19. 6-Hydroxydopamine Lesion Prevents Induction of Tyrosine Hydroxylase by Haloperidol in the Rat Substantia Nigra, Pars Reticulata

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Abstract: We investigated the number of tyrosine hydroxylase (TH)-positive neurons in the rat substantia nigra (SN) after unilateral lesion of SN dopamine neurons with intranigral 6-hydroxydopamine (6-OHDA) injection. Non-radioactive *in situ* hybridization procedure and immunohistochemistry detected much less (<5%) TH-positive neurons in the lesioned SN, compared with the contralateral side. Single administration of a dopamine antagonist haloperidol failed to increase TH labeling on the lesioned side, although it increased that in the contralateral SN pars reticulata (SNr), as in the case of unlesioned animals. These results indicate that 6-OHDA destroyed not only TH-positive SN neurons but also TH-negative but -inducible SNr neurons. It is probable that these "TH inducible" SNr neurons are dopaminergic neurons although they usually do not express TH at detectable levels.

Key words: Dopamine; substantia nigra; gene expression; *in situ* hybridization; immunohistochemistry.

Introduction. Nigral dopaminergic neurons release their neurotransmitter, dopamine, both from axon terminals in the striatum and from somata and dendrites in the substantia nigra (SN).^{6),9)} Most of these neurons are located in the SN pars compacta (SNc), on which most studies have been focussed, but the remaining 10–15% of tyrosine hydroxylase (TH)-immunoreactive neurons are found in the SN pars reticulata (SNr) with a number similar to that of nondopaminergic nigrostriatal neurons.^{3),7)} Dopamine has dual actions on striatal neurons via D₁ and D₂ receptors.^{4),13)} It inhibits the neighboring dopaminergic neurons in the SN^{2),6)} and activates some 30–40% of nondopaminergic neurons in the SNr.¹⁴⁾ Moreover, the released dopamine is thought to regulate dopamine synthesis, since acute blockade of dopamine receptors with haloperidol potentiates enzymatic activity of TH.^{1),11)} This regulation may, at least partly, be achieved by altering TH gene expression, since acute blockade of dopamine D₂ receptors with fluphenazine-N-mustard increases cellular levels of TH mRNA in SNc neurons.¹⁹⁾

Recently, we have demonstrated that haloperidol increases the number of TH-immunoreactive and TH cRNA-labeled neurons in the SNr by 70–80%.¹⁶⁾ The origin of these "TH-inducible" neurons could not be determined: it is probable that they are dopaminergic by nature with undetectable amounts of TH mRNA and protein, but it is also possible that nondopaminergic neurons switch their neurotransmitters to dopamine as

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occurs in some autonomic neurons.^{8),12)} In the present communication, this question was addressed by investigating the effect of haloperidol on 6-hydroxydopamine (6-OHDA)-lesioned SN. 6-OHDA exerts its selective toxicity on catecholaminergic neurons after being transported by the uptake system of these neurons specific for catecholamines and related substances.¹⁷⁾ Therefore, if TH-inducible cells are dopaminergic by nature, 6-OHDA will also destroy them and haloperidol will no longer be able to induce TH expression.

Methods. Six female Wistar rats (Wistar Shizuoka, 4 weeks old) received single 6-OHDA injections into the left SNc at a dose of 8 μ g in 4 μ l saline, delivered over 5 minutes, as described previously.¹⁰⁾ The effect of 6-OHDA was confirmed one and 2 weeks after the injection by observing turning responses to i.p. administration of methamphetamine (3 mg/kg body weight, Dainippon Pharmaceutical).¹⁷⁾ Lesioned animals were kept for another 4 weeks after the second methamphetamine injection in order to avoid any effects of this treatment on TH labeling. Then they received a single i.p. injection of 1 mg/kg haloperidol (Takeda; n=2) or saline at equivalent volumes (n=4) and were sacrificed by decapitation after 8 hours. Brains were taken out quickly and frozen with powdered dry ice, and coronal sections of 12 μ m thickness were prepared and investigated histologically as described previously.¹⁶⁾

Results and discussion. Five rats responded with more than 10 turns per minute to methamphetamine both 1 and 2 weeks after 6-OHDA treatment (Table I). Rat s2 turned 3–6 times per minute. This indicates effective lesion of SN dopamine neurons in at least 5 of 6 animals.¹⁷⁾

Cresyl violet staining revealed a reduction in the number of neurons in the 6-OHDA-injected SNc (not illustrated). In contrast, changes in the SNr were not recognized with this method, presumably due to the small percentage of dopaminergic cells in this area. Non-radioactive *in situ* hybridization histochemistry was performed using an

Table I. Turning responses and nigral TH⁺ cells in 6-OHDA-treated rats. One and 2 weeks after intranigral 6-OHDA injection, methamphetamine was administered intra-peritoneally to evaluate the nigral lesion. After another 4 weeks, haloperidol or saline was administered intraperitoneally to rats h1 and 2 or rats s1-4, respectively, 8 hours before sacrifice. ISH, *in situ* hybridization and IHC, immunohistochemistry

Animal	Turning response (turns/min)		Methods	Number of TH ⁺ cells (cells/15 sections)			
	Week 1	Week 2		Contralateral SNc SNr		Ipsilateral SNc SNr	
s1	10.1 $\pm$ 3.0	11.2 $\pm$ 4.7	ISH	1587	209	41 (2.6%)	0
			IHC	1560	207	36 (2.3%)	0
s2	6.2 $\pm$ 2.7	3.3 $\pm$ 1.8	ISH	1399	218	64 (4.6%)	0
			IHC	1420	223	58 (4.1%)	0
s3	14.2 $\pm$ 4.3	12.2 $\pm$ 4.1	ISH	1458	211	53 (3.6%)	3
			IHC	1483	205	52 (3.5%)	2
s4	15.8 $\pm$ 3.4	16.2 $\pm$ 4.4	ISH	1434	235	34 (2.4%)	0
			IHC	1410	229	37 (2.6%)	0
h1	16.3 $\pm$ 4.8	14.3 $\pm$ 6.4	ISH	1490	297	30 (2.0%)	1
			IHC	1510	274	38 (2.5%)	1
h2	10.2 $\pm$ 4.5	12.2 $\pm$ 3.6	ISH	1478	249	38 (2.6%)	0
			IHC	1452	241	39 (2.7%)	0

Fig. 1. TH mRNA-expressing cells revealed by *in situ* hybridization in the rostral SN after haloperidol treatment of an animal lesioned unilaterally by injection of 6-OHDA into the left SNc. On the injected side, no TH mRNA labeling was found in the SNr, whereas a few surviving TH positive cells could be seen in ventromedial aspects of the SNc (arrow). Contralateral to the injection site, TH positive neurons were labeled in both SNr and SNc. Bar, 1000 μ m.

antisense riboprobe prepared from a 380-bp Kpn I-EcoR I fragment of the rat TH gene.¹⁶⁾ Fluorescence immunohistochemistry was performed with a monoclonal mouse anti-TH antibody (Incstar).¹⁶⁾ *In situ* hybridization (Fig. 1) and immunohistochemistry (not illustrated) generally detected similar numbers of TH-positive (TH⁺) cells (Table I) and revealed similar distribution patterns in adjacent sections, as observed previously.¹⁶⁾ In all control animals which showed turning response of over 10 turns/min (s1, 3, 4), the number of TH⁺ neurons in the 6-OHDA lesioned SNc was less than 4% of that of the contralateral SNc (Table I). Only animal s2, which turned less than 10 times/min, showed a slightly higher percentage (4.6, 4.1%) of surviving TH⁺ neurons in the SNc. This result matches the observation that degeneration of 96% of SN dopamine neurons is necessary to evoke clear behavioral effects.¹⁷⁾ On the other hand, TH⁺ neurons in the SNr were almost completely removed by 6-OHDA treatment. Surviving TH⁺ cells were found usually in ventromedial aspects of the anterior SNc and exceptionally in the SNr of s3 and h1. Savasta *et al.*¹⁵⁾ have reported complete disappearance of TH oligonucleotide labeling after treatment with 6-OHDA, but 15% of SN neurons with catecholamine fluorescence survive nigral application of a smaller dose (4.8 μ g) of 6-OHDA.⁷⁾ This discrepancy may result from differences in the injection site, the dose of 6-OHDA, or detection sensitivity.

Haloperidol treatment did not alter TH labeling in the SNc either ipsi- or contralaterally, in accordance with previous observations on the non-lesioned animals.¹⁶⁾ It increased the number of TH⁺ cells by 30% compared with that of saline treatment in the contralateral SNr, but failed to induce TH labeling in the lesioned SNr (Table I). This change in the contralateral SNr differs from that in non-lesioned animals, where haloperidol increases the number of TH⁺ SNr neurons by some 80%. It is interesting to further investigate the reason for this discrepancy. However, the disappearance of TH⁺ neurons from lesioned SNr and the failure to induce them by haloperidol suggest that "TH inducible" SNr neurons are removed by 6-OHDA treatment, together with constitutively TH-expressing SN neurons. It is considered that "TH inducible" SNr neurons are able to take up and metabolize dopamine and, therefore, are dopaminergic, although they usually do not contain detectable amounts of TH protein or mRNA.

More than 95% of nigrostriatal neurons in the entire SN show glyoxylic acid-induced catecholamine fluorescence,^{7,18)} but only about 85% appear to be TH-immunoreactive.⁵⁾ Therefore, some 10% of nigrostriatal neurons would usually contain dopamine, but appear TH⁻ by immunohistochemistry and probably *in situ* hybridization. This population might include "TH inducible" neurons in the SNr.

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