Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Altered expression of dopamine receptors in cholinergic motoneurons of the hypoglossal nucleus in a 6-OHDA-induced Parkinson's disease rat model

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ARTICLE INFO

Article history:
Received 14 August 2014
Available online xxxx

Keywords:
Choline acetyltransferase
Tyrosine hydroxylase
Dopamine receptor
Hypoglossal nucleus
6-OHDA

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder that is often associated with weak tongue motility. However, the link between the degenerated dopaminergic neurons in the substantia nigra (SN) and lingual dysfunction remains unclear. In the present study, we investigated the localization of dopamine receptor 1 (D1) and dopamine receptor 2 (D2) and alternations in their expression in cholinergic motoneurons of the hypoglossal nucleus (HN) using double-label immunofluorescence, Western blotting and semi-quantitative reverse transcription and polymerase chain reaction (SqRT–PCR) in rats that received microinjections of 6-hydroxydopamine bilaterally into the SN (6-OHDA rats). The results revealed that a large population of choline acetyltransferase immunoreactive (ChAT-IR) neurons was distributed throughout HN and that almost all of the ChAT-IR motoneurons were also D1-IR and D2-IR. Several tyrosine hydroxylase (TH)-IR profiles were observed in a nonuniform pattern near the ChAT-IR, D1-IR or D2-IR somas, suggesting potent dopaminergic innervation. In the 6-OHDA rats, TH immunoreactivity in the SN was significantly decreased, but food residue was increased and treadmill occupancy time was shortened. In the HN, protein expression of TH and D2 was increased, whereas that of ChAT and D1 was decreased. A similar pattern was observed in mRNA levels. The present study suggests that dopamine may modulate the activity of cholinergic neurons via binding with D1 and D2 in the HN. Changes in the expression of ChAT, TH, D1 and D2 in the HN of 6-OHDA rats might be associated with the impaired tongue motility in PD. These findings should be further investigated.

1. Introduction

Parkinson's disease (PD) is a common and disabling neurodegenerative disorder that is characterized by a dramatic loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Swallowing impairment (dysphagia) is a frequent and often unrecognized complication in patients with PD. The prevalence of dysphagia in patients with PD has been reported to range from 50% to 100%, and this symptom is generally unresponsive to antiparkinson therapy [1,2]. A normal swallowing pattern includes lingual, pharyngeal and esophageal stages. Lingual dysfunction in dysphagia is often present in patients with PD [2], and the most common features include difficulty initiating swallowing, a segmented bolus swallow, prolonged lingual elevation, and subdued tongue movement during bolus propulsion [2–5]. 6-Hydroxydopamine (6-OHDA)-induced PD rats have impaired lingual motor function, which is consistent with deficits observed in patients with PD [6,7].

The hypoglossal nucleus (HN) is composed of cholinergic motoneurons, the axons of which innervate extrinsic and intrinsic muscles of the tongue and control its complex movements through the hypoglossal nerve. The HN is also innervated by noradrenergic [8], dopaminergic [9,10], serotonergic [8], peptidergic [11], and nitrergic fibers [12]. Tyrosine hydroxylase (TH) is the rate-limiting

Abbreviations: PD, Parkinson's disease; SN, substantia nigra; D1, dopamine receptor 1; D2, dopamine receptor 2; HN, hypoglossal nucleus; DMV, dorsal motor nucleus of the vagus; SqRT–PCR, semi-quantitative reverse transcription and polymerase chain reaction; 6-OHDA, 6-hydroxydopamine; ChAT, choline acetyltransferase; IR, immunoreactive; TH, tyrosine hydroxylase; DA, dopamine; NE, noradrenaline; DAPI, 4′,6-diamidino-2-phenylindole.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.104
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enzyme in the biosynthesis of dopamine (DA) and noradrenaline (NE). It is widely distributed throughout the HN [8,13]. In addition, abundant dopamine receptor 1 (D1) mRNA expression has been detected in the HN using *in situ hybridization* [14] and dopamine receptor 2 (D2) binding sites have been observed using autoradiography [15]. However, there is no further morphological evidence available to support dopaminergic regulation of HN motoneuron function. The purpose of the present study was to investigate the distributions of TH-immunoreactive (IR), choline acetyltransferase (ChAT)-IR, and dopamine receptor (DR)-IR in the HN using double-label immunofluorescence as well as to examine their response to 6-OHDA-induced destruction of dopaminergic neurons in the SN by assaying mRNA and protein expression in the HN, which may provide the experimental evidence that elucidates the mechanism underlying the lingual disorder suffered by patients with PD.

2. Materials and methods

2.1. Animals

We used fifty adult male Sprague–Dawley rats (Laboratory Animal Services Center of Capital Medical University, Beijing, China) that ranged in weight from 210 to 250 g. Every procedure was approved by the Animal Care and Use Committee of Capital Medical University and was conducted in accordance with the established guidelines of the National Institutes of Health (NIH, USA). All efforts were made to minimize the number of animals used and their suffering.

2.2. 6-OHDA-treated rat model

The methods employed in the present study have been described previously [16,17]. Briefly, one group of rats ($n = 30$) received bilateral infusions of 6-OHDA (Sigma, St. Louis, MO, USA). After being anesthetized with chloral hydrate (0.4 g/kg), rats were placed on a Kopf stereotaxic instrument. Two areas of the skull were exposed (coordinates: AP, −5.6 mm; ML, ±2.0 mm; DV, −7.5 mm) and 6-OHDA (4 μg in 2 μl of 0.9% saline containing 0.05% ascorbic acid) was injected with a 10 μl Hamilton syringe. A sham control group included 20 rats that were injected with 0.2% ascorbic acid/saline solution. Of the 30 rats treated with 6-OHDA, 16 presented a greater than 60% loss of dopaminergic neurons in the SN and were used for further study.

2.3. Immunohistochemistry

Rats were anesthetized with chloral hydrate (0.4 g/kg) and then perfused with chilled phosphate-buffered saline (PBS 0.1 M), followed by 4% paraformaldehyde (300 ml) in 0.1 M PBS. The brains were then removed and immersed in 4% paraformaldehyde overnight at 4 °C. They were then transferred into graded sucrose and subsequently frozen. Serial frontal sections (20 μm) were cut on a cryostat (Leica CM1850, St. Gallen, Switzerland) through brainstem blocks that contained the HN and then thaw mounted onto glass slides. All slides were stored at −80 °C until further testing.

The sections were brought to room temperature and washed in 0.01 M PBS (pH 7.4). Sections were then permeabilized with 0.3% Triton X-100 and immersed in 10 mM citrate buffer (pH 6.0) to perform microwave antigen retrieval. After blocking nonspecific binding, sections were incubated with a mixture of two primary antibodies overnight at 4 °C (Table 1). Sections were subsequently incubated with a mixture of the two secondary antibodies for 1 h at room temperature. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) before being mounted onto antifade media, and the slides were visualized under a confocal microscope (Olympus, FV1000).

2.4. Control experiments

To document the specificity of our staining, several control experiments were performed following the protocols of a previous study [18]: (I) two or three antibodies for ChAT, D1 and D2 from different companies were used, and the distribution features of the immunoreactive neurons or fibers were identical; (II) slides from the striatum were used as a positive control for TH, D1 and D2 antibodies; (III) a group that did not receive primary antibodies and a preabsorption group were used as negative controls; and (IV) cross-reactivity was not observed between antibody groups during double-label immunofluorescence.

2.5. Tissue preparation for protein and mRNA extraction

The SN and HN were collected on ice from the brains of control and 6-OHDA rats. Animals were sacrificed and decapitated, and the brainstems were quickly removed. The left and right hypoglossal nuclei were dissected on ice according to the protocol published by Yu [19] and then submerged in liquid nitrogen. Tissues were stored at −80 °C until further testing.

2.6. Western blot analysis

Tissues were homogenized in 50 μl of cold lysis buffer supplemented with protease inhibitors for protein extraction (1% Nonidet P-40; 10 mM Tris–HCl, pH 8.0; 5 μg/ml leupeptin; 150 mM NaCl; 1 mM EDTA; 2% SDS; 5 μg/ml aprotinin; 1 mM PMSF; 0.5% deoxycholic acid; and 1 mM sodium orthovanadate, all purchased from Sigma Company). Proteins (25 μg) were separated using a 10% SDS–PAGE gel. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane (NC membrane, Millipore, Billerica, MA, USA) at 4 °C, washed for 10 min in TBST (20 mM Tris–Cl, pH 7.4, containing 0.15 M NaCl, 2.7 mM KCl and 0.05% Tween 20) and blocked for 1 h at room temperature in blocking buffer (10% non-fat dry milk in TBST). The membrane was incu-

Table 1
First antibodies used in this study.

Antigen	Antibody	Dilution		Source/catalog no.
		Immunocytochemistry	Western blot	
TH	Mouse monoclonal	1:5000	1:100000	Sigma/T1299
ChAT	Mouse monoclonal	1:50	1:500	Millipore/MAB305
ChAT	Sheep polyclonal	1:50	1:500	Abcam/ab18735
D1	Rabbit polyclonal	1:100	1:500	Abcam/ab20666
D1	Rabbit polyclonal	1:50	1:500	Alomone/ADR-001
D2	Rabbit polyclonal	1:100	1:500	Abcam/ab21218
D2	Rabbit polyclonal	1:50	1:500	Abcam/ab32349
GAPDH	Mouse monoclonal	N/A	1:500	Beyotime/AG019

Please cite this article in press as: L. Zhou et al., Altered expression of dopamine receptors in cholinergic motoneurons of the hypoglossal nucleus in a 6-OHDA-induced Parkinson's disease rat model, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.08.104