Annals of Nuclear Medicine Vol. 11, No. 1, 21–26 1997

Effects of reserpine treatment on the dopamine receptor binding of [^{3}H/^{11}C]nemonapride in the mouse and rat brain

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We investigated the effect of reserpine treatment on the striatal uptake of a radiolabeled dopamine D_2-like receptor ligand nemonapride (NEM). In mice, the uptake of the [^{3}H]NEM in the striatum, cortex and cerebellum was enhanced by the reserpine pretreatment. Neither the ratio of striatum to cerebellum nor that to cortex was affected by the reserpine pretreatment. In rats, *ex vivo* autoradiography showed no effect of the reserpine treatment on the striatal uptake of [^{11}C]NEM or the striatum to cortex ratio. The results suggest that the receptor binding of NEM was not significantly influenced by reserpine-induced depletion of endogenous dopamine probably because of its high affinity for the receptors.

Key words: [^{11}C]nemonapride, reserpine, dopamine D_2-like receptor, *ex vivo* autoradiography

INTRODUCTION

FROM THE FIRST DEMONSTRATION of dopamine receptors of human brain by positron emission tomography (PET) with [^{11}C]*N*-methylspiperone,[1] several tracers have been used to investigate the dopaminergic system.[2,3] To date [^{11}C]raclopride[4] is widely used as a PET ligand to measure dopamine D_2 receptors. This benzamide compound has a selective affinity for D_2 (and D_3[5]) receptor subtypes, but because of relatively low affinity, the binding to the receptors is affected by the endogenous monoamine concentration *in vitro* and *in vivo*.[5–11] On the other hand, another benzamide compound ^{11}C-labeled nemonapride ([^{11}C]NEM, nemonapride is the registered name of YM-09151-2) has been proposed as a PET ligand.[12–17] NEM is a ligand for dopamine D_2-like (D_2, D_3 and D_4) receptors[5,18,19] with higher affinity and lower nonspecific binding than spiperone and raclopride.[20,21]

The aim of this study is to assess the effect of endogenous monoamine on the [^{3}H]NEM binding to the striatum in reserpinized mice according to the method of Inoue et al.[9] It is well known that reserpine depletes the monoamine in the presynaptic vesicle. The effect of endogenous dopamine on the receptor binding of raclopride and *N*-methylspiperone has been examined *in vivo* in reserpinized animals.[8,9] We also investigated the effect of reserpine treatment on [^{11}C]NEM binding in the rat striatum by *ex vivo* autoradiography (ARG).

Received August 22, 1996, revision accepted November 27, 1996.

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MATERIALS AND METHODS

[^{3}H]NEM ([^{3}H]YM-09151-2, 3,182 GBq/mmol) was purchased from NEN Research Products (Boston, MA). Reserpine and S(+)-butaclamol were obtained from Research Biochemicals International (Natick, MA). Desmethyl compound of NEM was obtained from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). Male ddY mice and male Wistar rats were supplied by Tokyo Laboratory Animals Co., Ltd. (Tokyo).

The animal studies were approved by the Animal Care and Use Committee of Tokyo Metropolitan Institute of Gerontology.

Radiosynthesis

[^{11}C]NEM was synthesized by the method of Hatano et al.[13] with slight modification. Briefly, [^{11}C]CH_3I prepared with the automated synthesis system[22] was trapped in 0.3

Table 1 Regional brain uptake of radioactivity after intravenous injection of [^{3}H]nemonapride into control and reserpine-treated ddY mice

	Uptake (% injected dose/g)						
	1 min	5 min	10 min	20 min	30 min	60 min	90 min
Control							
Plasma	1.59 ± 0.78	0.57 ± 0.35	0.57 ± 0.24	0.66 ± 0.17	0.82 ± 0.28	1.33 ± 0.13	1.46 ± 0.19
Striatum	4.05 ± 0.78	5.26 ± 1.92	6.87 ± 0.74	7.72 ± 0.92	8.38 ± 1.18	9.50 ± 1.33	11.10 ± 1.46
Cortex	3.76 ± 0.55	3.54 ± 0.39	2.81 ± 0.34	2.63 ± 0.33	2.24 ± 0.21	1.94 ± 0.26	1.59 ± 0.26
Cerebellum	3.36 ± 0.60	3.60 ± 0.36	2.63 ± 0.69	2.83 ± 0.31	2.33 ± 0.19	2.01 ± 0.26	1.78 ± 0.15
Reserpinized for 4 hours							
Plasma	2.47 ± 0.34	1.72 ± 0.11	1.51 ± 0.07	1.54 ± 0.18	1.71 ± 0.21	2.03 ± 0.15	2.33 ± 0.25
Striatum	4.27 ± 0.72	7.99 ± 0.36	9.89 ± 0.57	11.22 ± 0.59	12.00 ± 1.62	13.63 ± 1.70	14.99 ± 1.17
Cortex	3.83 ± 0.59	4.08 ± 0.76	4.42 ± 0.17	3.78 ± 0.20	3.52 ± 0.42	2.67 ± 0.27	2.50 ± 0.38
Cerebellum	3.46 ± 0.48	4.54 ± 0.11	4.36 ± 0.54	3.99 ± 0.11	3.52 ± 0.36	2.66 ± 0.25	2.49 ± 0.18
Reserpinized for 24 hours							
Plasma	3.56 ± 0.46	2.42 ± 0.25	2.06 ± 0.51	2.02 ± 0.15	2.26 ± 0.26	2.43 ± 0.80	3.39 ± 0.23
Striatum	7.47 ± 1.72	9.83 ± 1.41	11.06 ± 1.25	13.12 ± 2.00	15.21 ± 1.14	17.26 ± 1.63	17.84 ± 2.50
Cortex	6.26 ± 0.77	6.20 ± 0.66	5.38 ± 0.39	5.20 ± 0.65	4.33 ± 0.32	3.63 ± 0.59	3.37 ± 0.46
Cerebellum	5.57 ± 0.64	5.98 ± 0.82	5.56 ± 0.54	5.40 ± 0.69	4.81 ± 0.62	3.63 ± 0.38	3.33 ± 0.46

Mean ± S.D. (n = 4–6). Significant difference ($p < 0.02$, t-test) was observed between control and each of reserpinized groups at all time points of four tissues except for the data at 1 min in the 4 hour-reserpinized group.

Table 2 Selective uptake of radioactivity by the striatum after intravenous injection of [^{3}H]nemonapride into control and reserpine-treated ddY mice

	Uptake (% injected dose/g)						
	1 min	5 min	10 min	20 min	30 min	60 min	90 min
Control							
Str - Cor*	0.29 ± 0.38	1.72 ± 1.55	4.06 ± 0.74	5.48 ± 0.77	5.75 ± 1.37	7.56 ± 1.22	9.50 ± 1.39
Str - Cer#	0.69 ± 0.29	1.67 ± 1.65	3.34 ± 0.98	5.39 ± 0.78	5.55 ± 1.37	7.49 ± 1.17	9.31 ± 1.38
Reserpinized for 4 hours							
Str - Cor*	0.44 ± 0.20	3.91 ± 1.00¶	5.48 ± 0.54‡	7.45 ± 0.51†	8.47 ± 1.35¶	10.86 ± 1.51‡	12.49 ± 0.90‡
Str - Cer#	0.81 ± 0.26	3.44 ± 0.29¶	5.54 ± 0.76‡	7.23 ± 0.60‡	8.48 ± 1.30¶	10.97 ± 1.67‡	12.50 ± 1.08‡
Reserpinized for 24 hours							
Str - Cor*	1.44 ± 1.94	3.63 ± 0.89¶	5.81 ± 0.96‡	7.93 ± 2.10¶	10.88 ± 1.06†	13.63 ± 1.14†	14.47 ± 2.25†
Str - Cer#	1.90 ± 1.88	3.85 ± 0.77¶	5.62 ± 0.81‡	7.73 ± 1.92¶	10.39 ± 1.06†	13.62 ± 1.37†	14.51 ± 2.05†

Mean ± S.D. (n = 4–6).
*Difference in uptake between striatum and cortex. #Difference in uptake between striatum and cerebellum. †$p < 0.001$, ‡$p < 0.01$ and ¶$p < 0.05$ (t-test, between control and each of reserpinized groups)

Table 3 Uptake ratios of striatum to cerebellum and striatum to cortex after intravenous injection of [^{3}H]nemonapride into control and reserpine-treated ddY mice

	Uptake (% injected dose/g)						
	1 min	5 min	10 min	20 min	30 min	60 min	90 min
Control							
Striatum/Cerebellum	1.21 ± 0.08	1.46 ± 0.45	2.61 ± 0.98	2.72 ± 0.42	3.60 ± 0.26	4.73 ± 0.51	6.24 ± 0.63
Striatum/Cortex	1.08 ± 0.10	1.49 ± 0.41	2.44 ± 0.36	2.94 ± 0.71	3.74 ± 0.28	4.90 ± 0.67	6.98 ± 1.23
Reserpinized for 4 hours							
Striatum/Cerebellum	1.23 ± 0.05	1.76 ± 0.05	2.27 ± 0.35	2.81 ± 0.17	3.41 ± 0.21	5.12 ± 0.58	6.02 ± 0.41
Striatum/Cortex	1.11 ± 0.05	1.96 ± 0.53	2.24 ± 0.13	2.97 ± 0.15	3.41 ± 0.33	5.10 ± 0.37	6.00 ± 0.61
Reserpinized for 24 hours							
Striatum/Cerebellum	1.34 ± 0.38	1.65 ± 0.11	1.99 ± 0.18	2.43 ± 0.44	3.16 ± 0.36	4.75 ± 0.33	5.36 ± 0.15
Striatum/Cortex	1.19 ± 0.36	1.59 ± 1.12	2.06 ± 0.20	2.52 ± 0.55	3.51 ± 0.30	4.75 ± 0.40	5.29 ± 0.73

Mean ± S.D. (n = 4–6). No significant difference was observed between control and each of reserpinized groups (t-test).

Table 4 Association and dissociation constants and binding potential of [^{3}H]nemonapride in the control and reserpinized mice estimated with the two compartment analysis

	Reference region	k3 (min^{-1})	k4 (min^{-1})	k3/k4
Control	Cortex[a)]	0.090	0.016	5.66
	Cerebellum[b)]	0.088	0.016	5.39
Reserpinized for 4 hours	Cortex[c)]	0.108	0.024	4.48
	Cerebellum[d)]	0.104	0.022	4.68
Reserpinized for 24 hours	Cortex[e)]	0.088	0.020	4.39
	Cerebellum[f)]	0.085	0.020	4.26

Fitting errors against maximal difference between striatum and reference tissue were 3.5%[a)], 2.3%[b)], 6.7%[c)], 5.7%[d)], 2.7%[e)] and 5.3%[f)].

Table 5 Striatal uptake and striatum to cortex uptake ratio at 20 min after an injection of [^{11}C]nemonapride measured by *ex vivo* autoradiography in the control and reserpinized rats

	Uptake (PSL/mm^2/MBq)		Uptake ratio
	Striatum	Cortex	Striatum/Cortex
Control (n = 9)	4.56 ± 0.46	1.86 ± 0.70	2.50 ± 0.22
Reserpinized for 4 hours (n = 3)	5.04 ± 0.59	2.04 ± 0.26	2.47 ± 0.16
Reserpinized for 24 hours (n = 3)	4.50 ± 0.30	1.82 ± 0.22	2.48 ± 0.15

Mean ± S.D. No significant difference was observed between control and each of reserpinized groups (t-test).

mL of DMF solution, which contained 0.5 mg of desmethyl NEM and 1 mg NaH and was pre-heated at 37°C for 30 min. The solution was heated at 120°C for 1 min. After adding 1 mL of 0.1 M HCl, the reaction mixture was loaded onto a reverse phase column (YMC-Pack ODS-A, 20 mm i.d. × 150 mm length, YMC Co. Ltd., Kyoto, Japan). The mobile phase was a mixture of CH_3CN and 50 mM NaH_2PO_4 (4/6, v/v) at a flow rate of 15 mL/min. The [^{11}C]NEM fraction (retention time of 6.5–7.0 min) was collected and evaporated to dryness. The residue was dissolved in physiological saline. The specific activity was 28–88 GBq/μmol.

Kinetics of [^{3}H]NEM binding to the striatum in mice

Three groups of mice (eight weeks old) were used: the control and two groups of reserpinized mice which were administered intraperitoneally with 5 mg/kg of reserpine four or 24 hours before the tracer injection.

[^{3}H]NEM (74 kBq/23 pmol) was intravenously injected into the three groups of mice. They were killed by cervical dislocation at 1, 5, 10, 20, 30, 60 and 90 min (n = 4 to 7 for each group). The blood was removed by heart-puncture with a heparinized syringe followed by centrifugation to obtain the plasma. The brain was removed and the striatum, cerebral cortex and cerebellum were dissected out. Each tissue sample (approximately 15–100 mg) and the plasma (20 μL) was dissolved in Soluene-350, and the radioactivity was measured in a liquid scintillation counter. The level of radioactivity was expressed as the % injected dose per gram of tissue (% ID/g).

The kinetic analysis of [^{3}H]NEM binding to striatal D_2-like receptors was performed according to the method described by Inoue et al.[9] based on a compartment model, in which the striatal radioactivity was composed of free ligand and receptor-bound ligand. The concentration of the striatal free ligand was assumed to be the total radioactivity in the cerebellum or in the cerebral cortex, and the receptor-bound ligand was assessed as the difference between the total radioactivity in the striatum and that in the cerebellum or cortex. The optimal association (k3) and dissociation (k4) rate constants were obtained by means of a non-linear least square fitting procedure with the free ligand as input. The binding potential (k3/k4) of [^{3}H]NEM binding to striatal D_2 receptors was calculated.

[^{11}C]NEM autoradiographic study in rats

Rats were reserpinized (5 mg/kg i.p.) for four and 24 hours as described above. [^{11}C]NEM (100–200 MBq/0.8–2.3 nmol) was intravenously injected into a control and a reserpinized rat, and at 20 min after the injection the rats were killed by cervical dislocation. The brain was removed and the right hemisphere of the control and the left hemisphere of the reserpinized rat were put side by side for comparative demonstration. The hybrid brain was cut into 20 μm thickness and was apposed to an imaging plate (FUJIX BAS3000 IP-BAS TR for Bio Imaging Analyzer, Fuji Photo Film Co, Ltd., Tokyo, Japan), and the regional brain distribution was visualized. The density of the radioactivity in the region of interest was measured as the photo-stimulated luminescence (PSL), and expressed as PSL/mm^2/MBq in which the injected dose was decay-corrected at the contact time.

RESULTS

Regional brain distribution of the radioactivity after injection of [^{3}H]NEM into mice is summarized in Table 1. The time course in each brain region and in the plasma showed similar patterns in all three groups of mice, but their mean values were the highest in the 24 hours-reserpinized group followed by the 4 hours-reserpinized group and the control. Mean plasma radioactivity decreased for the first 20 min and then slightly increased in all three groups. Mean radioactivity in the striatum increased over 90 min, whereas those in the cerebral cortex and cerebellum gradually decreased. The receptor-specific uptake which was calculated as the striatal uptake minus the uptake in the reference region was increased by the reserpine treatment (Table 2). On the other hand, as shown in Table 3, the

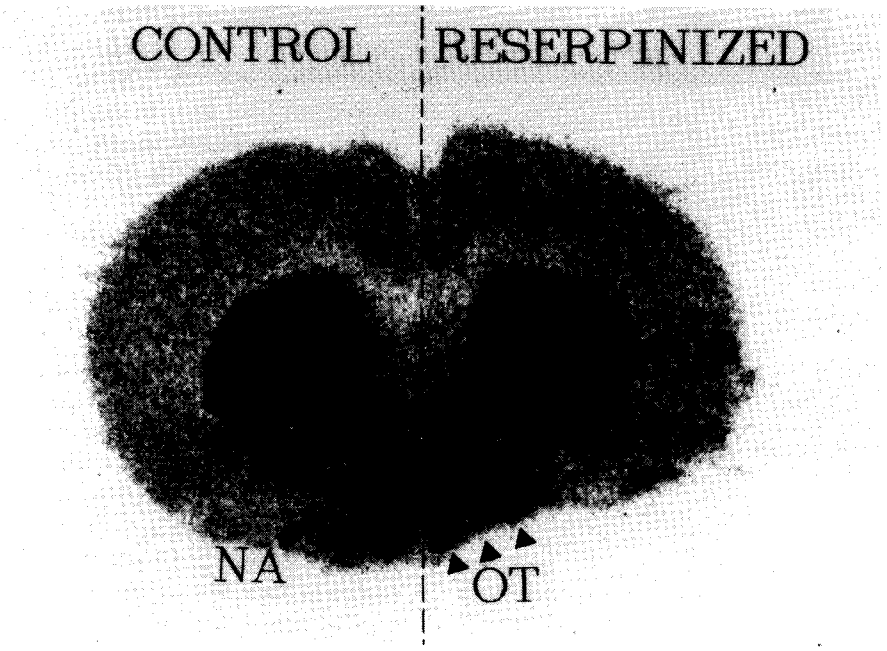

Fig. 1 Autoradiogram of the brain section at 20 min after an intravenous injection of [^{11}C]nemonapride into control and reserpinized rats. Left and right hemisphere shows control and reserpinized, respectively, rat brain. CP, caudate putamen; NA, nucleus accumbens; and OT, olfactory tubercle.

treatment did not affect the uptake ratios of striatum to cortex or striatum to cerebellum. Table 4 shows the result of the kinetic analysis with the compartment model. In the 24 hours-reserpinized brain, the association rate constant (k3) did not change, whereas it seems that the binding potential (k3/k4) gradually decreased with the reserpinized periods.

Figure 1 shows the [^{11}C]NEM ARG image of the brain sections of the rats at 20 min after the tracer injection. A high ^{11}C density was observed in the caudate putamen, nucleus accumbens and olfactory tubercle. The image of the control brain (left side, Fig. 1) is similar to that of the reserpinized brain (right side, Fig. 1). The striatal uptake and the striatum to cortex uptake ratios in all three groups were comparable (Table 5).

DISCUSSION

In the present study we investigated the effect of the reserpine-treated dopamine depletion on the radiolabeled NEM binding to the striatal dopamine D_2-like receptors in mouse and rat brain. In mice the uptake of [^{3}H]NEM was significantly enhanced not only in the striatum but also in the reference regions such as the cortex and cerebellum. Although the specific binding assessed by the differences in the uptake between the striatum and reference regions significantly increased, the ratios of striatum to reference did not change. In rats there was found no effect of the reserpine-treatment on the uptake and uptake ratio measured by *ex vivo* ARG. When the data obtained by the murine study were tentatively applied to a kinetic analysis according to the method of Inoue et al,[9] the association rate constant (k3) did not change and the binding potential seemed to be slightly decreased. This kinetic method is easily applied to experiments on small animals such as mice, but has relatively large data sampling errors. To detect small changes by kinetic analysis, sequential data sampling by PET is preferable. Anyhow, the present results suggest that endogenous dopamine does not substantially compete with the NEM in the receptor binding probably because of high affinity of NEM for the receptors. The characteristics of NEM are different from those of the other dopamine D_2 receptor ligands, i.e. raclopride and *N*-methylspiperone. Inoue et al. have shown with the same experimental protocol that the [^{3}H]raclopride binding was increased by more than twice by the reserpine treatment but that the [^{3}H]*N*-methylspiperone binding was decreased.[9] The former was mainly explained by the decreased dissociation rate, and the latter was explained by the decreased association rate. Several factors may explain these different responses of the three ligands to the reserpine treatment. The affinity of raclopride for the dopamine D_2 receptors is weaker than that of NEM and *N*-methylspiperone. Raclopride is selective for the D_2 and D_3 receptor subtypes,[5] whereas NEM and *N*-methylspiperone have similar affinity for D_2, D_3 and D_4 subtypes.[5,18,19] It is suggested that the binding mechanism of benzamide ligands differs from that of butyrophenone ligands.[23,24] *N*-methylspiperone also has an affinity for serotonin receptors. As far as NEM, serotonin and sigma receptors are also possible binding sites.[21,25,26] Changes in circulation and peripheral mechanisms caused by reserpine treat-

ment,[9] indicated by a slower radioactivity clearance (Table 1), could also affect ligand receptor binding besides depletion of synaptic dopamine stores.

We also confirmed the absence of a reserpine effect on the striatal uptake of [^{11}C]NEM in rats by *ex vivo* ARG (Fig. 1). The ARG was performed at 20 min after the tracer injection. No difference in the uptake or the uptake ratio was observed between the control and reserpinized rats at 90 min after a tracer injection by the tissue sampling method (the striatal uptake, 2.17 ± 0.20 and 2.09 ± 0.33 %ID/g for control and reserpinized mice, respectively; and the striatum to cortex ratio, 4.07 ± 0.14 and 3.97 ± 0.88 for control and reserpinized mice, respectively).

[^{11}C]NEM is an interesting ligand for PET studies. By *in vitro* membrane binding assay, Seeman and co-workers have defined the D_4-like binding sites in the postmortem human brain as the difference between the density of the [^{3}H]NEM binding site and the density of the [^{3}H]raclopride binding sites.[5,19] Three groups have found high D_4-like binding sites in postmortem striatum from patients with schizophrenia,[5,27-29] whereas Reynolds and Mason could not detect D_4-like binding sites.[30] These conflicting *in vitro* studies are discussed.[31] On the other hand, Wong et al.[32] reported high [^{11}C]*N*-methylspiperone binding sites in schizophrenia by PET, while Farde and co-workers could not find the elevated dopamine receptor density by using [^{11}C]*N*-methylspiperone and [^{11}C]raclopride.[33,34] Until now no trail has been done to measure the [^{11}C]NEM binding sites in patients with schizophrenia by PET.

In conclusion, the present study indicates that [^{11}C]NEM binding to dopamine D_2-like receptors was not affected by the endogenous dopamine-depletion produced by the reserpine-treatment. It is possible that this tracer could be used to assess the change in D_2-like receptors in the human brain.

ACKNOWLEDGMENTS

The authors thank Yamanouchi Pharmaceutical Co., Ltd. for supplying nemonapride and its desmethyl compound.

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