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A cytoarchitectonic and TH-immunohistochemistry characterization of the dopamine cell groups in the substantia nigra, ventral tegmental area and retrorubral field in the rock cavy (*Kerodon rupestris*)

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ABSTRACT

The 3-hydroxytyramine/dopamine is a monoamine of the catecholamine group and it is a precursor of the noradrenaline and adrenaline synthesis, in which the enzyme tyrosine hydroxylase acts as a rate-limiting enzyme. The dopaminergic nuclei retrorubral field (A8 group), substantia nigra *pars* compacta (A9 group) and ventral tegmental area (A10 group) are involved in three complex circuitries named mesostriatal, mesocortical and mesolimbic, which are directly related to various behavioral manifestations such as motor control, reward signaling in behavioral learning, motivation and pathological manifestations of Parkinson's disease and schizophrenia. The aim of this study was to describe the delimitation of A8, A9 and A10 groups and the morphology of their neurons in the brain of the rock cavy (*Kerodon rupestris*), a typical Brazilian Northeast rodent belonging to the suborder Hystricomorpha, family Caviidae. Coronal and sagittal sections of the rock cavy brains were submitted to Nissl staining and TH immunohistochemistry. The organization of these dopaminergic nuclei in the rock cavy brain is very similar to that found in other animals of the Rodentia order, except for the presence of the tail of the substantia nigra, which is found only in the species under study. The results revealed that, apart some morphological variations, A8, A9 and A10 groups are phylogenetically stable brain structures.

1. Introduction

In the 1950s, 3-hydroxytyramine/dopamine (DA) was described as a neurotransmitter in the central nervous system, in addition to its role as a precursor of the noradrenaline and adrenaline synthesis (Carlsson et al., 1958; Björklund and Dunnett, 2007a). As such, DA is a monoamine included in the catecholamine group, is a major neurotransmitter in the modulation of brain function and plays a crucial role in the adaptation of animal behavior throughout evolution (Smeets and González, 2000; Jones and Pilowski, 2002; Yamamoto and Vernier, 2011).

The first detailed description of the distribution of neurons containing catecholamine substances identified 12 neuronal groups, designated A1–A12 in caudorostral direction in the rat encephalon (Dahlström and Fuxe, 1964). Subsequent studies added five more cell groups, A13–A17 (Hökfelt et al., 1984). Of all these, the caudal groups A1–A7 are mainly noradrenergic,

Abbreviations: 3N, oculomotor nucleus; Aq, cerebral aqueduct; Cli, caudal linear nucleus of the raphe; cp, cerebral peduncle; csc, commissure of the superior colliculus; fr, fasciculus retroflexus; Hb, habenular nucleus; IF, interfascicular nucleus; IP, interpeduncular nucleus; ml, medial lemniscus; MN, mammilary nucleus; ns, nigrostriatal bundle; PAG, periaqueductal gray; PBP, parabrachial pigmented nucleus; pc, posterior commissure; PIF, parainterfascicular nucleus; PN, paranigral nucleus; RLi, rostral linear nucleus; RN, red nucleus; RRF/A8, retrorubral field; rs, rubrospinal tract; SN/A9, substantia nigra pars compacta (nuclear complex); SNCD, substantia nigra dorsal tier; SNCL, substantia nigra lateral cluster; SNCM, substantia nigra medial cluster; SNCV, substantia nigra ventral tier; SNR, substantia nigra reticulate; STh, subthalamic nucleus; SuM, supramammilary nucleus; tSN, tail of the substantia nigra; VTA/A10, ventral tegmental area (nuclear complex); VTAR, ventral tegmental area rostral part; ZI, zona incerta.

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A8–A11 cells are mainly dopaminergic, whereas the TH-positive cells in A12, A14 and A15 indicate absence of aromatic amino acid decarboxylase (AADC), the dopamine producing enzyme (Björklund and Dunnett, 2007b). A8, A9 and A10 are developed from the neuromere midbrain, with expansion to prosomere p1 (diencephalon), rostrally, and to isthmus-rhombomere 1 region (I-r1), caudally (Marín et al., 2005). These groups are coincident with the retrorubral field (RRF), the substantia nigra (SN), and the ventral tegmental area (VTA), respectively. The neurons of these groups express tyrosine hydroxylase (TH), but not dopamine beta-hydroxylase, which is an enzyme active in the conversion step for noradrenaline/adrenaline. Because of this, the A8, A9 and A10 groups are considered typically dopaminergic groups (Björklund and Dunnett, 2007b). Beside their DA content, these neuronal clusters can be divided on cytoarchitectonic and chemoarchitectonic grounds, as described in the mouse (Fu et al., 2012). It is known that these nuclei are involved in three complex circuitries, named mesostriatal, mesolimbic and mesocortical (François et al., 1999; Smith and Kieval, 2000; Björklund and Dunnett, 2007b) which are involved with motor control, motivation, cognition, reinforcement learning and some neurological/psychiatric disorders, such as Parkinson's disease and schizophrenia (Chudasama and Robbins, 2004; Nicola et al., 2005; Fields et al., 2007; Cohen et al., 2012).

Given the functional and pathological relevance of dopamine we believe it is necessary to expand studies on these neuronal groups in order to reach the greatest number of species.

The rock cavy (*Kerodon rupestris*) is a rodent inhabiting the semiarid Caatinga of the Brazilian Northeast, although it can be also found in the Southeast region as far as the south state of Minas Gerais (Cabrera, 1961). This species reaches adulthood at 200 days, and can reach up to 50 cm in length and 1 kg in body weight (Roberts et al., 1984). According to traditional taxonomy, the rock cavy belongs to the order Rodentia (Carleton and Musser, 2005). According to classifications using the skull shape as a primary characteristic – Anomaluromorpha, Castorimorpha, Hystricomorpha, Myomorpha and Sciuromorpha (Carleton and Musser, 2005), the rock cavy is part of the suborder Hystricomorpha, infraorder Caviomorpha, superfamily Cavioidea, family Caviidae, subfamily Caviinae. Morphological (Silva Neto, 2000) and molecular biology (Rowe and Honeycutt, 2002) studies have connected the genus *Kerodon* with the genus *Hydrochaeris*, which includes the capybara (family Hydrochaeridae), and is closely related to the genus *Dolichotis* of the subfamily Dolichotinae, whose representative in South America is the Patagonian hare (*Dolicothis patagonum*). The order Rodentia is the most diverse among placental mammals. A new nuclear gene analysis supports the division of this order in "squirrel-related", "mouse-related" clades, as well as the "Ctenohystrica clade", in which the suborder Hystricomorpha is included (Blanga-Kkanfi et al., 2009; Fabre et al., 2012).

The rock cavy has a predominantly crepuscular behavior (Sousa and Menezes, 2006) and is adapted to the Brazilian Northeast ecological conditions such as heat, water and food scarcity, especially in periods of severe drought. It inhabits rocky places with numerous crevices where it takes shelter from predators and spends much of its time. Moreover, rock cavies are excellent jumpers and can climb rocks and tree branches where they draw food, consisting mainly of tree bark, unlike other terrestrial caviinaes that eat grass (Carvalho, 1969; Lacher, 1981; Mendes, 1985).

For a while this species has been used as an experimental model for studies on the nervous system, for example in research on retinal projections to thalamic nuclei (Nascimento Jr. et al., 2008, 2010a) and circadian centers (Nascimento Jr. et al., 2010b) and the serotonergic system in the brain (Soares et al., 2012).

The present study aimed to describe the morphology of A8, A9 and A10 dopamine groups in the rock cavy by TH immunohistochemistry. It provides a foundation for future research on hodological and functional aspects of these neuronal groups in this species, broadening the basis for understanding evolutionary processes associated with the nuclear organization of this neuronal system.

2. Materials and methods

Four young adult rock cavies (two males and two females), weighing between 300 and 400 g, from rural municipalities in the state of Rio Grande do Norte, Brazil, were used. Animal capture was authorized by the Brazilian Environmental Agency (IBAMA, license 21440-1). Approval for the experiments was obtained from the local Animal Experimentation Ethics Committee (Protocol 015/2009-addendum) in compliance with National Institute of Health (NIH) guidelines. All efforts were made to minimize the number of animals and their suffering.

Individuals were housed for a short adaptation period in 3.00 × 2.00 × 2.60 m masonry cages consisting of four wire screen walls, ceramic tile ceilings and natural soil floor, with creeping vegetation and rocks to simulate their natural habitat. The animals were exposed to environmental temperature, air humidity and light, with unlimited access to food and water. Each individual was pre-anesthetized with an intramuscular injection of tramadol chloridrate and xylazine, both 5 mg/kg and maintained with gas isofluoran and 100% oxygen. Upon deep anesthesia, they were perfused through a cannula positioned in the ascending aorta, and connected to a peristaltic pump (Cole-Parmer). After cutting the right auricula, 300 ml of 0.9% saline solution in 0.1 M phosphate buffer, pH 7.4, containing heparin 5000 IU/ml (Parinex, Hipolabor, Sabará, MG, Brazil, 2 ml/1000 ml of saline solution) were injected for approximately 5 min. Next, 700 ml of a 4% paraformaldehyde, 2% picric acid and 0.05% glutaraldehyde fixative solution in 0.1 M phosphate buffer, pH 7.4 (Zamboni and De Martino, 1967) was administered. A flow rate of 70 ml/min was applied for half the solution and 17.5 ml/min for the other half, totaling 30 min for the entire procedure.

After perfusion, two animals were placed in the stereotaxic frame and the incisor bar was adjusted until the lambda and bregma were at the same height. The skull bones were removed to expose the dorsal surface of the encephalon, which was sectioned into 3 blocks by means of two coronal sections: one at the bregma level and the other at the lambda level. Finally, the encephalon was removed from the skull, stored in 30% sucrose solution in 0.1 M phosphate buffer, pH 7.4, for 24–48 h, and then sectioned by dry ice freezing in a sliding microtome, obtaining coronal sections of 30 μm. The brains of the other two animals were sectioned at the sagittal plane. In both cases, the sections were collected sequentially into 6 compartments, each containing one of every 6 sections, thereby representing a serial sequence with a distance of 180 μm between the sections.

Sections from one series were immediately mounted on gelatin coated glass slides and Nissl stained with thionin, to visualize the cytoarchitectonic delimitation of neuronal groups. Sections from another series were submitted to immunohistochemistry to reveal TH. All the immunohistochemical procedures were performed at room temperature. Free-floating sections, previously submitted to pre-treatment with sodium borohydride and hydrogen peroxide (H_2O_2), were placed in contact with the mouse anti-TH antibody (Sigma, 1:10,000) and 2% normal goat serum in 0.4% Triton X-100 for 18 h, in a rotator. This was followed by incubation in the secondary antibody, consisting of 1:1000 biotinylated donkey anti-mouse (Jackson Immunoresearch Labs.) under gentle shaking in a rotator, for 90 min. In order to visualize the reaction, the sections underwent 90-min incubation in an avidin–biotin–HRP complex (Vector Elite ABC kit), followed by the final reaction in a medium containing H_2O_2 as substrate and 3,3′-diaminobenzidine tetrahydrochloride as chromogen. H_2O_2 was offered indirectly, by mixing oxidase glucose and β-D-glucose into the solution, causing a reaction in which the former acting on the latter releases H_2O_2 (Itoh et al., 1979). The sections were thoroughly washed with a 0.1 M phosphate buffer, pH 7.4, at the beginning, between each step and at the end. Sections were mounted on previously gelatinized glass slides, which, after drying at room temperature, were rapidly submerged in a solution of 0.05% osmium tetroxide to enhance the visibility of the reaction product. The sections were dehydrated in a battery of gradually increasing alcohols, cleared, and then coverslipped in an Entellan® mounting medium.

With respect to staining specificity, a number of sections were submitted to immunohistochemical reactions omitting the primary or secondary antibodies. In these cases, no TH-immunoreactivity was obtained.

TH-immunostained coronal sections of the rock cavy brain were analyzed using an optical microscope (Olympus BX41) under bright field illumination. Digital images were obtained from representative sections using a digital video camera (Nikon DXM1200) coupled to the microscope. The digitized images were converted to a gray scale, corrected minimally for brightness and contrast, and mounted using Adobe Photoshop CS5 software (Adobe Systems, Mountain View, CA, USA). Diagrams were obtained from images of Nissl-stained coronal and sagittal sections with Adobe Illustrator CS5 software (Adobe Systems, Mountain View, CA, USA).

3. Results

In this study, TH immunohistochemistry was used to delimit dopaminergic neuronal groups A8, A9 and A10 in the rock cavy