorphine in dose of 0.2 mg/kg than controls, although other doses did not induce a significant change between controls and treated groups.

2. Pilocarpine-induced Catalepsy

Pilocarpine (100 mg/kg and 150 mg/kg) given to the 28-day-old rats from both controls and haloperidol-treated groups induced catalepsy in controls (Table I). The effect was dose-dependent and the drug-induced catalepsy reached a peak 30 to 45 min after pilocarpine administration. Pilocarpine produced catalepsy less effectively in treated-animals.

3. [³H]Spiperone Binding in the Striatum

The specific binding of [³H]spiperone to striatal homogenates was saturable and plateaued at 0.5–0.7 nM. From a Scatchard analysis, the mean values for saturable parameters for [³H]-spiperone were calculated as; $K_D = 0.404$

± 0.010 nM and $B_{max} = 0.495 \pm 0.015$ pmol/mg (Table II). The kinetic parameters in haloperidol-treated animals were; $K_D = 0.385 \pm 0.032$ nM, $B_{max} = 0.670 \pm 0.021$ pmol/mg. The subchronic treatment with haloperidol did not alter K_D but increased B_{max} by 34.0% ($p < 0.01$) of controls.

4. [³H]QNB Binding in the Striatum, Mesolimbic Region and Hippocampus

The specific [³H]QNB binding revealed to be saturable in the striatal, mesolimbic and hippocampal regions. From a Scatchard analysis, kinetic parameters in each region were calculated

FIG. 1. Influence of Subchronic Haloperidol Treatment on Apomorphine-induced Locomotor Hyperactivity in the 28-day-old Rat

Apomorphine 0.05, 0.2, 0.5, 1.0 and 2.0 mg/kg was i.p. injected to the 28-day-old animal in the control and haloperidol-treated group 4 days after cessation of the drug treatment. Locomotor activity (counts/15 min) is shown as the mean $\pm$ S.E. of 6 to 10 animals. $\circ$ — $\circ$, control; $\bullet$ ---- $\bullet$, haloperidol treated. Significance, * $p < 0.05$ vs. control.

TABLE I. *Influence of Subchronic Haloperidol Treatment on Pilocarpine-induced Catalepsy in Developing Rats*

Dose	Control							
	Number of animals	15	30	45	60	75	90	120
Saline	6	0	0	0	0	0	0	0
100 mg/kg	9	3	5	4	3	3	0	0
150 mg/kg	16	7	9	10	8	6	3	1

Dose	Haloperidol							
	Number of animals	15	30	45	60	75	90	120
Saline	6	0	0	0	0	0	0	0
100 mg/kg	9	2	5	3	3	1	0	0
150 mg/kg	19	6	8	7	4	2	1	0

Developing rats were subchronically treated with haloperidol (0.5 to 5.0 mg/kg) twice daily for 10 days (15 to 24 days after birth). On day 28, animals from both controls and treated groups were used for the test of pilocarpine-induced catalepsy. Results show the number of animals displaying catalepsy at various intervals following an injection of pilocarpine.

TABLE II. *Influence of Subchronic Haloperidol Treatment on Saturation Constants for Specific Bindings of [³H]Spiperone and of [³H]QNB in the Brain Regions of 28-day-old Rats*

Ligand	Region	Constant	Control	Haloperidol	Significance
[³ H]Spiperone	Striatum	K_D (nM)	0.404 ± 0.010	0.385 ± 0.032	N.S.
		B_{max} (pmol/mg)	0.495 ± 0.015	0.670 ± 0.021	$p < 0.01$
[³ H]QNB	Striatum	K_D	0.734 ± 0.029	0.723 ± 0.058	N.S.
		B_{max}	0.713 ± 0.014	0.694 ± 0.029	N.S.
	Mesolimbic	K_D	0.749 ± 0.032	0.735 ± 0.043	N.S.
		B_{max}	0.652 ± 0.015	0.595 ± 0.027	$p < 0.10$
	Hippocampus	K_D	0.332 ± 0.007	0.320 ± 0.006	N.S.
		B_{max}	0.186 ± 0.002	0.188 ± 0.002	N.S.

Saturation parameters were determined by Scatchard analysis of specific bindings of [³H]spiperone and [³H]QNB. Each value shows the mean $\pm$ S.E. in a linear regression line from 2 to 4 separate experiments, each performed in triplicate. K_D , dissociation constant. B_{max} , the maximal number of binding sites. N.S., not significant vs. controls.

and summarized in Table II. Both saturation parameters of K_D and B_{max} were similar in the striatum and in the mesolimbic area, but values in the hippocampus were lower than those in other regions. Haloperidol-treatment did not significantly affect any constants in these regions, although the treatment reduced B_{max} value in the mesolimbic area by 8.8% of control.

DISCUSSION

The results presented here show that a low dose of apomorphine 0.2 mg/kg induced more effectively locomotor hyperactivity in 28-day-old rats subchronically treated with haloperidol than in controls, suggesting hypersensitivity of central DA receptors at the withdrawal stage of haloperidol treatment. This is consistent with our pre-

vious findings that haloperidol treatment enhances the apomorphine-induced reduction of the striatal homovanillic acid content 4 days after cessation of the drug in 24-day-old rats.⁷⁾ In biochemical experiments, Scatchard analysis of the specific [³H]spiperone binding indicated that haloperidol treatment produced a significant increase in the maximum number of binding sites ($B_{\max}$) labelled with [³H]spiperone in the striatal homogenates without altering dissociation constant (K_D) of the ligand to binding sites. A significant increase (43.8%, $p < 0.05$) of apomorphine (0.2 mg/kg)-induced locomotor hyperactivity in treated animals seems to be due to an increase of maximum number of binding sites of [³H]spiperone. Central DA receptors are sensitive to DA agents at 15–24 days after birth.^{16,22)} This factor may be responsible to haloperidol-induced hypersensitivity of DA receptors in young rats as well as in adult ones reported so far.⁸⁾ Furthermore, central DA receptors which are not fully sensitive to DA agents on day 7 reach functional maturity by 20 days of the postnatal life in rats.²³⁾

Pilocarpine was less effective in inducing catalepsy in haloperidol-treated animals than in controls, suggesting muscarinic cholinergic hyposensitivity in the CNS. Since muscarinic cholinergic response is detectable between day 15 and 20 in rats,^{17,22)} cholinergic transmission may be modified by long-term haloperidol at this stage through rapidly developing DA-ACh interrelationship. The continuous ACh release during repeated haloperidol treatment evokes a decrease in receptor sensitivity, if DA is presumed to exert an inhibitory influence on ACh interneurons in several regions such as the striatum.^{24,25)} In adult rats, pilocarpine-induced locomotor hypoactivity is retarded and hyperactivity produced by dextimide, an anticholinergic agent, is enhanced following repeated treatment with haloperidol.³⁾ Moreover, less depression of locomotor activity by physostigmine has also been documented in haloperidol-treated mice than in controls.¹⁾

By contrast, Scatchard analysis of the specific [³H]QNB binding showed that subchronic halo-

peridol treatment altered neither the maximum number of binding sites nor affinity of a ligand to receptor sites in the striatum, mesolimbic area and hippocampus at the interval of 4 days after cessation of the drug treatment. The results are inconsistent with behavioral observation concerning haloperidol-induced muscarinic cholinergic hyposensitivity. This discrepancy is not clearly understood but following explanations are presumable: 1) decrease in [³H]QNB binding in other areas not examined presently (*e.g.* neocortex), 2) physiological significance of 8.8% ($p < 0.10$) reduction of [³H]QNB binding in the mesolimbic area. Some previous studies showed that the specific [³H]QNB binding in the striatum and mesolimbic area is not changed by long-term haloperidol administration in adult rats.^{9,10)} Only significant reduction in $B_{\max}$ in [³H]QNB binding was observed in the hippocampus 14 days after withdrawal of the neuroleptic.¹⁰⁾

In conclusion, muscarinic ACh receptors hyposensitivity as well as DA receptors hypersensitivity probably involves in the extrapyramidal side effects, namely, tardive dyskinesia after long-term haloperidol treatment in young developing rats. Behavioral ACh receptor hyposensitivity by chronic haloperidol seems to be not due to a decrease in the striatal [³H]QNB binding. It remains to be studied in the future as to how and where muscarinic ACh receptor hyposensitivity is caused by chronic haloperidol in the rat brain.

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REFERENCES

- 1) R. Dunstan and D.M. Jackson: The demonstration of a change in adrenergic receptor sensitivity in the central

- nervous system of mice after withdrawal from long-term treatment with haloperidol, *Psychopharmacology*, **38**, 105–114 (1976).
- 2) G.Gianutsos, M.D.Hynes and H.Lal: Enhancement of apomorphine-induced inhibition of striatal dopamine-turnover following chronic haloperidol, *Biochem. Pharmacol.*, **24**, 581–582 (1975).
 - 3) G.Gianutsos and H.Lal: Alteration in the action of cholinergic and anticholinergic drugs after chronic haloperidol: Indirect evidence for cholinergic hyposensitivity, *Life Sci.*, **18**, 515–520 (1976).
 - 4) G.Gianutsos and K.E.Moore: Dopaminergic supersensitivity in striatum and olfactory tubercle following chronic administration of haloperidol or clozapine, *Life Sci.*, **20**, 1585–1592 (1977).
 - 5) G.Gianutsos, R.B.Drawbaugh, M.D.Hynes and H.Lal: Behavioral evidence for dopaminergic supersensitivity after chronic haloperidol, *Life Sci.*, **14**, 887–898 (1974).
 - 6) K.L.Davis, L.E.Hollister and W.C.Fritz: Induction of dopaminergic mesolimbic receptor supersensitivity by haloperidol, *Life Sci.*, **23**, 1543–1548 (1978).
 - 7) Y.Nomura, T.Komori, S.Okuda and T.Segawa: Developmental change in striatal concentration of homovanillic acid and 3,4-dihydroxyphenylacetic acid in response to apomorphine and haloperidol treatment, *Archs. Int. Pharmacodyn. Thér.*, **237**, 25–30 (1979).
 - 8) D.R.Burt, S.J.Enna, I.Creese and S.H.Snyder: Dopamine receptor binding in the corpus striatum of mammalian brain, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 4655–4659 (1975).
 - 9) P.Muller and P.Seeman: Dopaminergic supersensitivity after neuroleptics: Time course and specificity, *Life Sci.*, **21**, 1751–1758 (1977).
 - 10) R.M.Kobayashi, J.Z.Fields, R.E.Hruska, K.Beaumont and H.I.Yamamura: "Brain Neurotransmitter Receptors and chronic Antipsychotics Drug Treatment: A Model for Tardive Dyskinesia" in "Animal Models in Psychiatry and Pharmacology," ed. by I.Hanin and E.Usdin, Pergamon Press, Oxford, 1978, pp. 405–409.
 - 11) F.Owen, A.J.Cross, J.L.Waddington, M.Poulter, S.J.Gramble and T.J.Crow: Dopamine-mediated behaviour and ³H-spiperone binding to striatal membranes in rats after nine months haloperidol administration, *Life Sci.*, **26**, 55–59 (1980).
 - 12) G.Bartholini, M.Stadler, M.Gadea-Ciria and K.G.Lloyd: "Interaction of Dopaminergic and Cholinergic Neurons in the Extrapyramidal and Limbic Systems" in "Advances in Biochemical Psychopharmacology," vol. 16, ed. by E.Costa and G.L.Gessa, Raven Press, New York, 1977, pp. 391–395.
 - 13) L.L.Butcher: "Acetylcholinesterase in Rat Nigro-Neostriatal Neurons: Experimental Verification and Striatum and Evidence for Cholinergic-Dopaminergic Interactions in the Substantia Nigra and Caudate-Putamen" in "Cholinergic-Monoaminergic Interactions in the Brain," ed. by L.L.Butcher, Academic Press, New York, 1978, pp. 25–95.
 - 14) H.Ladinski, S.Consolo, S.Bianchi, D.Ghezzi and R.Samanin: "Link between Dopaminergic and Cholinergic Neurons in the Striatum as Evidenced by Pharmacological, Biochemical and Lesion Studies" in "Interactions between Putative Neurotransmitters in the Brain," ed. by S.Garattini, J.Pujol and R.Samanin, Raven Press, New York, 1978, pp. 3–21.
 - 15) L.A.Baez, N.K.Eskridge and R.Schein: Postnatal development of dopaminergic and cholinergic catalepsy in the rat, *Eur. J. Pharmacol.*, **36**, 155–162 (1976).
 - 16) Y.Nomura and T.Segawa: Apomorphine-induced locomotor stimulation in developing rats treated with 6-hydroxydopa, *Eur. J. Pharmacol.*, **50**, 153–156 (1978).
 - 17) Y.Nomura and T.Segawa: Muscarinic hyposensitivity in the developing rat pretreated with 6-hydroxydopa, *Eur. J. Pharmacol.*, **50**, 431–435 (1978).
 - 18) Y.Nomura, H.Kajiyama, Y.Nakata and T.Segawa: Muscarinic cholinergic binding in striatal and mesolimbic areas of the rat: Reduction by 6-hydroxydopa, *Eur. J. Pharmacol.*, **58**, 125–131 (1979).
 - 19) P.Seeman, K.Westman, P.Miroslav, J.Jilek, P.C.Jain, A.K.Saxena, N.Anad, L.Hubel and A.Philipp: Neuroleptic receptors: Stereoselectivity for neuroleptic enantiomers, *Eur. J. Pharmacol.*, **56**, 247–251 (1979).
 - 20) Y.Nomura, F.Naitoh and T.Segawa: Regional changes in monoamine content and uptake of the rat brain during postnatal development, *Brain Res.*, **101**, 305–315 (1976).
 - 21) O.H.Lowry, J.N.Rosebrough, A.L.Farr and R.J.Randall: Protein measurement with the Folin reagent, *J. Biol. Chem.*, **193**, 265–275 (1951).
 - 22) B.A.Campbell, L.D.Lytle and H.C.Fibiger: Ontogeny of adrenergic arousal and cholinergic inhibitory mechanisms in rats, *Science*, **166**, 635–636 (1969).
 - 23) Y.Nomura, K.Oki and T.Segawa: Apomorphine- and haloperidol-induced change in 3,4-dihydroxyphenylacetic acid content in the mesolimbic-striatum of the developing rat, *J. Pharmacobio-Dyn.*, **3**, 111–116 (1980).
 - 24) H.McLennan and D.H.York: The action of dopamine on neurons of the caudate nucleus, *J. Physiol. Lond.*, **189**, 393–402 (1967).
 - 25) P.L.McGeer, D.S.Grewaal and E.G.McGeer: Influence of noncholinergic drugs on rat striatal acetylcholine levels, *Brain Res.*, **80**, 211–217 (1974).