Original Paper

Brain, Behavior and Evolution

Brain Behav Evol 387
DOI: 10.1159/0000XXXXX

Received: May 30, 2002
Returned for revision: July 15, 2002
Accepted after revision: September 3, 2002

The Distribution and Morphological Characteristics of Catecholaminergic Cells in the Brain of Monotremes as Revealed by Tyrosine Hydroxylase Immunohistochemistry

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Key Words
Mammals · Monotremes · Platypus · Echidna · Dopamine · Noradrenaline · Adrenaline · Sleep

Abstract
The present study describes the distribution and cellular morphology of catecholaminergic neurons in the CNS of two species of monotreme, the platypus *(Ornithorhynchus anatinus)* and the short-beaked echidna *(Tachyglossus aculeatus)*. Tyrosine hydroxylase immunohistochemistry was used to visualize these neurons. The standard A1–A17, C1–C3 nomenclature was used for expediency, but the neuroanatomical names of the various nuclei have also been given. Monotremes exhibit catecholaminergic neurons in the diencephalon (A11, A12, A13, A14, A15), midbrain (A8, A9, A10), rostral rhombencephalon (A5, A6, A7), and medulla (A1, A2, C1, C2). The subdivisions of these neurons are in general agreement with those of other mammals, and indeed other amniotes. Apart from minor differences, those being a lack of A4, A3, and C3 groups, the catecholaminergic system of monotremes is very similar to that of other mammals. Catecholaminergic neurons outside these nuclei, such as those reported for other mammals, were not numerous with occasional cells observed in the striatum. It seems unlikely that differences in the sleep phenomenology of monotremes, as compared to other mammals, can be explained by these differences. The similarity of this system across mammalian and amniote species underlines the evolutionary conservatism of the catecholaminergic system.

Introduction

Catecholaminergic (CA) neurons are found throughout the central nervous system of all classes of vertebrates. The phylogeny of the CNS CA system of vertebrates has been comprehensively reviewed [Smeets and Reiner, 1994; Smeets and González, 2000], and the reader is referred to these reviews to obtain detailed descriptions of the CA system in the various vertebrate species studied to

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date. For ease of description and reading, the classical A1–A17/C1–C3 terminology is used throughout the present text, as this is in common usage [Smeets and Reiner, 1994; Smeets and González, 2000]. However, Smeets and González [2000] have indicated that this terminology might not be the most correct, so where the anatomy of the monotremes [Hines, 1929] lacks ambiguity in comparison to other mammals, anatomical names have been given in the heading of the description of each CA cell group.

CA neurons have been identified in the mammalian retina (A16) and olfactory bulb (A17) [Smeets and Reiner, 1994]. CA cell groups in the mammalian diencephalon have been divided into several subgroups [Tillet, 1994], namely A11, A12, A13, A14, and A15. CA cell groups in the mammalian midbrain and brainstem have also been divided into several subgroups [Kitahama et al., 1994] classified as A9, A9v (ventral), A9l (lateral), A10, A10dc (dorsal, caudal), and A10c (caudal). CA neurons in the rostral rhombencephalon are classified into four subgroups, A4, A5, A6 (locus coeruleus), and A7 (subcoeruleus), and those CA neurons in the caudal rhombencephalon are subdivided into six groups, A1, A2, A3, and C1, C2, and C3. Similar distributions of the majority of these cell groups have been described for a range of vertebrate species [Smeets and Reiner, 1994; Smeets and González, 2000].

In addition to these main groups, CA cells have been reported in various regions of the vertebrate CNS. In mammals, these include CA neurons located in the cerebral cortex, striatum, basal forebrain, and habenular region, with varying reports of CA neurons in the spinal cord [Smeets and Reiner, 1994; Smeets and González, 2000]. The occurrence of these groups in other vertebrate species is variable. Of significance for the present study is the occurrence of CA cells in the spinal cord, pretectum, and hypothalamic periventricular organ, and the lack of CA cells in the habenular region, cortex, striatum, and basal forebrain of reptiles [Smeets and González, 2000].

The majority of immunohistochemical studies of the distribution of catecholaminergic neurons in the brains of mammals have been restricted to placental mammals, including primates, rodents, carnivores and ungulates [Kitahama et al., 1994; Tillet, 1994; Smeets and González, 2000]. The present study provides a detailed anatomical analysis of the distribution of catecholaminergic neurons in the brain of monotremes, which, as an early branch of mammalian evolution, provides interesting insights into the evolution of the mammalian CA system.

Materials and Methods

The brains of three adult platypus *(Ornithorhynchus anatinus)* and three adult short-beaked echidna *(Tachyglossus aculeatus)*, obtained from previous experimentation [Siegel et al., 1996, 1998, 1999], were used in this study. While under deep barbiturate anesthesia, the animals were perfused via the heart with 0.9% cold saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer.

Serial 50-μm sections of the brains were made in coronal and sagittal planes. A one in five series of stains was made for Nissl, fibers [Gallyas, 1979], choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) and serotonin. The results of ChAT and serotonin immunohistochemistry are presented elsewhere. For TH staining, the sections were rinsed 3 times in 0.1 *M* Trizma-buffered saline (TBS) followed by a 48-hour incubation at 4°C with a 1/500 dilution of primary rabbit antiserum to TH (Eugene Tech International Inc, Ridgefield Park, New Jersey). The dilutions were prepared with a solution of 1% normal goat serum (NGS) and 0.25% Triton X-100 in 0.1 *M* Tris-saline. This was followed by a 2.5-hour incubation with biotinylated goat anti-rabbit IgG (Vector Labs, Burlingame, California) diluted 1/200 with 1% NGS in Tris-saline. The tissue was then incubated for 2 h with the avidin-biotin complex diluted 1/100 with 1% NGS in Tris-saline (Vector). Between each incubation the sections were rinsed 3 times with 1% NGS in Tris-saline. The sections were then treated for 6 min with a 0.05% solution of 3,3′diamino-benzidine and 0.01% hydrogen peroxide, rinsed in phosphate buffer, mounted on gel-coated slides, cleared in xylene and coverslipped with Depex mounting medium.

The stained sections were examined under a low-power dissecting microscope, cell bodies marked using a camera lucida, and then matched to architectural boundaries determined from the adjacent Nissl and fiber stained sections. High-power photomicrographs were taken of approximately 100 cells in each architectonic region and the somatal area determined using the program Image Tools. Only cells in which a clear nucleolus could be seen were used in this analysis. This research was carried out according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes under Queensland National Parks and Wildlife permits T00803 and K01782.

Results

Tyrosine hydroxylase immunohistochemistry revealed a variety of positively stained somata throughout the CNS of the monotremes. These ranged from the olfactory bulb through to the junction of the medulla oblongata and the spinal cord. For ease of description and in keeping with previously published works, the main CA cells groups have been named according to the A1–A17/C1–C3 nomenclature as proposed by Dahlstrom and Fuxe [1964]. Traditional anatomical names have also been given where possible. These main CA cell groups have also been segregated in an anatomical sense, dividing these groups into olfactory bulb, diencephalon, midbrain, rostral rhombencephalon and caudal rhombencephalon [Smeets and González, 2000]. This parcellation is in accord with previously

published studies in vertebrates and will make cross study comparisons easier [Smeets and Reiner, 1994; Smeets and González, 2000]. The presence or absence of TH-positive neurons outside the main CA cell groups [Smeets and González, 2000] are given at the end of the description of the main CA cell groups. The distribution of the cell groups was basically identical in both species of monotremes, thus the following description is applicable to both species. Any specific differences are noted.

Olfactory Bulb (A16)

A16, Olfactory Bulb. Typically mammalian olfactory bulbs are present in both species of monotremes, however, in the echidna this organ has become greatly enlarged. In the echidna the expansion of the olfactory bulb has progressed to such a point as to require gyrification of the layers of this structure [Griffiths, 1978]. We did not examine the platypus olfactory bulb in the present study. In the echidna TH immunohistochemistry revealed a substantial population of cells located deep to the glomerular layer. These TH-positive cells had very small, triangular somata and their dendrites formed a dense network around the glomeruli (fig. 3A). These cells formed an almost distinct layer in the olfactory bulb. Occasional TH-positive cells were found between the glomeruli and in the external plexiform layer, however, this was more the exception than the rule.

Diencephalon (A11–A15)

A series of TH-positive neurons were found in the diencephalon, from the level of the anterior commissure to the posterior pole of the dorsal thalamus (fig. 1: 8–11; 2: 1–2). Within placental mammals these have been classified into groups numbered A11–A15. In the monotremes we were able to identify all groups. The distribution of these cells within the diencephalon is slightly altered in comparison to that of placental mammals, due to the enormous anterior commissure in this region of the brain. However, despite this difference the general location is similar enough to allow us to describe these cell groups with the normal nomenclature.

A15. In the rat, this division of the diencephalic CA system has been further divided into two divisions, a dorsal and ventral component [Hökefelt et al., 1984]. In both the platypus and echidna these two components were evident, however, this group contained very few cells (fig. 1: 8; 2: 1). The dorsal component was located immediately ventral to the anterior commissure and had an antero-posterior extent of only 500 μm. Similarly, the ventral component had a restricted antero-posterior extent and was found in the medial corner of the hemisphere, ventral to the dorsal component. The cellular morphology was similar in both components, with small triangular shaped soma and usually three primary dendrites oriented away from the midline.

A14, Rostral Periventricular Cell Group. As with all other species described [Tillet, 1994], the cells of the A14 division were adjacent to the wall of the third ventricle. In this location they formed a dorso-ventral column of a few cells thickness, which stretched from the roof of the third ventricle almost to its floor (fig. 1: 9–11; 2: 1). The anterior limit of this column is coincident with the posterior margin of the anterior commissure, and its posterior limit is adjunct to the cells of the A10 group (see below). The cells of this division exhibit fusiform soma parallel to the ventricular wall and appear to be bipolar. Two primary dendrites are seen to ramify parallel with the wall of the ventricle.

Abbreviations

ac	anterior commissure
amb	nucleus ambiguus
bc	brachium conjunctivum
c/p	caudate/putamen
cer	cerebellum
ctx	cerebral cortex
d. th.	dorsal thalamus
dec. bc	decussation of the brachium conjunctivum
gp	globus pallidus
Hb	habenula
hip	hippocampus
i. olive	inferior olive
ic	inferior colliculus
IIIn	oculomotor nucleus
lat. sept.	lateral septal nucleus
m.teg.	medullary tegmental area
med. sept.	medial septal nucleus
n. acc.	nucleus accumbens
olf. tr.	olfactory tract
olf. tub.	olfactory tubercle
pc	posterior commissure
Sc	superior colliculus
V mes	fifth mesencephalic nucleus
V mot	trigeminal motor nucleus
V sens	trigeminal sensory nucleus
VIIn	facial nucleus
vh	ventral horn
Xn	dorsal motor vagus nucleus
XIIn	hypoglossal nucleus

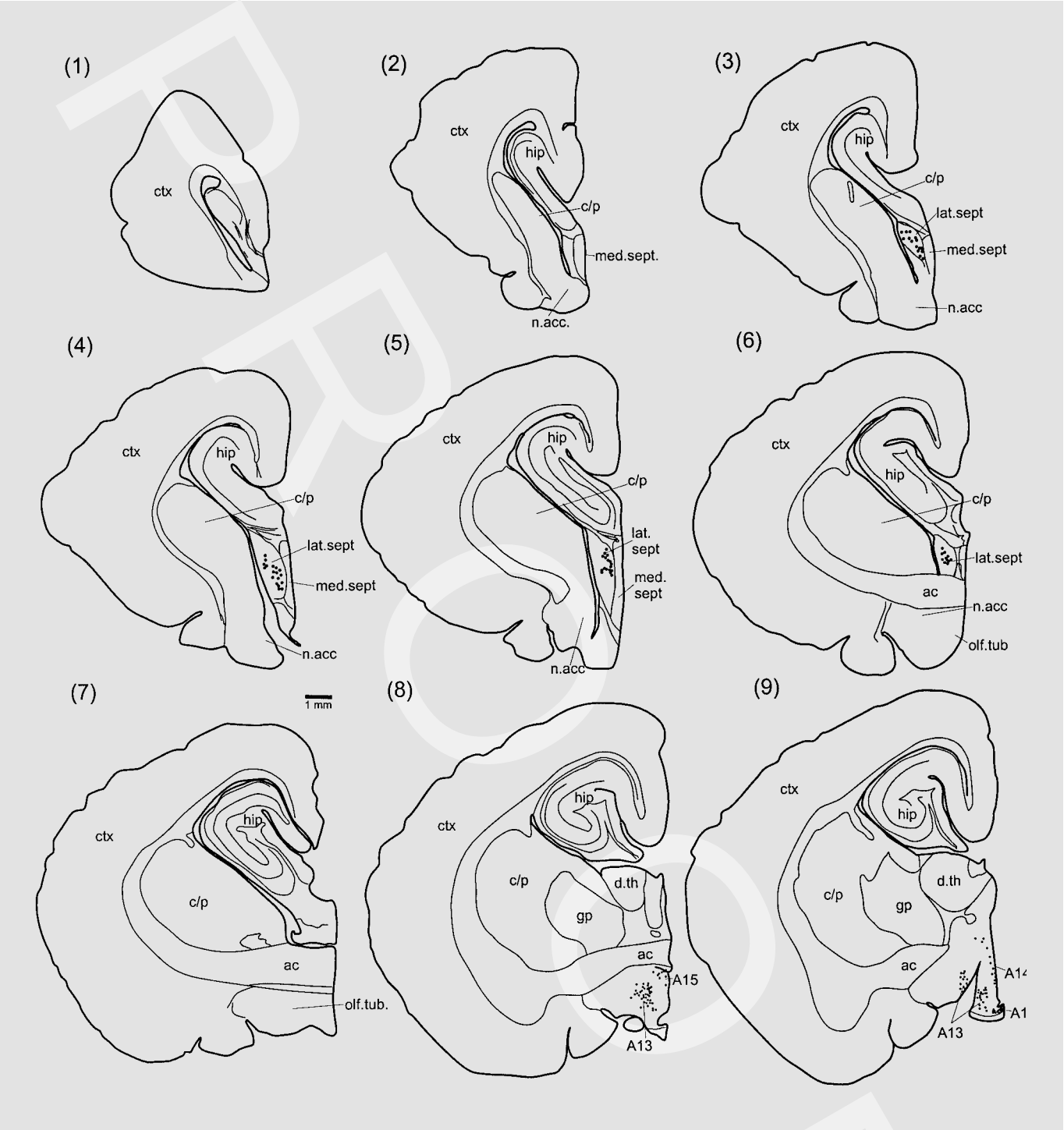
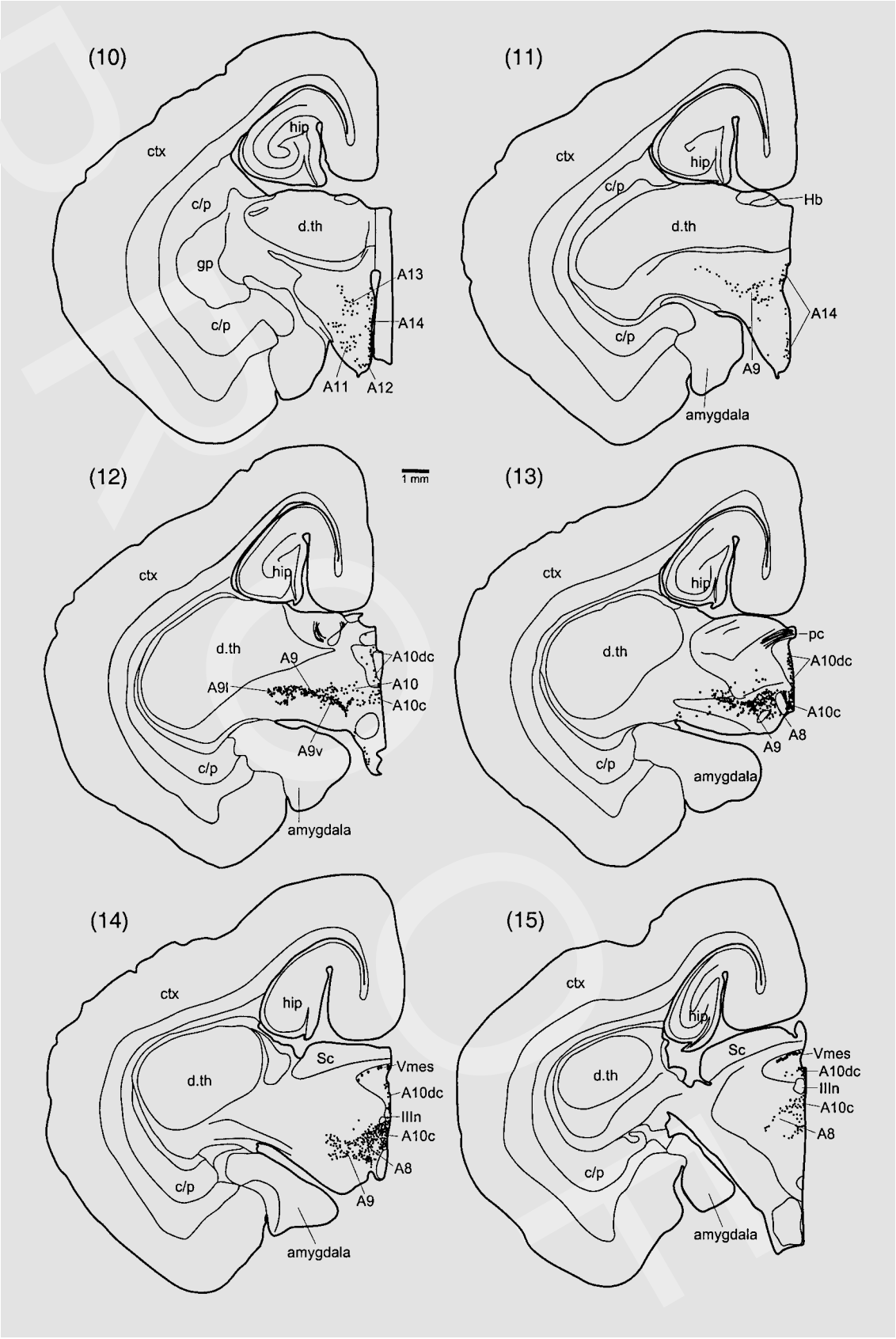


Fig. 1. Serial drawings of coronal sections through the platypus brain showing the distribution of catecholaminergic cells (small black dots). Those dots in the lateral septal nucleus, diagrams 3–6, indicate the location of pericellular baskets. Each section is approximately 1000 μm apart. The number on each diagram corresponds to the number on the diagrams in the accompanying papers [Manger et al., 2002a,b]. See list for abbreviations.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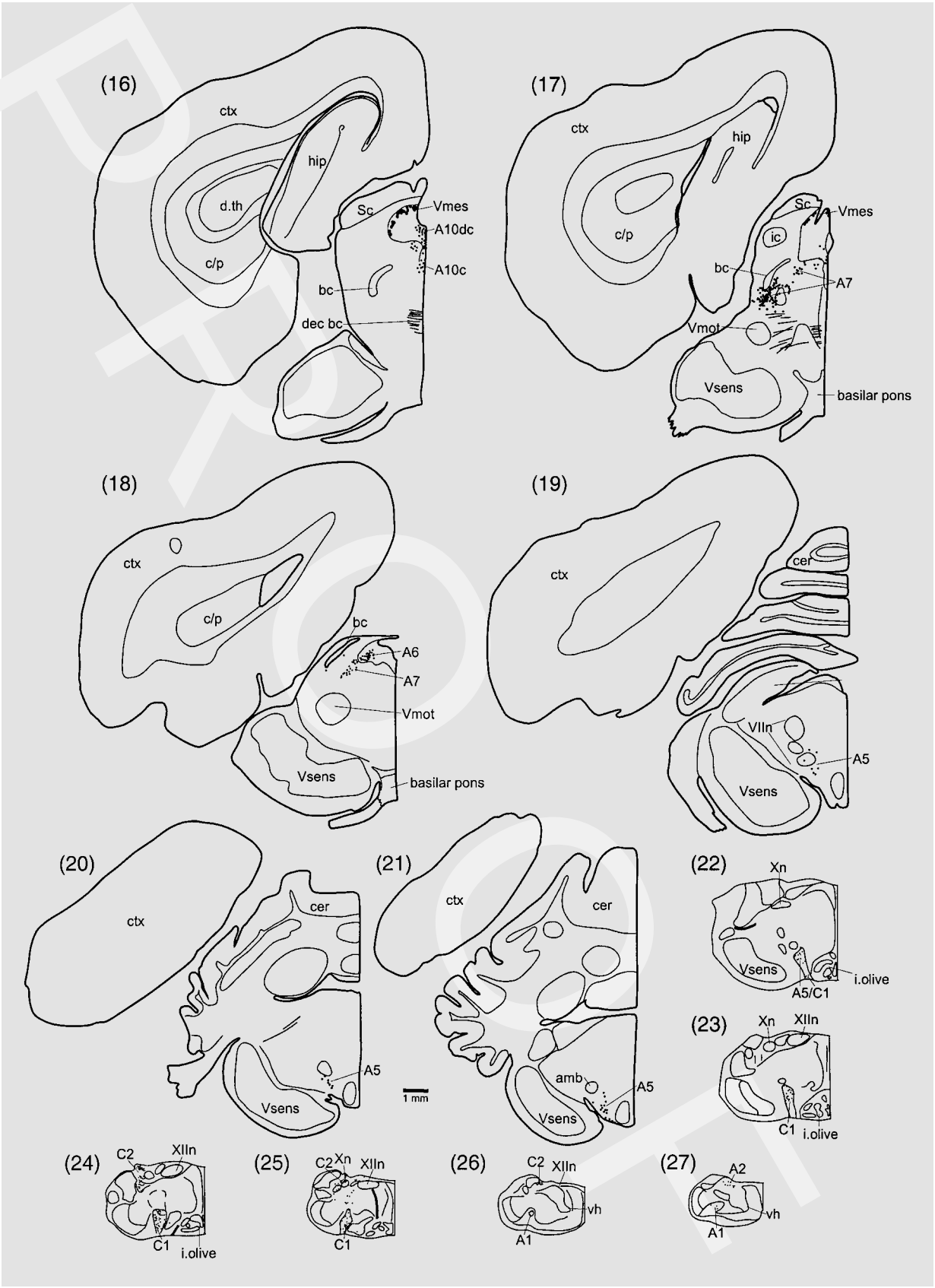
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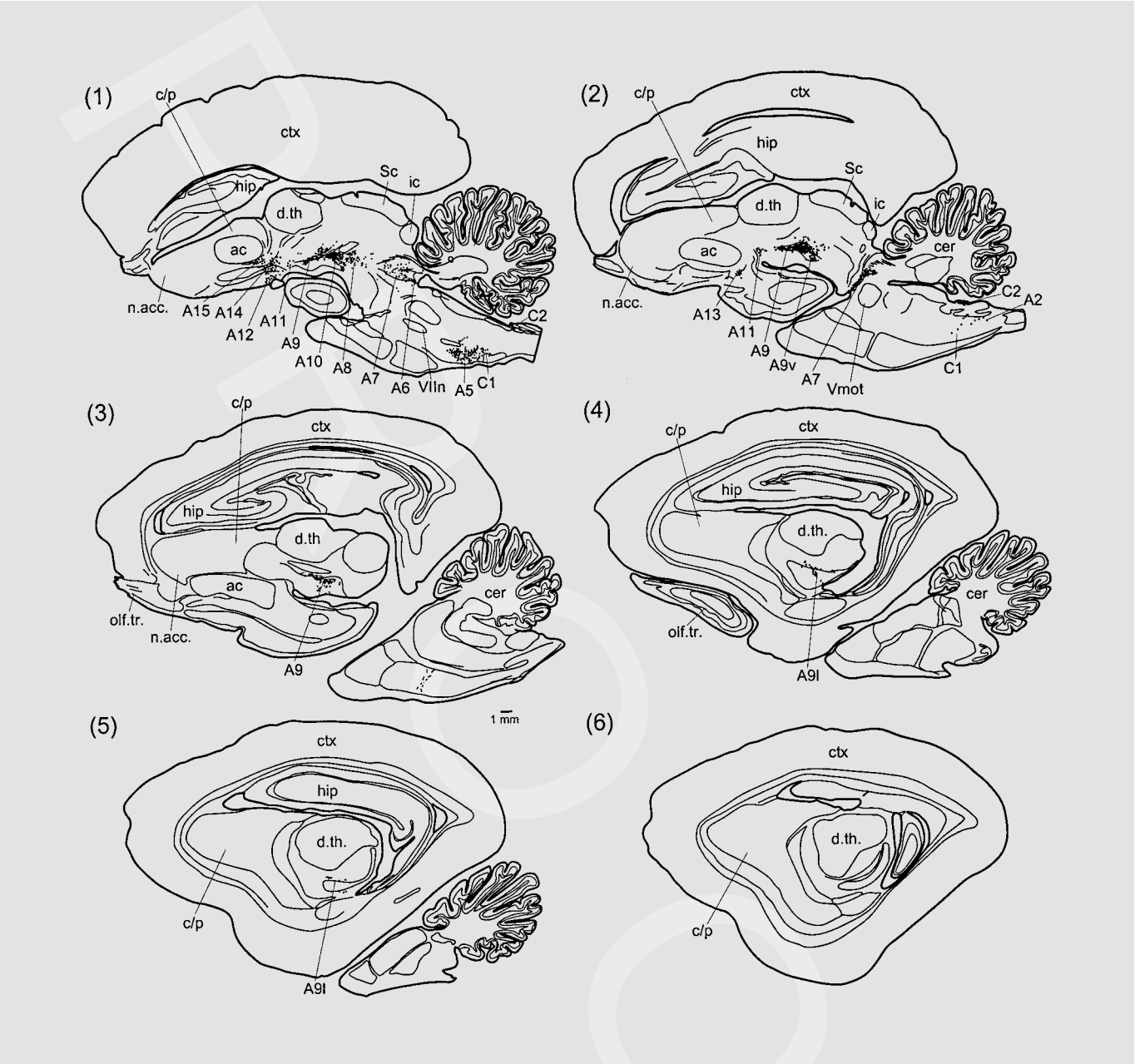

Fig. 2. Diagrammatic series of drawings in the sagittal plane from the brain of the platypus, demonstrating the location of catecholaminergic neurons. Each section is approximately 500 μm apart. The first drawing in the series is located closest to the midline. The number on each diagram corresponds to the number on the diagrams in the accompanying papers [Manger et al., 2002a, b]. See list for abbreviations.

A13, Zona incerta. This division constitutes the most expansive of the diencephalic CA system of the monotremes and exhibits the greatest number of cells. The cells of this division are found lateral to the A14 column, and ventro-lateral to the dorsal component of A15 (fig. 1: 8–10; 2: 2). The anterior limit of this group is defined by the olfactory tubercle, whereas the cells in the posterior part of this group merge with those of the A11 division (see below). The cellular density is moderate. The cells of this division exhibit small triangular soma with approximate-

ly three primary dendrites emanating from each corner of the triangle, similar in appearance to those of the cat [Tillet, 1994, his figure 9.6c].

A12, Tuberal Cell Group. In the monotremes a small group of TH-positive neurons, which constitute the A12 division of the diencephalic CA system are located immediately dorsal to the optic chiasm (fig. 1: 9–10; 2: 1) in a position similar to that seen in eutherian mammals [Tillet, 1994]. This division extends slightly caudal to the optic chiasm by a distance of around 1 mm. The cells of this division are found close to the wall of the third ventricle and the floor of the hypothalamus. The cellular density is high within this restricted distribution. The somata are small and circular, bipolar, with short dendrites.

A11, Caudal Diencephalic Group. This division initially appears as a caudal continuation of A13, in particular the ventral part of the A13 cell group (fig. 1: 10; 2: 1–2). It lies lateral to the A14 and A12 divisions and the cells are bordered laterally by the edge of the hypothalamus. The number of cells is moderate and shows a density similar to that of A13. The posterior limit of this group is close to the anterior limit of A9 (see below). The soma of these CA neurons were small and spherical and exhibit three primary dendrites.

Midbrain (A8–A10)

Additional CA groups are found in the midbrain of all mammals studied to date [Kitahama et al., 1994]. These have been classified as groups A8–A10, with some intragroup divisions. These occur from the level of the posterior pole of the dorsal thalamus through to the anterior limit of the brachium conjunctivum. In the monotremes we identified groups A8, A9, A9v, A9l, A10, A10c and A10dc (fig. 1: 11–16; 2: 1–5).

A10, Ventral Tegmental Area. The A10 clusters of TH-positive cells in both species of monotremes was readily subdivided into three components, A10, A10c (caudal) and A10dc (dorsal, caudal) (fig. 1: 12–16; 2: 1). The largest subdivision of these was the A10 group which was located between the red and interpeduncular nuclei. The soma of these cells was polygonal in shape and had 3–4 primary dendrites (fig. 3D). The dendrites of these cells formed a dense local plexus. Closer to the midline we were able to discern the A10c group. These cells were found mixed with those of the median raphe nucleus adjacent to the midline of the brain. They formed a rough dorso-ventral column, conforming to the shape of the median raphe. This group extended from the mid-dorsal most part of the interpeduncular nucleus to the ventral most part of the periaqeuductal grey at a level close to the oculomotor nucleus. The soma were spherical with 3–4 primary dendrites. Dorsal to this group and located entirely within the periaqueductal gray matter was the A10dc group. The cells of this group were found along the midline of the periaqueductal gray, dorsal to the oculomotor nucleus, adjacent to the floor of the aqueduct. At the aqueduct, the cell column would separate and some cells would fan out along the edge of the aqueduct, but often not for any significant distance. The soma of these cells were quite large (table 1), and only 2 primary dendrites emanated from each cell.

A9, Substantia nigra. As with all other mammals studied the A9 group of cells is co-extensive with the substantia nigra. This group forms a horizontally elongated band extending from the A10 subdivision lateral to the mid-level of the dorsal thalamus (fig. 1: 11–14; 2: 1–5). The A9 group of TH-positive cells in the midbrain of monotremes could be readily partitioned into three subdivisions: A9, A9v (ventral), and A9l (lateral). The main component is the A9 subdivision, which is found within the pars compacta of the substantia nigra. In this division there is a high density of cells, and an intricate local plexus of dendrites. The axons of these cells accumulate and appear to form a projection extending from the lateral most portion of this nucleus towards the striatum. The soma of these cells were elongated in a horizontal plane, with two horizontally oriented primary dendrites (fig. 3C). Within the substantia nigra pars reticulata small, scattered clusters of TH-positive neurons were located. These distinct clusters are grouped together as the A9v subdivision. The soma were triangular in configuration with three primary dendrites. A further group of TH-positive cells, the A9l subdivision, are found in the substantia nigra pars lateralis. These cells are loosely dispersed and appear to be an extension of the main A9 subdivision but they are separated from this main subdivision by the caudal most part of the medial lemniscus. As with the cells of A9, the soma of A9l are horizontally elongated but not to the same degree. Each cell had two horizontally oriented primary dendrites.

A8, Retrorubral. A group of scattered TH-positive cells were found slightly dorsal and medial to the A9 group, in a location posterior to the red nucleus, in the retrorubral region of the midbrain (fig. 1: 13–15; 2: 1). These cells form a weak horizontal band and merge ventrally with A9. They do not extend any further dorsal than the level of the oculomotor nucleus. This division was larger in the echidna than in the platypus, however, cellular density was lower in the echidna. The soma were triangular with three primary dendrites (fig. 3B).

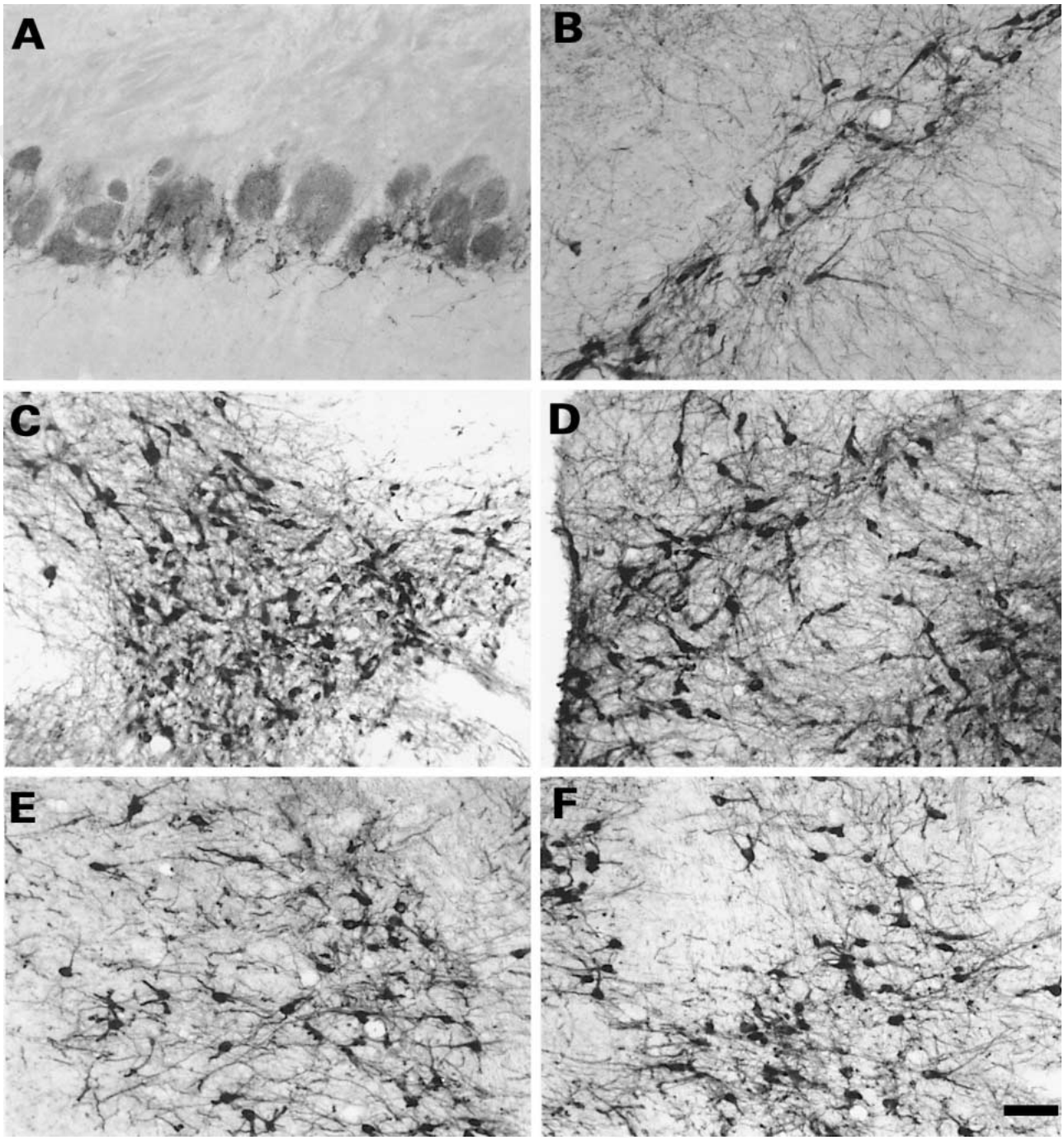

Fig. 3. Photomicrographs of catecholaminergic cells in the olfactory bulb of the echidna, and the midbrain, and rostral rhombencephalon of the platypus. A Neurons of the A16 group in the olfactory bulb of the echidna are high in density and appear to form a layer at the base of the glomeruli. B Neurons of the A8 group located at the caudal end of the midbrain catecholaminergic neurons. C Neurons of the A9 group located in the substantia nigra. D Neurons of the A10 group, adjacent to the wall of the third ventricle. E Neurons of the A6 group, the locus coeruleus, located within the periaqueductal gray matter of the brainstem. F Neurons of the A7 group, nucleus subcoeruleus, found adjacent to the brachium conjunctivum. Scale bar = 100 μm (for all photomicrographs).

Table 1. Data on the somatal areas of the catecholaminergic neurons in μm^2 (and standard deviation) of the various subdivisions of the platypus and echidna

TH+ nucleus	Somatal area in platypus (μm^2)	Somatal area in echidna (μm^2)
A1	259.15 (65)	540.40 (93)
A2	145.65 (42)	254.82 (51)
A5	260.03 (71)	468.83 (88)
A6	362.27 (51)	689.03 (139)
A7	292.93 (30)	529.30 (80)
A8	350.12 (49)	431.22 (110)
A9	358.84 (92)	389.51 (85)
A9v	381.74 (75)	717.31 (221)
A9l	256.74 (77)	446.83 (79)
A10	380.47 (36)	440.49 (106)
A10c	401.11 (117)	428.65 (116)
A10dc	710.11 (106)	1,241.97 (102)
A11	216.70 (56)	765.71 (114)
A12	120.83 (24)	288.78 (80)
A13	181.48 (36)	237.32 (36)
A14	194.56 (39)	240.04 (59)
A15	173.11 (42)	236.67 (48)
A16	–	223.64 (35)
C1	212.93 (38)	362.17 (60)
C2	120.04 (21)	398.30 (118)

Note the consistently larger size of the echidna soma compared to the platypus.

Rostral Rhombencephalon (A5–A7), the Locus coeruleus Complex

For most placental mammals, the CA neuronal clusters of the rostral rhombencephalon are divided into four groups, A4, A5, A6 (locus coeruleus), and A7 (subcoeruleus) [Kitahama et al., 1994]. In both species of monotremes we found the A5, A6 and A7 divisions (fig. 1: 17–22; 2: 1–2). No evidence for the A4 division could be found.

A5, Fifth Arcuate Nucleus. A series of TH-positive somata were identified in the region of the monotreme brain defined as the fifth arcuate nucleus by Hines [1929]. This region is co-extensive with the region designated as A5 in studies of the CA neurons of other mammals [Kitahama et al., 1994]. In both species of monotremes, the cluster of TH-positive cells that make up the group A5, are located in the ventromedial portion of the medulla (fig. 1: 19–22; 2: 1). They are found from just lateral to the anterior border of the inferior olive, to a level adjacent to the posterior limit of the trigeminal motor nucleus. The cells are not numerous and form a loose latticework of interconnected dendritic branches through which large bundles of fibers pass. The cells are found from the floor of the medulla extending dorsal to the lowest limit of the facial nucleus. The soma are small and triangular in shape with three primary dendrites.

A6, Locus coeruleus. In both species of monotremes the locus coeruleus is located in the posterior region of the ventro-lateral quadrant of the periaqueductal gray matter (fig. 1: 18; 2: 1). It has an antero-posterior dimension of approximately 2 mm, and at its anterior pole, locus coeruleus is bordered by the lateral wing of the dorsal raphe nucleus. Its posterior pole is located adjacent to the lateral dorsal tegmental nucleus. Locus coeruleus is bordered laterally, ventrally and posteriorly by the margins of the periaqueductal gray matter. At the more caudal level of this nucleus it is bordered superiorly by the floor of the fourth ventricle. The somal size of locus coeruleus neurons in the platypus was found to be 362.27 μm^2 (SD 51), and is smaller than those of the echidna which were found to be 689.03 μm^2 (SD 139). The shape of the soma in both species was round, but suggestive of a triangular shape due to the emergence of the 2–4 primary dendrites (fig. 3E).

A7, Subcoeruleus. The subcoeruleus in both species of monotremes was found to be associated with the brachium conjunctivum. The subcoeruleus is seen as a ventral and lateral projection of the cells that form the locus coeruleus, and it forms an arc within the tegmentum on the medial side of the brachium conjunctivum (fig. 1: 17–18; 2: 1–2). In the platypus, this arc is restricted to within 1 mm of the medial side of the brachium conjunctivum, but in the echidna the cells of the subcoeruleus are more scattered and spread further medially than those of the platypus. In both species the anterior border of the subcoeruleus is coincident with the decussation of the brachium conjunctivum, and the posterior border is found at the level of the anterior pole of the trigeminal motor nucleus. Very few cells of the subcoeruleus were found lateral to the brachium conjunctivum. The inferior portion of the subcoeruleus is bordered by the medial lemniscus. The subcoeruleus has an antero-posterior dimension of approximately 3 mm. The cells of the subcoeruleus had somal areas of 292.93 μm^2 (SD 30) in the platypus, and were smaller than those of the echidna, which were found to be 529.3 μm^2 (SD 80). The shape of the soma are roughly spherical, but this shape is distorted by the emergence of the 2–4 primary dendrites (fig. 3F).

Caudal Rhombencephalon (A1, A2, C1, C2)

In many mammals the CA neuronal clusters within the medulla oblongata have been divided into six groups, A1,

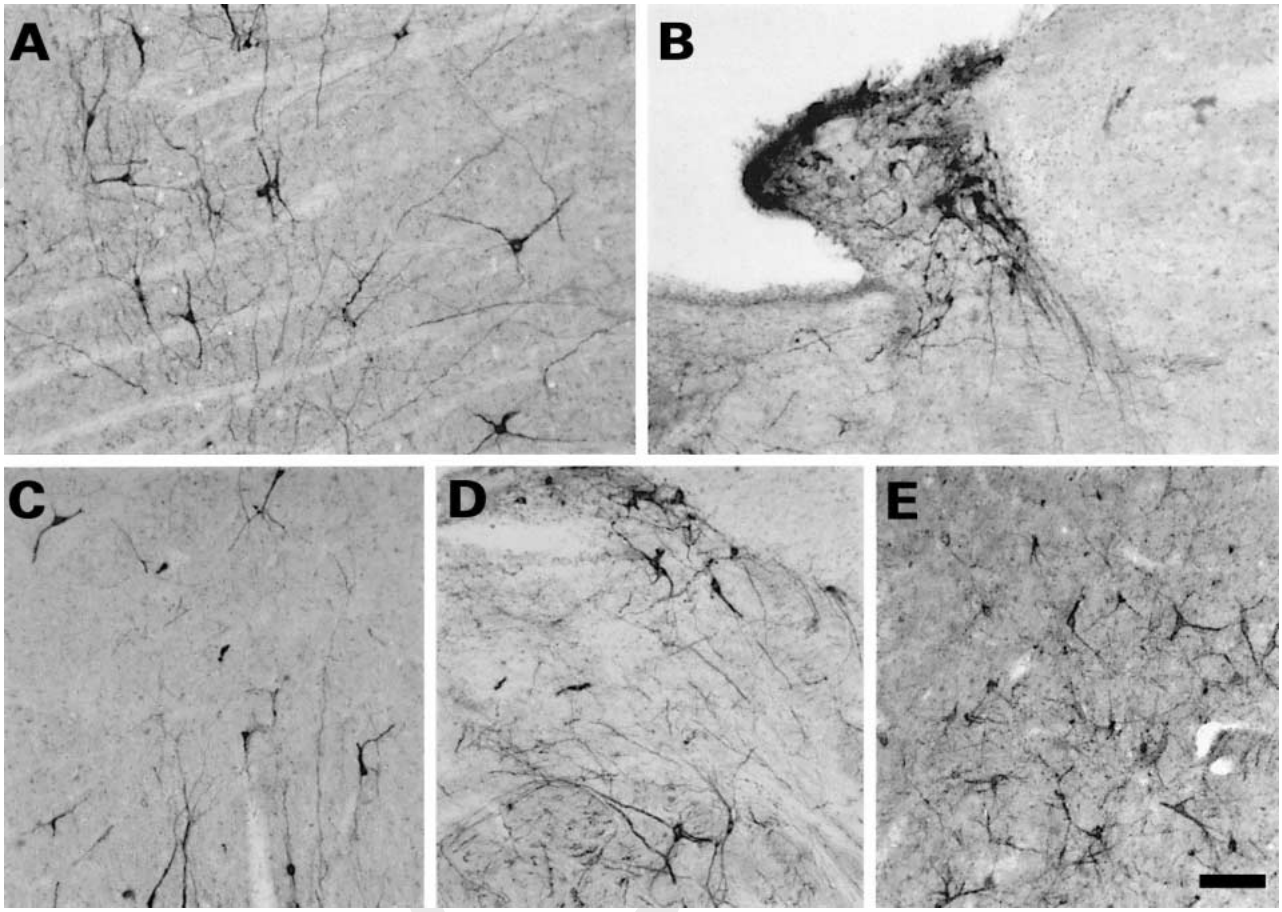

Fig. 4. Photomicrographs of catecholaminergic cells in the medulla oblongata of the platypus. A Neurons of the C1 group. B Neurons of the A2 group in area postrema. C Neurons of the A1 group immediately rostral to the spinal cord. D Neurons of the A2 group located immediately posterior to the dorsal motor vagus nucleus. E TH-positive terminals in the lateral septum of the platypus. The terminals are so densely packed around certain cells in this region that the morphology of the cells is clearly visible. Scale bar = 100 μm (for all photomicrographs).

A2, A3, C1, C2, and C3 [Kitahama et al., 1994]. Four of these subdivisions, A1, A2, C1, and C2, were found in both species of monotremes (fig. 1: 22–27; 2: 1–2). The description of these groups is based purely in TH immunohistochemstry, thus the divisions given here based on this stain might not be perfect. PNMT immunohistochemistry might be required to provide a more accurate description of these divisions, thus a word of caution must be given regarding the present subdivisions.

C1, Rostral Ventrolateral Tegmental Group. The TH-positive cells of the C1 group appear as a caudal continuation of the A5 group. In other mammals, similarities in neurotransmitter staining have been seen for the cells of C1 and A5 [Kitahama et al., 1994]. In both species of monotremes, these cells have an antero-posterior span along the ventral floor of the medulla that is coincident with the inferior olive (fig. 1: 22–25; 2: 1–2). The cells of C1 are seen as a loosely packed column, from the floor of the medulla extending dorsal to the level of the nucleus ambiguus. Posterior to the nucleus ambiguus, this cell column extends further dorsal and appears to mingle with the most ventral cells of the C2 group (see below). The somatal shape and dendritic branching is similar to that seen for the A5 group (fig. 4A).

A1, Caudal Ventrolateral Tegmental Group. The TH-positive cells of the A1 group can be recognized as a caudal continuation of the cell column of the C1 group. It is apparent that there is a continuous rostro-caudal column of TH-positive cells within the ventral lateral tegementum that is formed by the A5, C1 and A1 groups. However, the

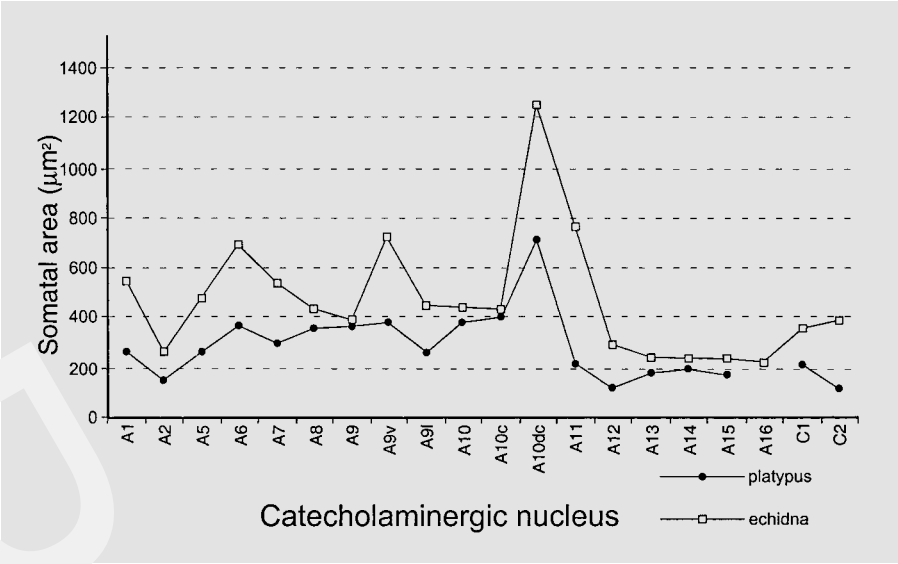

Fig. 5. Graphical representation of the data on somatal areas of monotreme catecholaminergic neurons given in table 1. Note the consistently larger size of the soma of neurons in the echidna as compared to the platypus. This size difference, coupled with differences in brain size is suggestive of an allometric relationship.

exact borders between these groups could not be fully ascertained in the present study as specific markers for adrenergic and noradrenergic cells were not used, thus the divisions given here are tentative. This column was seen in both monotreme species and is described in most mammals studied to date [Kitahama et al., 1994]. The column of A1 cells has as its anterior limit the cells of C1, at the posterior-most level of the inferior olive (fig. 1: 26–27). This cellular column extends caudal to the level of the cervical spinal cord. The ventral restriction of this column is the floor of the medulla and the dorsal limit is found at the midlevel of the medulla. Cellular shape and dendritic branching is similar to both A5 and C1 (fig. 4C), and as in both these groups, a loose latticework of dendrites is seen to surround large bundles (up to 100 μm diameter) of fibers.

C2, Rostral Dorsomedial Group. A small number of TH-positive cells, found just ventral and medial to the dorsal motor vagus nucleus (DMV), were seen in both species of monotremes (fig. 1: 24–25; 2: 1–2). These cells have been designated as those that make up the C2 group. The antero-posterior length of this group was found to be less than 2 mm. These cells were found adjacent to the most dorsal portion of the cell column of the C1 group caudal to nucleus ambiguus. Somatal morphology and dendritic branching was similar to that of the C1 group.

A2, Caudal Dorsomedial Group. The TH-positive cells that form the A2 group have been subdivided on the basis of neurochemistry and anatomical location in several species of mammals [Kitahama et al., 1994]. The A2 group in monotremes is found from the midlevel of DMV through to the cervical spinal cord (fig. 1: 27; 2: 2). In both species of monotremes, three subdivisions of the A2 group could be readily made. These subdivisions include those cells dorsal and lateral to the DMV (in area postrema), those within the DMV, and those located posterior to the DMV. Within area postrema, the cells are found densely packed in a column approximately 200 μm from the edge of the neural tissue (fig. 4B). A dense matrix of dendritic branching of the TH-positive somata is evident within area postrema. The column continues ventrally to the level of the DMV. Scattered cells of the A2 group are found in the posterior half of the DMV. A loose network of dendritic branching was found. Posterior to the DMV, a group of TH cells were seen to form a small, loose column extending caudal to the obex (fig. 4D). A weak network of dendritic branching originated from these cells.

Somatal Areas of Catecholaminergic Neurons in the Main Cell Groups

In both species of monotremes the somatal areas of the neurons were calculated. The results are presented in table 1. The smallest cells were found in the C2 and A12 nuclei in the platypus where the somatal areas were 120 μm^2. The largest were located in the A10dc division of the echidna (1,241 μm^2). When presented graphically (fig. 5), an interesting feature emerges. It is apparent that all cell groups in the echidna have larger somatal areas than the homologous cell groups in the platypus. There appears to be a reasonably consistent difference in the somatal areas between these two species, suggestive of an

allometric relationship. Howerver, certain cell groups were approximately equal in somatal area, whereas others were as much as two or three times larger in the echidna.

CA Neurons in Non-Classified Groups

Eutherian mammals have CA cells within the spinal cord (variously reported), habenular region, cortex and basal forebrain, and not in the pretectum and periventricular hypothalamic organ. Reptiles have CA cells in the spinal cord, pretectum and periventricular hypothalamic organ, but not in the habenular region, cortex (variously reported), or basal forebrain [see review of Smeets and González, 2000]. Thus, we closely examined these regions of the monotreme brain for TH immunoreactive cells.

Only the most rostral portion of the cervical spinal cord was included in the present study, and a small number of TH-immunoreactive cells that appear as a caudal continuation of the A2 group of cells were noted. No TH-immunoreactive cells were evident in the pretectum of either monotreme. A dense TH-immunoreactive fasciculus was evident immediately ventral to the habenular nuclei (fig. 6A), however, no immunoreactive cells were seen in this region. The periventricular hypothalamic organ was clearly delineated with serotonin immunoreactivity [Manger et al., 2002b], however, no TH-immunoreactive cells were found in this region of the monotreme brain. No TH-immunoreactive cells were found in the cerebral cortex, or the basal forebrain, despite axonal staining in these regions. Occasional TH-immunoreactive cells were found in the striatum of both species of monotremes (fig. 6).

Pericellular Baskets in the Lateral Septal Nucleus

Within this nucleus numerous TH-negative somata are surrounded by dense networks of TH-positive terminals forming pericellular baskets (fig. 1: 3–6). The immunoreactivity was so intense that it allowed visualization of the shape of the somata and the proximal dendritic branches (fig. 4E) [see Gall and Moore, 1984, for a full description of the nature of the lateral septal pericellular baskets in rats]. Similar pericellular baskets were seen in this nucleus of the monotremes with serotonin immunohistochemistry [Manger et al., 2002b].

Discussion

The present study details the distribution and morphological characteristics of CA neurons in the brain of two species of monotreme, the platypus *(Ornithorhynchus anatinus)* and the echidna *(Tachyglossus aculeatus)*, as determined by tyrosine hydroxylase immunohistochemistry. These main CA groups were readily categorized into the standard A1–A17/C1–C2 divisions used to describe this system. We searched for other, non-classified CA cell groups but these were not readily identified.

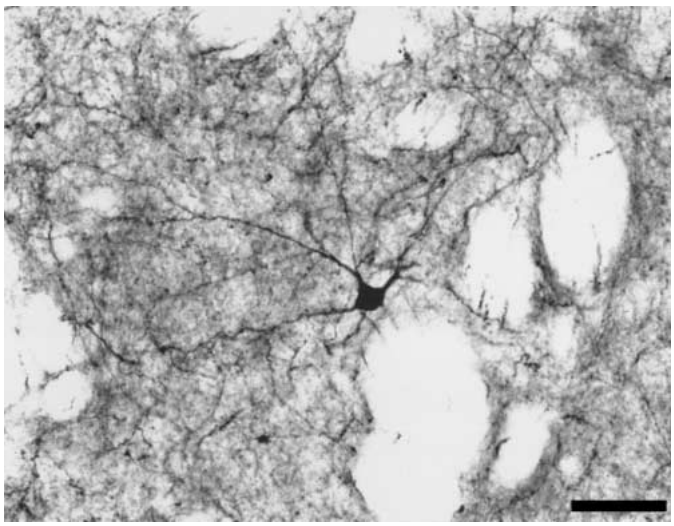

Fig. 6. Photomicrograph of a TH-immunoreactive cell in the striatum of the platypus. Scale bar = 100 μm.

Comparison of the Main CA Cell Groups to Other Mammalian Species

A comparison of the subdivisions of the CA systems, according to the standard terminology [Dahlstrom and Fuxe, 1964] between that of the monotremes and other mammals previously studied is given in table 2. The data for the monotremes is derived from the present study and that of other mammals from reviews in Smeets and Reiner [1994] and Smeets and González [2000]. In the diencephalon of most mammals, the divisions A15, A14, A13, A12, and A11 have been recognized [Tillet, 1994]. All of these divisions were found in both species of monotreme. Some of the extra components of these divisions that have been identified in rats [Hökfelt et al., 1984] were not identified in monotremes, but these are not often identified even in other rodent species [Tillet, 1994].

Within the midbrain of most mammalian species, the CA cell groups classified as A10, A9 and A8 have been described [Kitahama et al., 1994]. In both species of monotremes these groups were readily identifiable, on both anatomical and morphological bases. More specifically, the A10 and A9 midbrain groups have been parti-

Table 2. Comparative summary of the catecholaminergic subdivisions in various amniotes

Division	Birds	Reptiles	Monotremes	Eutherian mammals
A1	+	+	+	+
A2	+	+	+	+
A3	–	–	–	±
A4	±	–	–	±
A5	+	±	+	+
A6	+	+	+	+
A7	?	±	+	+
A8	+	+	+	+
A9	+	+	+	+
A10	+	+	+	+
A11	+	+	+	+
A12	+	+	+	+
A13	+	?	+	?
A14	+	+	+	+
A15	+	+	+	+
A16	+	+	+	+
A17	+	+	?	+
C1	+	+	+	+
C2	+	±	+	±
C3	–	–	–	±
Spinal cord	+	+	+	±
Pretectum	+	+	–	–
Habenula	–	–	–	+
OPH	+	+	–	–
Cortex	–	±	–	+
Striatum	?	?	+	+
Basal forebrain	–	–	–	+

Data derived from the present study and the reviews in Smeets and Reiner [1994], and Smeets and González [2000].

tioned into A10, A10c, A10dc, A9, A9v, and A9l subdivisions [Kitahama et al., 1994] all of which were identified in the monotremes.

The rostral rhombencephalon contains the locus coeruleus complex. This complex has been divided into four main groups, A4, A5 (fifth arcuate nucleus), A6 (locus coeruleus) and A7 (subcoeruleus) [Kitahama et al., 1994]. Monotremes were only found to differ from other mammals by the lack of the A4 group. This group is not present in all mammals studied and was not identified in sheep [Tillet and Thibault, 1989]. Thus, the lack of an A4 group in the monotremes does not appear to be of major significance.

The caudal rhombencephalon of mammals contains numerous divisions of CA cells which have been classified as the A1, A2, A3, C1, C2, and C3 groups [Kitahama et al., 1994]. We were able to identify the A1, A2, C1 and C2 groups in both species of monotremes, however, the A3 and C3 groups were not apparent. The C3 group has only been identified previously in the rat and hamster [Howe et al., 1980], and has not been identified in other mammals [Kitahama et al., 1994]. An A3 group has only been described in rats [Dahlstrom and Fuxe, 1964], but has not been identified with immunohistochemical techniques, thus it is questionable whether this group exists [Smeets and Reiner, 1994]. Thus, the monotremes differ very little from other mammals in regards to subdivisions of CA neurons of the caudal rhombencephalon.

Comparison of the Main CA Cell Groups to Other Amniotes

Due to the early divergence of the monotremes from other mammalian species [Musser and Archer, 1998] it is of interest to compare the anatomy of the CA system of monotremes with those of reptiles and birds. In a series of reviews, published under the same cover [Smeets and Reiner, 1994], the anatomy of the CA system has been described for several vertebrate species.

Within the diencephalon of birds, CA divisions of A11 through A15 have been described, but in reptiles the A13 division was not found [Smeets and Reiner, 1994; Smeets and González, 2000; see table 2]. The monotremes, as with birds, contain all the diencephalic subdivisions. It is interesting that all mammals and birds studied exhibit the A13 division despite the lack of this group in modern reptiles. Given the evolutionary relationships of these three groups it is possible that this division might have arisen twice in vertebrate evolution, once in mammals and once in birds, or that it was present in early reptiles and lost in subsequent evolution.

Reptiles [Smeets, 1994] and birds [Reiner et al., 1994] contain the A8–10 divisions found in the midbrain of monotremes. However, unlike monotremes and other mammals, the subdivision of these cells is not readily made. Thus, both reptiles and birds show less nuclear differentiation of the midbrain CA system than that of mammals. Within the rostral rhombencephalon of the monotremes, we described three subdivisions of the CA system, A5, A6 (locus coeruleus), and A7 (subcoeruleus). All reptiles and birds exhibit an unambiguous locus coeruleus (A6). In some species of birds, as in some species of mammals but not monotremes or sheep, there appears to be an A4 division [Smeets and Reiner, 1994]. The A5 division has been found in all birds studied, but is not always identified in reptiles [Smeets, 1994]. The existence of the A7 group in birds is questioned [Reiner et al., 1994], and is

occasionally reported for reptiles [Smeets, 1994]. Within the caudal rhombencephalon, the monotremes were found to have A1, A2, C1 and C2 divisions. All these divisions are found in birds and reptiles. Monotremes were lacking the A3 and C3 divisions, as are birds, reptiles, and some species of mammals.

Comparison of Non-Classified CA Cell Groups Across Amniote Species

Six groups of CA neurons that do not fall into the classical subdivisions of this system have been variously reported across vertebrate species. These include CA cells within the spinal cord, pretectum, habenular region, hypothalamic periventricular organ, cortex and basal forebrain. These are of interest in the phylogenetic sense, as eutherian mammals appear to have groups that are not found in reptiles, and vice versa [see table 7 of Smeets and González, 2000]. Due to the particular evolutionary position of the monotremes, it might be hypothesized that study of the CA system in these species could lead to insights into these differences. Do the monotremes have the same groups as reptiles, or as eutherian mammals? Or do they possess all, or none, of these groups? The answer seems to be that monotremes possess the spinal cord group, in common with all other vertebrate species, however, they lack the remainder of these non-classified cell groups.

CA cells have been reported in the habenular region, cerebral cortex, striatum, and basal forebrain of eutherian mammals [Smeets and González, 2000], however, excepting the striatum, no TH-immunoreactive cells were located in these regions of the monotreme brain. These regions have not been specifically examined for CA neurons in any species of marsupial [Smeets and González, 2000], thus, it is unclear whether the lack of CA cells is specific to monotremes, or whether marsupials also lack these CA neuronal groups. We found occasional CA cells within the striatum of the monotremes. CA cells have been reported in the striatum of rats [Tashiro et al., 1989] and primates [Betarbet et al., 1997], however, these do not appear to have been reported for non-mammalian species [Smeets and González, 2000], thus, striatal CA cells might be a purely mammalian feature, or could have been overlooked in the studies of non-mammals.

Reptiles have been shown to exhibit CA neurons in the pretectum, hypothalamic periventricular organ, with occasional reports in the cortex [Smeets and González, 2000]. No CA cells were found in the pretectum of the monotremes. However, as the marsupial pretectum has not been examined for CA cells it is unclear whether a lack of CA cells in this region is a monotreme-specific feature. In an accompanying paper [Manger et al., 2002b], we describe the existence of serotonergic neurons in the hypothalamic periventricular organ. TH immunohistochemistry did not reveal labeled cells in this region, but TH immunohistochemistry rarely reveals the CA cells of this organ [Meek, 1999]. CA cells have not been investigated in this region of marsupials and have not been reported for eutherians [Smeets and González, 2000], so again, it is unclear whether this is a monotreme-specific feature.

The present study then indicates that the non-classified CA cell groups present in eutherian mammals might be particular to these species and could have evolved late in mammalian evolution. Second, it suggests that those non-classified CA cell groups in reptiles and other vertebrates that are not found in eutherian mammals were lost during the evolution of the earliest mammalian species. Of course we must consider that the results of the present study might be false negatives, as only TH immunoreactivity was used, and that more stringent antibodies might reveal some or all of these cell groups in monotremes. However, given the observation of TH-immunoreactive cells in the striatum, the dense TH-immunoreactive plexus ventral to the habenula, and the ready identification of the main CA cell groups, we can only conclude for the present that our results are correct. A detailed analysis of the marsupial CA system is required to demonstrate if the non-classified cell groups in eutherians are specific for these species, and if those in reptiles were truly lost in the reptile-mammal transition.

Somatal Areas of Catecholaminergic Neurons

One interesting difference found in the present study is the consistently larger size of the soma of echidna CA neurons as compared to the platypus (table 1, fig. 5). On average, the somatal area of platypus neurons were 62% (SD 19) that of the echidna. This feature is indicative of an allometric relationship of the somatal areas of CA neurons with brain size. The echidna brain weighs approximately 27.5 g, whereas that of the platypus is approximately 9.2 g [Pirlot and Nelson, 1978]. Although no directly proportional correlation is evident, this is not usually the case with allometric relationships, which generally follow a power function [e.g., Bauchot, 1978; Pirlot, 1987]. In the two companion papers to this, regarding cholinergic and serotonergic cells of monotremes [see Manger et al., 2002a, b], we found no such relationship. Woolf [1991] proposed a relationship between cholinergic cell size and brain width, but our comparison [Manger et

al., 2002a] does not support this hypothesis. Unfortunately, the data on somatal areas of CA neurons from different species is limited. Until more data is generated we will not be able to determine if indeed there is an allometric relationship between brain and soma size for the CA system, although our findings indicate this is a distinct possibility.

Functional Significance of the Catecholaminergic System in Monotremes

The observations of the present study suggest that, in general, the function of the CA divisions of the monotremes can be predicted from studies of more typical laboratory animals. Although the exact details of the function of monotreme CA neurons might differ from studies of laboratory animals [see for example the firing of locus coeruleus neurons during the sleep-wake cycle of the echidna; Siegel et al., 1996], the global inferences that can be made are of importance. For example, CA neurons of the diencephalon are involved in regulating reproduction. Each of the various subdivisions have specific projections with specific functional implications [Tillet, 1994]. As the monotremes have similar subdivisions of diencephalic CA neurons, the principles that underlie control of reproduction in other mammals must also apply to the monotremes.

The present study of the monotreme CA system represents an extreme data point for use in comparisons of this system across mammals and amniotes. The extensive similarities of this system with other mammals and also with birds and reptiles underlies the conclusions drawn by Smeets and Reiner [1994] and Smeets and González [2000], that: (1) the evolution of this system is very conservative; and (2) evolutionary trends of this system are difficult to discern.

Acknowledgments

This work was supported by the Medical Research Service of the Veterans Administration, USPHS grants NS32819 and NS14610, and the Australian Research Council Special Research Centres Budget.

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