Brain Research Bulletin 124 (2016) 129–135

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Cytoarchitecture of nitrergic neurons in the human striatum and subthalamic nucleus

Bruno Lopes dos Santos-Lobato[a,d], Elaine Del-Bel[a,b,d], José Eymard Homem Pittella[c], Vitor Tumas[a,d,*]

[a] Department of Neurosciences and Behavioral Sciences, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil
[b] Department of Morphology, Physiology and Basic Pathology, Ribeirão Preto Dentistry School, University of São Paulo, Ribeirão Preto, SP, Brazil
[c] Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil
[d] Center for Research Support on Applied Neuroscience (NAPNA-USP), Brazil

ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form 4 April 2016
Accepted 5 April 2016
Available online 6 April 2016

Keywords:
Nitric oxide
Basal ganglia
Striatum
Subthalamic nucleus
Human brain

ABSTRACT

Background: Nitric oxide (NO) is a gaseous molecule that modulates several physiological processes, including signal transmission in the central nervous system. There is evidence supporting NO as a major neurotransmitter involved in motor and emotion/behavior control. We investigated the distribution and morphology of nitrergic neurons in the two main input structures of the basal ganglia of human brain: the striatum and subthalamic nucleus.
Methods: We studied samples of striatum (caudate and putamen) and subthalamic nucleus of 20 human brains from subjects without neurological/psychiatric diseases. The tissues were stained by histochemistry for nicotinamide adenine dinucleotide phosphate diaphorase activity and by immunohistochemistry for neuronal NO synthase (nNOS). Subsequently, we analyzed the nitrergic neuronal profile and its morphometric parameters.
Results: Our data corroborate that approximately 2% of neurons in striatum express nNOS and these exhibited morphology characteristic of interneurons. Posterior regions of the striatum have a higher nitrergic neuronal profile than anterior regions of this nucleus suggesting an anteroposterior gradient of nitrergic neurons. Posterior limbic-associated areas of the striatum have a higher nitrergic neuronal profile compared to other functional subdivisions. Also, approximately 90% of neurons in the subthalamic nucleus express nNOS.
Conclusions: A remarkable presence of nitrergic neurons in the human striatum and subthalamic nucleus suggests that NO may play a critical role in the physiology of these nuclei.

1. Introduction

The basal ganglia (i.e., striatum, globus pallidus, subthalamic nucleus and substantia nigra) are complex structures responsible for motor control, motor learning, executive functions, behaviors and emotions. These structures are regulated by classical neurotransmitters, including glutamate, acetylcholine and dopamine (Lanciego et al., 2012), but the role of new substances on these nuclei has been recently explored. One of these molecules is nitric oxide (NO), which is a gaseous molecule responsible for many essential physiological processes, including signal transmission in the central nervous system (Garthwaite et al., 1988). NO is generated in neurons by neuronal NO synthase (nNOS).

Nitrergic neurons are located in many regions of the central nervous system, particularly in the basal ganglia, where the physiological actions of NO are poorly understood. NO synthase inhibition diminishes levodopa-induced dyskinesia in animal models of parkinsonism, suggesting that NO may modulate the action of dopamine in the striatum and in other nuclei of the corticobasothalamic pathways (Padovan-Neto et al., 2009, 2011, 2015). Previous reports have described the presence of nitrergic neurons in human basal ganglia (Hirsch et al., 1987; Kowall et al., 1987; Geula et al., 1993; Morton et al., 1993; Böckelmann et al., 1994; Egberongbe et al., 1994; Mufson and Brandabur, 1994; Nisbet et al., 1994; Selden

Abbreviations: NADPHd, nicotinamide adenine dinucleotide phosphate diaphorase; NBT, nitroblue tetrazolium; NeuN, neuronal nuclear antigen; NNP, nitrergic neuronal profile; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PB, phosphate buffer; STN, subthalamic nucleus.

* Corresponding author at: Department of Neurosciences and Behavioral Sciences, Ribeirão Preto Medical School, University of São Paulo, Av. Bandeirantes 3900, CEP: 14049-900 Ribeirão Preto, SP, Brazil.
E-mail addresses: bruls4@usp.br (B.L.d. Santos-Lobato), eadelbel@forp.usp.br (E. Del-Bel), pittella@medicina.ufmg.br (J.E.H. Pittella), tumasv@rnp.fmrp.usp.br (V. Tumas).

http://dx.doi.org/10.1016/j.brainresbull.2016.04.003
0361-9230/

et al., 1994; Norris et al., 1996; Eve et al., 1998; Ohyu and Takashima, 1998; Johannes et al., 2003; Bernácer et al., 2005; Lauer et al., 2005; Fritzen et al., 2007).

Despite these reports, data about presence and morphology of nitrergic neurons in human subthalamic nucleus (STN) are unknown. Likewise, more elucidation about their distribution on human striatum is necessary. Aiming to explore possible roles of these neurons in basal ganglia circuitry, the objective of this study was to investigate the distribution and morphology of nitrergic neurons in the human striatum and STN.

2. Methods and materials

2.1. Subjects and sample collection

Post-mortem brain samples were obtained from 20 adult individuals with no clinical or pathological evidence of neurological or psychiatric disorders, brain death or human immunodeficiency virus infection. Demographic and clinical data from the subjects were recorded. Tissue was collected at autopsy and was free of significant macroscopic pathological alterations, as indicated by hematoxylin-eosin and Nissl staining. Brains were sectioned at a thickness of 0.5 cm and fixed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 48 h at 4 °C. Brain slices were immersed in 30% sucrose in PB at 4 °C until sedimentation, frozen using isopentane and dry ice, and subsequently stored at −80 °C until further assay. Samples from the striatum (i.e., caudate and putamen) and STN were sectioned using a cryostat to generate sections with a thickness of 30 μm and serially collected in a cryoprotective solution until staining. The study was approved by the Ribeirão Preto Medical School Hospital Ethics Committee, and tissue was kindly provided by the Department of Pathology of Ribeirão Preto Medical School.

2.2. NADPH-diaphorase histochemistry

Striatal sections were processed to visualize nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) enzymatic activity, which correlates with the presence of nNOS in neurons (Matsumoto et al., 1993), using an adapted protocol (Vincent and Kimura, 1992). Free-floating sections were rinsed for 30 min in PB and subsequently incubated in a staining solution containing 0.1 M PB (pH 8.0) with 0.3% Triton X-100, 1 mg/ml of β-NADPH (Sigma, Saint Louis, Missouri, U.S.A.) and 0.1 mg/ml of Nitroblue Tetrazolium (NBT) (Sigma). Incubation was performed in the dark at 37 °C for 180 min. After incubation, sections were rinsed thoroughly in PB, mounted onto gelatin-coated slides, and air-dried overnight. Sections were then dehydrated using increasing concentrations of alcohol (70%, 95%, 100%, 100% + xylene) and xylene 100%, and covered with Entellan® mounting medium (MERK, Darmstadt, Germany).

2.3. Immunohistochemistry

The NADPHd technique for STN sections showed poor staining, and immunohistochemistry was performed to label nNOS, a specific marker of nitrergic neurons. Immunohistochemistry for NeuN, a marker of the neuronal nucleus, was performed to label all neuronal types in the striatum, whereas Nissl staining was used to label all neurons in the STN. Antigen recovery was performed by heating sections for 30 min in a water-bath at 90 °C in a 0.1 M citrate buffer solution at pH 6.0. Next, sections were allowed to cool to room temperature for one hour. Inactivation of endogenous peroxidase activity was performed by treating the sections for 30 min with 0.3% H_2O_2 in 0.1 M phosphate-buffered saline containing 0.15% Triton X-100. Non-specific binding sites were blocked using 2% bovine serum albumin (BSA, Jackson Immuno Research, U.S.A.) containing 5% normal rabbit (for nNOS) or normal horse (for NeuN) serum for 1 h at room temperature.

Following these procedures, free-floating sections were incubated overnight with a 1:15,000 dilution of a sheep anti-nNOS antibody (kindly donated by Prof. Dr. Piers C. Emson, Babraham Institute, Cambridge, U.K.) and a 1:200,000 dilution of a mouse anti-NeuN monoclonal antibody (Chemicon, Temecula, California, U.S.A.). To detect primary antibodies, the following biotinylated secondary antibodies were applied for 90 min: rabbit anti-sheep for nNOS and horse anti-mouse for NeuN. All biotinylated secondary antibodies were used at a dilution of 1:400 and were obtained from Vector Laboratories (Burlingame, California, U.S.A.). Sections were incubated in avidin-biotin-peroxidase complex for 120 min (Vector Laboratories) and 3,3′-diaminobenzidine for 10 min (Sigma).

At each level, the first two consecutive sections were stained using NADPHd and NeuN (striatum) and the nNOS and Nissl method (STN), respectively, to estimate the proportion of nitrergic neurons in total number of neurons in these nuclei.

2.4. Analysis of the striatum and subthalamic nucleus

The striatum was subdivided into 10 levels along the anteroposterior axis, from the most anterior portion of the caudate to the most posterior portion of the putamen, using the following coordinates, with the anterior commissure as point zero (Mai et al., 2004): section 1, −20.0 mm; section 2, −15.0 mm; section 3, −10.0 mm; section 4, −5.8 mm; section 5, −2.0 mm; section 6, +2.0 mm; section 7, +9.3 mm; section 8, +14.6 mm; section 9, +19.9 mm; and section 10, + 25.2 mm (Fig. 1).

Further on, the caudate and putamen were also subdivided, using the anterior commissure as a landmark, into three (i.e., precommissural head, postcommissural head and body of the caudate) and two territories (i.e., precommissural and postcommissural putamen) respectively, as previously described (Bernácer et al., 2005).

In addition, we also examined two specific regions of the striatum: the posteroventral putamen, localized in the most ventral portion of the posterior postcommissural putamen, which is considered the “limbic-related” striatal area (Bernácer et al., 2005; Fudge and Haber, 2002), and the nucleus accumbens, with boundaries defined by Selden et al. (1994).

Functionally, we analyzed the striatum based on its cortical inputs, which was divided into three functional territories: sensorimotor (all postcommissural putamen sections, except the posteroventral putamen), associative (dorsomedial sector of the whole caudate) and limbic (posteroventral putamen and nucleus accumbens) territories (Bernácer et al., 2007).

The gyrus and tail of the caudate were not analyzed due to technical difficulties in dissection and staining.

The STN was subdivided into three anteroposterior levels at specific coordinates (Mai et al., 2004) (i.e., anterior: at the level of the mammillary bodies, +13.3 mm; middle: next to the SN pars compacta, +16.0 mm; and posterior: at the level of the red nucleus, +18.6 mm) and into three functional territories (i.e., sensorimotor, associative and limbic), based on a previously described method proposed by Parent and Hazrati (1995).

Sections were examined using an Axiophot II microscope (Carl Zeiss, Jena, Germany) equipped with a 3.0 megapixel Axiocam MRc digital camera and a microcomputer with AxioVison LE software, version 4.8.1.0 (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany). Whole section images were scanned at high resolution and 100× magnification using “MosaiX” module of AxioVison LE software. The final rendering of each drawing was generated using Adobe Photoshop CS5 64-bit for Windows (Adobe Systems, San