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Selective destruction of nigrostriatal dopaminergic neurons does not alter [^{3}H]-ryanodine binding in rat striatum

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Abstract

Dopamine nigrostriatal neurons are important for motor control and may contain a particularly dense population of ryanodine receptors involved in the control of dopamine release. To test this hypothesis, we used a classical model of unilateral selective lesion of these neurons in rats based on 6-hydroxydopamine (6-OHDA) injection into the substantia nigra. Binding of [^{3}H]-GBR 12935, used as a presynaptic marker since it labels specifically the dopamine uptake complex, was dramatically decreased by 83-100% in striatum homogenates after 6-OHDA lesion. On the contrary, no reduction of [^{3}H]-ryanodine binding was observed. The present data indicate that [^{3}H]-ryanodine binding sites present in rat striatum are not preferentially localized in dopaminergic terminals.

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The presynaptic mechanisms which control and modulate dopamine release are very important mainly for locomotor function, but also for some aspects of cognitive and associative functions. The corpus striatum, particularly, contains dopaminergic terminals of nigrostriatal neurons that are important for the control of movements, as well exemplified by their role in the genesis of Parkinsonism (1). As a consequence, the mechanisms modulating dopamine release in these cells need to be further investigated. In neurons, transient rises in the intracellular calcium level are fundamental for neurotransmitter release and occur mainly by influx through voltage-operated calcium channels present in the plasma membrane (2). However, an alternative or complementary way to increase intracellular calcium is by activating one of the two calcium release channels that control the neuronal intracellular pools: the inositol-1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors (RyR). The latter, in particular, may participate in dopamine release as indirectly suggested by the beneficial effect of dantrolene in the neuroleptic malignant syndrome (NMS), a rare but serious idiosyncratic reaction to neuroleptics (3,4). Indeed, dantrolene, which is mainly used as a skeletal muscle relaxant in the treatment of malignant hyperthermia, acts peripherally by inhibiting the release of calcium from internal stores controlled by ryanodine-sensitive channels (4,5). Since there

are indications that dantrolene modulates dopamine release in the central nervous system of patients with NMS (6) as well as in rat striatum (7), dopaminergic terminals of nigrostriatal neurons may contain a particularly dense population of RyR involved in the control of dopamine release.

This hypothesis was tested in rats with unilateral, selective, 6-hydroxydopamine (6-OHDA)-induced lesion of the dopaminergic nigrostriatal pathway, a well-known model of hemi-parkinsonism (8,9), in which the decrease of several markers of the dopaminergic nerve terminals has been reported. Following the lesion, [^{3}H]-ryanodine and [^{3}H]-GBR 12935 binding, used as a presynaptic marker, was measured in striatal homogenates.

Under chloral hydrate anesthesia (500 mg/kg), male Wistar rats (250-300 g) were placed in a David Kopf stereotaxic frame. A unilateral injection of 6-OHDA (12 µg of 6-OHDA hydrobromide in 4 µl aqueous solution of 0.9% (w/v) NaCl containing 0.1% (w/v) sodium metabisulfite) was delivered into the substantia nigra pars compacta at stereotaxic coordinates measured from bregma: lat = -1.8 mm; post = -5.2 mm; prof = -7.8 mm (10). The injection time for the 6-OHDA solution was 4 min and the injection cannula was left in place for a further 5 min before being slowly withdrawn. In order to enhance the specificity of 6-OHDA for dopaminergic neurons and to protect this neurotoxin from enzymatic oxidation, respectively, all rats were injected (*ip*) with a mixture of 25 mg/kg desipramine and 5 mg/kg pargyline 30 min prior to surgery.

Fourteen days after the injection of 6-OHDA, the striata were dissected out, immediately frozen in liquid nitrogen and stored at -80°C for up to 3 weeks. On the day of the assay, left and right striata were allowed to thaw at room temperature and were homogenized with a teflon/glass homogenizer in 5 ml ice-cold 50 mM Tris buffer, pH 7.4. The homogenates were centrifuged at 100,000 *g* for 1 h at 4°C and the pellets were resuspended in 1 ml ice-cold 50 mM Tris buffer, pH 7.4. Protein concentration was determined using the Coomassie dye reagent (11).

The binding of [^{3}H]-GBR 12935 was performed at 22°C in plastic tubes containing 30 µg protein resuspended in a final volume of 1 ml. Binding buffer contained 50 mM Tris, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 0.1% sodium metabisulfite, and 0.1% bovine serum albumin. Non-specific binding was measured in the presence of 10 µM nomifensine. Incubation was performed for 30 min and was terminated by the addition of 3 ml ice-cold washing buffer (50 mM Tris, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl). The suspension was immediately filtered through glass fiber filters (Whatman GF/B presoaked for 1 h in 0.5% (w/v) polyethylenimine; Maidstone, Kent, UK) and washed twice with the same washing buffer. Filters were immersed in 5 ml Aqualuma (Lumac, Groningen, The Netherlands) before determination of radioactivity.

To measure [^{3}H]-ryanodine binding, 70 µg protein was incubated for 1 h at 37°C, a time sufficient to reach equilibrium (12), in a medium (0.5 ml) containing 1.5 M KCl, 10 mM ATP, 0.8 mM $CaCl_2$ (107 µM free Ca^{2+}), 10 mM HEPES, pH 7.4, and 1 nM [^{3}H]-ryanodine, essentially as previously described (13). Incubations were terminated by dilution of the samples with 5 ml of ice-cold buffer (150 mM KCl, 10 mM Tris, pH 7.4) followed by rapid filtration under vacuum on glass fiber filters (Whatman GF/C). Filters were further washed twice with the same washing buffer. Non-specific binding was measured in the presence of 10 µM unlabelled ryanodine.

[^{3}H]-GBR 12935 (40 Ci/mmol) and [^{3}H]-ryanodine (92 Ci/mmol) were purchased from Amersham (Buckingham, UK). Bovine serum albumin, pargyline hydrochloride and 6-OHDA hydrobromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Desipramine HCl and nomifensine maleate were obtained from RBI (Research Biomedicals Inc., Natick, MA, USA).

Table 1 shows that unilateral lesion of the dopaminergic nigrostriatal pathway led, in the denervated striatum, to a dramatic decrease (83-100%) of the binding sites for [^{3}H]-GBR 12935, a specific ligand of dopamine uptake complex used as a presynaptic marker. This indicates that dopaminergic terminals were effectively destroyed by 6-OHDA injection. On the contrary, no decrease in [^{3}H]-ryanodine binding was observed as a consequence of this treatment.

Table 1 - Specific binding of [^{3}H]-ryanodine and [^{3}H]-GBR 12935 to crude membrane preparations from striatum of 6-OHDA-lesioned rats.

Specific binding of [^{3}H]-ryanodine and [^{3}H]-GBR 12935 in right striatum (corresponding to the side of the substantia nigra lesioned by 6-OHDA injection) is reported as percent of specific binding measured in the contralateral striatum (control side). Specific binding accounts for 62 and 51% of total binding for [^{3}H]-ryanodine and [^{3}H]-GBR 12935, respectively.

Rats	[^{3}H]-Ryanodine binding (% control side)	[^{3}H]-GBR 12935 binding (% control side)
1	92.6	3.7
2	114.7	0
3	106.8	0
4	114.3	17.2
5	91	13.8
6	114.7	1.6
Mean ± SEM	105.7 ± 4.6	6.0 ± 3.1

These results are consistent with previous reports showing an important loss of striatal dopamine uptake binding sites following degeneration of nigrostriatal dopamine neurons in humans or in rats (14,15). The binding of [^{3}H]-neurotensin, a modulator of dopamine release, was also found to be nearly abolished in the neostriatum after this treatment, indicating a specific localization in dopaminergic terminals (16). On the contrary, no decrease in [^{3}H]-ryanodine binding was observed in the neostriatum, indicating that RyR are not mainly located in nigrostriatal dopaminergic terminals. In fact, either the RyR are not present in dopaminergic terminals, or their density is very low in these cells compared with the whole population of RyR present in the striatum, so that a putative reduction should not be detected. Note that recent work by Martone et al. (17) indicating an apparent absence of RyR in presynaptic boutons did not rule out the possibility of a particularly rich density of the RyR_3 isoform in nigrostriatal dopaminergic terminals since the antibodies used by the authors only recognized the other two isoforms (RyR_1 and RyR_2) and the RyR_3 isoform was expressed particularly in specific regions such as the corpus striatum (18). The absence of a high density of [^{3}H]-ryanodine binding labelling all three RyR isoforms in striatal dopaminergic terminals is in accordance with the presence of RyR in nearly every cell of the corpus striatum (17).

In this case, which calcium pool should be sensitive to dantrolene in the striatum, if such pools are thought to be the targets for the central effect of this drug? Interestingly, large aspiny cholinergic cells, assumed to correspond to the cholinergic interneurons that are so important in the "indirect inhibitory ENK/D_2 pathway" (19), are intensely labelled by anti-RyR antibodies but contain very few, if any, IP_3R (17). Although totally speculative, we are inclined to think that acetylcholine release in these interneurons could be particularly sensitive to RyR blocking by dantrolene, an effect that could help the D_2 dopamine receptor agonist bromocriptine, successfully used in the treatment of NMS, in blocking the release of acetylcholine in these neurons.

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