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Distribution of Tyrosine Hydroxylase-Immunoreactive Neurones in the Diencephalon and Mesencephalon of the Coypu (*Myocastor coypus*)

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With 5 figures and 2 tables

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Summary

The aim of the present study was to examine the catecholaminergic neurones located within the midbrain of the coypu, a South American hystricomorph rodent. The neuronal distribution of the catecholaminergic systems and morphological parameters of the immunostained cell bodies and fibres were investigated, using an immunohistochemical method. The brains of five coypu were fixed, immersed in gelatine–glycerol and cut in 40-μm slices using a freezing microtome. Samples were processed with ultrasound-based antigen retrieval and stained with labelled antityrosine hydroxylase monoclonal antibody. An image analyser was used to measure the neuronal bodies. The catecholaminergic neurones of the tuberoinfundibular system were mainly observed in the arcuate and periventricular nuclei with their axons projecting towards to the median eminence; they represented 28% of the global population of tyrosine hydroxylase-immunoreactive cells observed. Significant morphological differences were observed in comparison with the other two studied systems. Fifty per cent of total catecholaminergic neurones were detected in the nigrostriatal system distributed in the reticular and compact substance nigra. Most neuronal bodies had a fusiform aspect. The immunoreactive neurones of the mesolimbic system represented 22% of the total population. They were distributed around the interpeduncular nucleus. Two types of morphologically different catecholaminergic systems of the brain were established: hypothalamic neurones located in the periventricular and arcuate nuclei and mesencephalic neurones located in the substance nigra and interpeduncular nuclei. These systems showed morphological and probably physiological-pharmacological differences.

Introduction

The central monoaminergic systems were first described in rats using the histofluorescence method (Dahlström and Fuxe, 1964) and several studies have been conducted in this species and other species of rodents (German et al., 1983). More recently, immunohistochemical methods have been used to investigate the distribution and morphology of central catecholaminergic neurones in several species of mammals, including humans (Fallon and Moore, 1978; Tanaka et al., 1982; German et al., 1983; Pearson et al., 1983). Antibodies raised against tyrosine hydroxylase (a cathecolamine-synthesizing enzyme) have been widely used to examine the monoaminergic neurones within the brain. However, based upon these studies in the mesencephalon and hypothalamus of numerous species of mammals mainly dopaminergic neurones have been observed which are grouped in the systems (Ganong, 1986): tuberoinfundibular, nigrostriatal and mesolimbic.

In the tuberoinfundibular system the neuronal bodies are located in the tuberal region, the arcuate nucleus (ARC) and the hypothalamic periventricular nucleus (PeVH). The dopamine released by these cells in the median eminence (ME) inhibits secretion of prolactin hormone (PRL). In the nigrostriatal system the neuronal bodies are located in the substance nigra (SN), projecting their axons towards the caudate nucleus (CA), putamen (PUT) and globus pallidus (GP), allowing the modulation of motor activity. It has been shown that the cell's degeneration of this system is related to the motor disorder associated with Parkinson's disease (McGeer et al., 1977). Although the function of these cells in motor control is not completely understood, they generally ease the initiation of motor responses to environmental stimuli. In the mesolimbic system the neuronal bodies surround the interpeduncular nucleus (IPN) and the ventral tegmental area (VTA). This system has been related to the activating of certain adapted behaviours, such as pleasant sensations induced by the addiction to drugs (Bozarth, 1991). It has also been associated with psychiatric disorders such as schizophrenia (Mackay et al., 1982).

In this context, the identification and neuroanatomical location of tyrosine hydroxylase-immunoreactive (TH-ir) neurones in the midbrain of hystricomorph rodents might add important data on the evolution of catecholaminergic systems in mammals. This is due to the fact that the anatomical features of these rodents are similar to those of the oldest extinct rodents (Hershkovitz, 1969; Patterson and Pascual, 1972). Moreover, some authors consider this as the mother group of all rodents (Novasek, 1992). Their origin presumably occurred in Africa during the Eocene and some of them migrated across the South Atlantic to South America on rafts during the Oligocene (Lavocat, 1974). Our animal, *Myocastor coypus*, belongs to this suborder and is commonly used in the fur industry and has been slowly introduced as an experimental model in different lines of investigation (Jurado et al., 1997; Silva et al., 1997).

On the basis of the above-mentioned data, the aim of this study was to investigate the distribution and morphometric parameters of TH-ir neurones, by means of immunohistochemistry, in the diencephalon and mesencephalon of the coypu.

Materials and Methods

Animals

The brains of five mature coypus (6200 g average body weight) from an established colony of animals of the Institute of Anatomy at the School of Veterinary Medical Sciences of La Plata National University were used. Animals were perfused with buffered saline under general anaesthesia and bled to whiteness. Then, they were fixed by perfusion of paraformaldehyde-picric acid solution (Zamboni and Martino, 1967) through the carotid arteries. The brains were extracted, measured, postfixed in the same solution and immersed in gelatin–glycerol. The blocks containing the diencephalon and mesencephalon were cut in 40-μm slices, using a freezing microtome, and divided into a series of sections at 240-μm intervals. Three series of sections were used for each animal. The first was stained with toluidin blue to identify nuclei and neuronal areas. The other two series were immunostained to show the catecholaminergic neurones (antityrosine hydroxylase).

Immunohistochemistry to catecholaminergic neurones

An antigen retrieval process based on ultrasound (Portiansky and Gimeno, 1996) was applied to all the sections in order to counteract the time-dependent cross-linking effects that the formaldehyde has on proteins (Leong and Gilham, 1989). Briefly, section slides were dipped into a glass dish containing 400 ml citrate buffer. The tip of an ultrasound disrupter (Branson Ultrasonics model 250, Danbury, CT) was immersed in the solution. The blocks were exposed to 40 W potency for 40 s. The blocks were then transferred to phosphate-buffered saline. Serial sections were processed using a monoclonal antibody antityrosine hydroxylase (Calbiochem-Novabiochem Corporation, La Jolla, CA). A commercial kit (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA) was used as a detection system. The horseradish peroxidase activity was demonstrated using 3,3-diaminobenzidine tretrahydrochlorate, as a chromogen. The sections were finally countercoloured with toluidine blue, dehydrated and mounted for their observation under an optic microscope.

Image processing and analysis

The images of the different portions of the midbrain were captured by an analogical RGB video camera (Sony DXC-151 A CCD, Tokyo, Japan) attached to a microscope (Olympus BX50, Tokyo, Japan) with a microscopic magnification of 200×. The images were then digitized with a frame grabber (Flashpoint 128, Integral Technologies Inc, Indianapolis, IN, USA) connected to the computer (PC Pentium II, 266 MHz, 64Mb RAM; Software: ImagePro Plus for Windows95 v4.00 – Media Cybernetics, Silver Spring, MA, USA), with a pixel depth of 24 bits, RGB and TIFF format. The resolution was set to 640×480 pixels and the corresponding yield was 0.625 μm/pixel.

A background correction operation was performed on each image in order to meaningfully compare the optical density (OD) of the different slides. To separate the immunostained TH-ir neurones (brown stain) from the counterstained objects (Toluidin blue stain) the Color Segmentation operation was applied. The brownish stain was selected with a sensitivity of 4 (maximum 5). A mask was then applied in order to make a permanent colour separation. The images were then transformed into an 8-bit grey scale TIFF format. After spatial and intensity of light calibration of the images the immunohistochemically stained area (expressed in μm^2) and the OD of the labelled reaction, defined by the antigen–antibody complex (Wells et al., 1993), were obtained. Values registered from the histograms, obtained from at least 25 images of each slide, were exported to a spreadsheet in order perform the statistical analysis.

The selected objects were then characterized by means of morphometric values such as major and minor axis measured in μm, aspect and roundness. Table 1 shows the morphometric values evaluated.

Statistical analysis

The analysis of variance was used to evaluate differences between TH-ir neuronal systems. Tukey's method (Zar, 1984) was used as a post hoc test. Significant differences between the slices were defined as those with an error probability < 0.05. Highly significant differences were defined as those with a P value < 0.01. To determine the significance of the OD data Student's t-test for differences between paired groups was applied.

Results

Distribution of TH-ir neurones and fibres of the tuberoinfundibular system

The TH-ir neurones of this system represented 28% of the global population of immunoreactive cells present in the midbrain (diencephalon and mesencephalon.); 23% of cell bodies were observed grouped within the ARC, and the remaining 5% were observed in the PeVH, closer to the ventricular wall (Fig. 1). The average size of the cellular bodies found in the ARC was $12\ \mu m \times 18\ \mu m$ and in the PeVH it was $13\ \mu m \times 17\ \mu m$ (Fig. 2a and b). A significant difference was found in the minor axis, roundness and neuronal area regarding the other two systems studied; however, the major axis and aspect did not show such differences (Table 2). The TH-ir axons of this system were identified in the ME forming a net in the external layer of the same

Table 1. Morphometric parameters used to characterize the immunoreactive neurones

Major axis	Reports the length of the main axis of the ellipse equivalent to the object (i.e. an ellipse with the same area, first and second degree moments).
Minor axis	Reports the length of the minor axis of the ellipse equivalent to the object (i.e. an ellipse with the same area, first and second degree moments).
Aspect	Reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object (i.e. an ellipse with the same area, first and second degree moments), as determined by major axis/minor axis.
Roundness	Reports the roundness of each object, as determined by the following formula: $(\text{perimeter}^2)/(4 \times \pi \times \text{area})$. Circular objects will have a roundness = 1; other shapes will have a roundness > 1.
Area	Reports the area of each object (minus any holes).

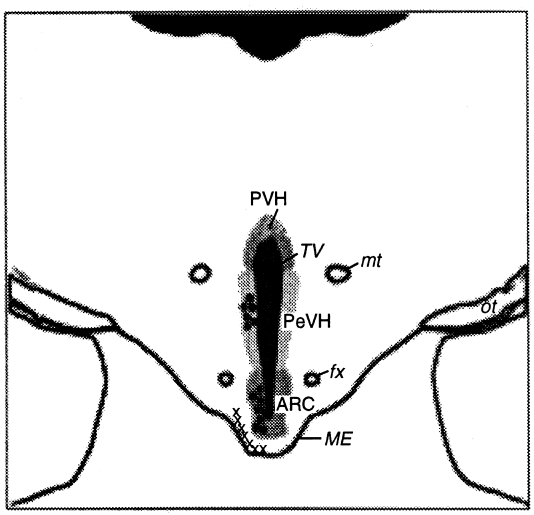

Fig. 1. Schematic drawing of a crown-like section of the diencephalon of the coypu at the levels of the arcuate nucleus, showing the distribution of the TH-immunoreactive neuronal bodies (•) and immunoreactive fibres (X) on the left half of the outline. Abbreviations: PVH, hypothalamic paraventricular nucleus; TV, third ventricle; mt, mamilothalamic tract; PeVH, hypothalamic periventricular nucleus; fx, fornix; ARC, arcuate nucleus; ME, median eminence; ot, optic tract.

area, in connection with the primary capillary of the portal vessels. In this area, the OD exceeded the middle of the scale (Fig. 3), showing a great affinity of the antibody used (Wells et al., 1993).

Distribution of TH-ir neurones of the nigrostriatal system

The population of TH-ir neurones in this system represented 50% of the total amount of catecholaminergic cells found along the midbrain. These neurones were abundant in the pars compacta (CSN) and they were observed forming clusters within the pars reticulata (RSN) of the substance nigra (Fig. 4). The majority of neuronal bodies had a lengthened fusiform aspect and their average size was 7 μm × 18 μm (Table 2) (Fig. 2c).

Distribution of TH-ir neurones of the mesolimbic system

The population of TH-ir neurones in this system represented 22% of the total amount of immunoreactive cells observed in the coypu midbrain. The neuronal bodies were mainly distributed surrounding the IPN (Fig. 5) in a concentrated area at the lateral portion of the nucleus and more diffusely towards its dorsal sector. Only a few groups of TH-ir neurones were observed in the VTA but always in the vicinity of the IPN. The neurones of this system mostly presented a lengthened fusiform aspect with an average size of 8 μm × 17 μm (Table 2) (Fig. 2d).

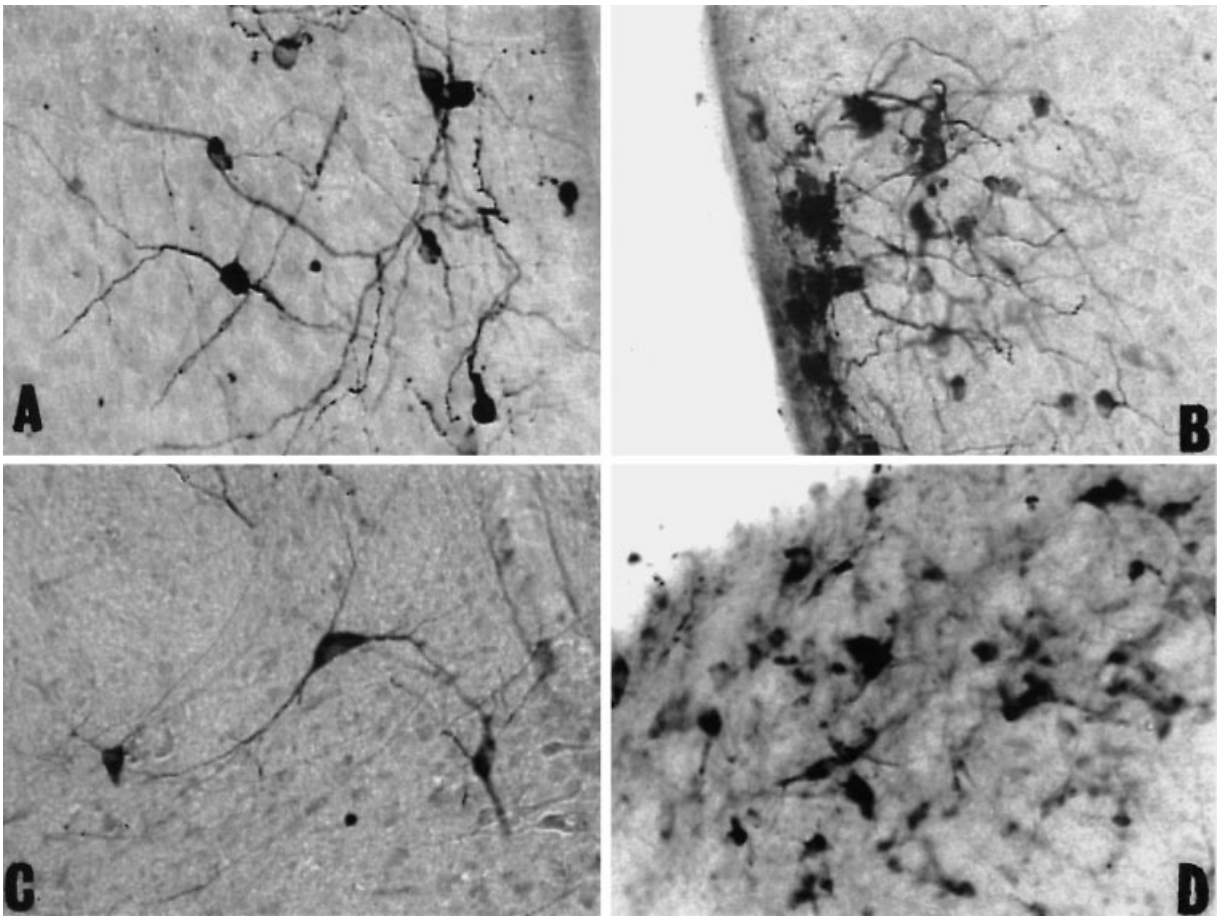

Fig. 2. (a) TH-ir neurones in the arcuate nucleus; (b) periventricular nucleus neurones; (c) reticular substance nigra neurones; (d) neurones surrounding the interpeduncular nucleus. Magnification: ×400.

Table 2. Morphometric parameters corresponding to neuronal bodies of different monoaminergic systems

Parameter	Tuberoinfundibular system (ARC)	Nigrostriatal system	Mesolimbic system
Cellular area	321 ± 24.5 **	154 ± 15.5	150 ± 12.7
Major axis	18 ± 0.7	18 ± 1.1	17 ± 1.4
Minor axis	12 ± 0.5*	7 ± 0.8	8 ± 0.6
Aspect	2.2 ± 0.11	2.6 ± 0.15	2.1 ± 0.14
Roundness	2.6 ± 0.16*	1.8 ± 0.12	1.6 ± 0.13

Each value represents the average of the neuronal bodies of each monoaminergic systems ± SD. *Significant ($P < 0.05$); ** highly significant ($P < 0.01$).

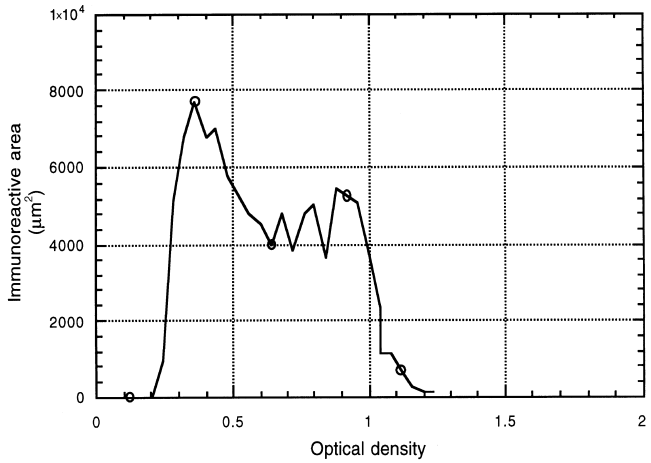

Fig. 3. Immunoreactive area of the antityroxine hydroxylase monoclonal antibody, distributed according to the optical density of the labelled reaction in the ME.

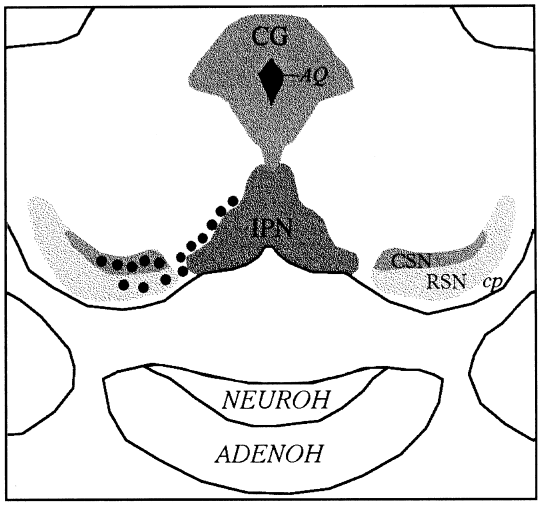

Fig. 4. Schematic drawing of a crown-like section of the coypu rostral mesencephalon at the interpeduncular nucleus (IPN) level showing the distribution of the TH-immunoreactive neuronal bodies (•) in the left half of the outline. Abbreviations: CG, central grey; AQ, aqueduct; IPN, interpeduncular nucleus; CSN, compact substance nigra; RSN, reticular substance nigra; cp, cerebral peduncle; NEUROH, neurohypophysis; ADENOH, adenohypophysis.

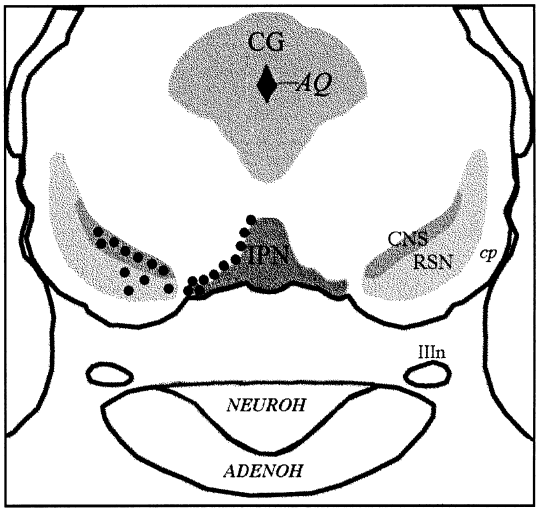

Fig. 5. Schematic drawing of a crown-like section of the coypu median mesencephalon at the interpeduncular nucleus (IPN) level showing the distribution of the TH-immunoreactive neuronal bodies (•) in the left half of the outline. Abbreviations: CG, central grey; AQ, aqueduct; IPN, interpeduncular nucleus; CSN, compact substance nigra; RSN, reticular substance nigra; cp, cerebral peduncle; NEUROH, neurohypophysis; ADENOH, adenohypophysis; IIIn, oculomotor nerve.

Discussion

The population of coypu TH-ir neurones was identified specifically by applying immunohistochemistry, using an antityroxine hydroxylase monoclonal antibody, and thus it was possible to determine their localization and their percentage of the global population of the midbrain. The localization of the neurones observed in this study resembles that reported for other species, not only rodents but also rabbits, dogs, primates and humans (Shimada et al., 1976; German et al., 1983; Pau et al., 1997). Therefore, it is dangerous to extrapolate information from one species to another. In this case, specific knowledge of these neuronal systems seems to be indispensable.

The dopamine nigrostriatal system gathers the most TH-ir neuronal bodies, which were observed in the SN with their projection fibres toward the CA, PUT and GP nuclei, coinciding with data reported for the rat brain (German et al., 1983). There were differences in the size of the immunoreactive neuronal bodies of the coypu compared with humans (20 μm × 70 μm), rats (14 μm × 18 μm) and mice (9 μm × 17 μm) (German et al., 1983; Pearson et al., 1983). However, the localization of

the neuronal bodies as well as the efferent connections of the system showed a high degree of homology, suggesting a common physiological role for dopamine as an encephalic neurotransmitter.

The neuronal bodies of the mesolimbic system were distributed in a different pattern, as in the rat, because few TH-ir neurones were observed in the VTA and the vast majority of catecholaminergic neurones were around the IPN nucleus. The morphometric parameters of these neurones were very similar to those of the dopamine cells of the nigrostriatal system. However, there were morphometrically significant differences in the neuronal bodies of the tuberoinfundibular system with regard to the other two systems, with a more rounded form in these neurones, typically multipolar, due probably to the role that they have in different functional circuits. It is known that neurones of the tuberoinfundibular system act as inhibitors of PRL and of the pulsatile secretion of gonadotropin-releasing hormone (GnRH), for diverse mechanisms. The dopamine transported and liberated in the ME inhibits, via the portals vessels, the lactotropic cells of the adenohypophysis. However, the action on the system of the gonadotropic hormones is due to the narrow relationship of the dopaminergic terminal that inhibits the GnRH terminal at the level of the ME (Thiéry and Martin, 1991). On the other hand, the stimulating effect of the pre-ovulatory peak of LH shown in the rat (Thiéry et al., 1989, 1995) should not be discarded. The function of this dopamine system in the control of reproduction is similar in several vertebrate groups (Thiéry et al., 1995) and it can suggest an old phylogenetic route in the regulation of the mammal reproductive activity.

The present work suggests that there are two types of morphologically different TH-ir midbrain systems, the hypothalamic neurones located in the ARC and PeVH nucleus (tuberoinfundibular system) and the mesencephalic neurones located in the SN and IPN (nigrostriatal and mesolimbic system). These systems appear to be similar in the rodentia order but they show morphological and physiological differences. Moreover, pharmacological studies (Bunney, 1979; Freed et al., 1984; Hökfelt et al., 1984) confirm the hypothesis of two different dopamine populations of neurones that should be characterized in more detail based on their embryological origin and their ultrastructure. In consequence, future work should be carried out to clarify these aspects in this rodent and in other South American hystricomorph rodents, as well as to clarify the changes that take place due to age or specific illnesses that affect the different midbrain mono-aminergic systems.

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