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The Distribution and Morphological Characteristics of Cholinergic Cells in the Brain of Monotremes as Revealed by ChAT Immunohistochemistry

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Key Words
Mammals · Monotremes · Platypus · Echidna · Acetylcholine · Choline acetyltransferase · Sleep

Abstract
The present study employs choline acetyltransferase (ChAT) immunohistochemistry to identify the cholinergic neuronal population in the central nervous system of the monotremes. Two of the three extant species of monotreme were studied: the platypus *(Ornithorhynchus anatinus)* and the short-beaked echidna *(Tachyglossus aculeatus)*. The distribution of cholinergic cells in the brain of these two species was virtually identical. Distinct groups of cholinergic cells were observed in the striatum, basal forebrain, habenula, pontomesencephalon, cranial nerve motor nuclei, and spinal cord. In contrast to other tetrapods studied with this technique, we failed to find evidence for cholinergic cells in the hypothalamus, the parabigeminal nucleus (or nucleus isthmus), or the cerebral cortex. The lack of hypothalamic cholinergic neurons creates a hiatus in the continuous antero-posterior aggregation of cholinergic neurons seen in other tetrapods. This hiatus might be functionally related to the phenomenology of monotreme sleep and to the ontogeny of sleep in mammals, as juvenile placental mammals exhibit a similar combination of sleep elements to that found in adult monotremes.

Introduction

In several eutherian mammalian species the cholinergic system has been shown to globally innervate the entire brain, from the olfactory bulbs to the spinal cord, and to form an interconnected network amongst its own subdivisions via dendritic connections [for review of the cholinergic system see Woolf, 1991]. The cholinergic system can be partitioned into seven cell groups, based on location, morphology and connections: cerebral cortical neurons, striatal interneurons, basal forebrain neurons, diencephalic neurons, pontomesencephalic neurons, medullary neurons, and motor neurons of the cranial nerve nuclei and spinal cord. The distribution and morphological characteristic of each of these cholinergic cell groups has been

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described using choline acetyltransferase (ChAT) immunohistochemistry in rat, cat, monkey, and human [Palkovits and Jacobowitz, 1974; Kimura et al., 1981; Armstrong et al., 1983; Satoh et al., 1983; Mesulam et al., 1984, 1989; Satoh and Fibiger, 1985a, b; Sofroniew et al., 1985; Mizukawa et al., 1986; Parnavelas et al., 1986; Jones and Beaudet, 1987; Reiner and Vincent, 1987; Everitt et al., 1988; Shiromani et al., 1988; Tago et al., 1989; Reiner, 1991]. Studies of the distribution and morphological characteristics of the cholinergic cell groups in other mammals and other vertebrates are more limited [teleosts – Ekstrom, 1987; Brantley and Bass, 1988; eel – Molist et al., 1993; amphibia – Marin et al., 1997; caiman – Brauth et al., 1985; lizard *Gallotia* – Medina et al., 1993; lizard *Gekko* – Hoogland and Vermeulen-Vander-Zee, 1990; pigeon – Medina and Reiner, 1994].

The cholinergic nuclei have been shown to project to virtually every structure in the mammalian brain [Sofreniew et al., 1985; Woolf and Butcher, 1986, 1989]. It is the diverse set of projections of these nuclei that have lead to proposals that cholinergic cell groups function to modulate behavior and cognition [e.g., Bartus et al., 1982; Smith, 1988]. Cholinergic neurons have been shown to be essential for the normal sleep-wake cycle [Webster and Jones, 1988], and have been implicated in the determination of EEG pattern [Stewart et al., 1984; Braun et al., 1997]. This is of particular interest as in monotremes it has been shown that standard mammalian pattern of sleep phenomenology is altered; brainstem neurons appear to show discharge rates indicating REM sleep (coincident with behavioral signs such as rapid eye movements and skeletal muscle twitches), while the cortical EEG exhibits slow waves [Siegel et al., 1996, 1998, 1999]. The pontomesencephalic cholinergic cell groups have also been implicated in the planning and execution of complex motor control [Garcia-Rill et al., 1987; Kelland and Asdourian, 1989]. Furthermore, by co-ordination with other cholinergic cell groups in the brain these cells have been proposed to provide a basis for cognitive behaviors such as learning and memory [Bartus et al., 1982; Vanderwolf, 1987].

Literature on the neuroanatomy of monotremes is very limited. Two comprehensive papers, one on the platypus [Hines, 1929], and one on the echidna [Abbie, 1934], describe in detail the anatomy of the brain using classical neuronal and fiber stains. Both of these authors describe little difference in the anatomy of the brain of monotreme species. Each author lists differences and similarities to other mammals and also to reptiles and birds, both sustaining the notion that the monotreme brain exhibits a somewhat intermediate form between that of other mammals and reptiles.

The present study describes the distribution of cholinergic cells in the brain of the platypus *(Ornithorhynchus anatinus)* and short-beaked echidna *(Tachyglossus aculeatus)*. The evolutionary history of monotremes [Musser and Archer, 1998; Kirsch and Mayer, 1998] marks the present study as an extreme data point in the neuroanatomy of the mammalian cholinergic system. Behavioral and physiological differences of the monotremes [e.g., Griffiths, 1978; Grigg et al., 1992; Siegel et al., 1996, 1998, 1999] compared with eutherian mammals allows for an interesting comparison across species, especially when comparing proposed functional and evolutionary attributes of the cholinergic system [Woolf, 1991]. ChAT immunohistochemistry, which is specific for cholinergic cells, was used in the present investigation.

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 anaesthesia, the animals were perfused via the heart with 0.9% cold saline, followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer. The brains were post-fixed overnight in the second perfusate, and then equilibrated in 30% sucrose in 0.1 *M* phosphate buffer, frozen in dry ice and stored at –20°C until sectioning on a freezing microtome.

Serial 50 μm sections of the brains were cut in the coronal and sagittal planes. A one in five series of stains was made for Nissl, fibers [Gallyas, 1979], ChAT, tyrosine hydroxylase (TH) and serotonin. The results of the TH and serotonin immunohistochemistry are presented elsewhere [Manger et al., 2002a, b]. For ChAT staining, the sections were rinsed three times in 0.1 *M* tris-buffered saline (TBS) prior to a 40 min incubation in 3% normal rabbit serum (NRS) and 0.3% Triton X-100 (TX) diluted in 0.1 *M* TBS. This was followed by a 48-hour incubation at 4°C in 1:80 goat anti-ChAT antibody (Chemicon International Inc., Temecula, Calif.). The sections were then incubated for 1 h in 1:50 non-biotinylated rabbit anti-goat IgG (Sternberger Monoclonals Inc., Baltimore, Md.) followed by a 1-hour incubation in 1:40 goat peroxidase-anti-peroxidase (PAP) (Sternberger). The sections were rinsed after each incubation in 1% NRS in TBS, which was also used for diluting antibodies and PAP. The sections were then treated for 6 min with a 0.05% solution of 3,3′ diaminobenzidine and 0.01% hydrogen peroxide, rinsed, mounted on gel coated slides, dehydrated in a graded series of alcohol, 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 ChAT immunoreactive cells in each architectonic region and the somatal area was determined using the

Table 1. Somatal areas of cholinergic cells in μm² (and standard deviation) in various species of mammals

	Platypus	Echidna	Human[1]	Monkey[3]	Cat[2]	Cat[3]	Rat[3]
Islands of Calleja	165 (47)	153 (37)		158 (22)		100 (5)	52 (5)
Olfactory tubercle	160 (53)	148 (62)		247 (26)		227 (22)	111 (7)
Nucleus accumbens	276 (58)	254 (67)		219 (5)		199 (8)	116 (11)
Caudate nucleus	247 (59)	309 (77)		241 (10)		233 (13)	116 (11)
Medial septal n.	233 (55)	437 (50)		303 (24)		235 (10)	117 (7)
Horiz. band n.	not found	not found		302 (11)		255 (14)	117 (4)
Nucleus basalis	258 (53)	213.13 (87)		342 (13)		305 (14)	119 (6)
Lat. hypo. area	not found	not found		167 (17)		92 (4)	67 (7)
Medial habenula	122 (35)	97 (22)		128 (8)		103 (11)	48 (6)
Parabigem nucleus	not found	not found		141 (14)		108 (3)	54 (2)
PPN	272 (58)	222 (60)	521.2 (130.6)	383 (38)	160.72 (3.4)	354 (24)	56 (16)
LDT	583 (110)	347 (65)	609.8 (198.2)	380 (20)	284.86 (8.4)	324 (19)	148 (9)
III	545 (130)	537 (95)		543 (23)	311.13 (19.1)	451 (20)	181 (14)
IV	647 (125)	527 (145)			363.27 (27.8)		
V mot	920 (183)	907 (176)			387.72 (16.1)		
VI	487 (124)	484 (127)					
VII dors	906 (199)	1,172 (237)			407.24 (11.6)		
VII vent	753 (120)	1,019 (292)			407.24 (11.6)		
amb	870 (269)	956 (230)			650.81 (48.3)		
X	470 (85)	301 (69)			134 (3.1)		
XII	704 (148)	862 (175)			358 (9.8)		
v. horn	1,360 (213)	1,182 (380)		565 (54)		504 (36)	264 (23)

Data in this table are derived from the present study and those of Mesulam et al. [1989][1], Shiromani et al. [1988][2], and Woolf [1991][3].

program Image Tools. Only cells in which a clear nucleus could be seen were used for the calculation of somatal areas. 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

As described earlier, there are several subdivisions of the cholinergic cell system in the brain of mammals [Woolf, 1991]. The distribution and cellular morphology of these groups in the monotremes are presented here. As there is little difference in the distribution and morphology of these cells between the two species of monotreme investigated, this general description is applicable to both. The cholinergic neurons identified in the present study could be classified into six different groups: striatal, basal forebrain, diencephalic, pontomesencephalic, medullary tegmental field and those of the cranial nerve nuclei and spinal cord. We were unable to identify cholinergic cells in the cerebral cortex or hypothalamus of either species of monotreme. Somatal areas are given in table 1 and presented graphically in figure 6.

Distribution and Morphology of Striatal Cholinergic Interneurons

Within the striatum of mammals, three groups of cholinergic cells have been defined. These include those of the islands of Calleja and the olfactory tubercle, the nucleus accumbens, and the caudate-putamen complex [Woolf, 1991]. All of these cell groups were identified in both species of monotreme, each showing approximately the same distribution and morphological characteristics as that of other mammals.

Islands of Calleja and Olfactory Tubercle. Both of these structures are found in the region ventral to the anterior commissure (fig. 1: 6–9; 3: 1–3). The olfactory tubercle is bordered dorsally by either the nucleus accumbens (see below) or the anterior commissure. Medially, the olfactory tubercle is bordered by the medial wall of the telencephalic hemisphere. Ventrally, the olfactory tubercle is bordered by the islands of Calleja, anteriorly, by the anterior olfactory nucleus, and posteriorly, by the subcommissural region. The islands of Calleja are seen as densely packed cell aggregations forming medio-lateral bands along the ventral aspect of the olfactory tubercle. These bands form the floor of the hemisphere in this region.

The neuronal cell bodies within the olfactory tubercles were ovoid in shape and had three to five primary dendrites emerging from the ends of the ovoid. The dendrites formed a local plexus. The soma of the cholinergic neurons in the islands of Calleja were also ovoid in shape, however, with a more flattened appearance and were oriented parallel to the floor of the hemisphere. On average, two primary dendrites emerged from either end of the cell body and traversed a short distance parallel to the long axis of the cell body before forming a local plexus.

Nucleus accumbens. The nucleus accumbens of monotremes is large in comparison to other mammals. It appears as an anterior continuation of the caudate-putamen complex (see below), bordering the anterior portion of this complex medially and ventrally (fig. 1: 2–6; 3: 1–3). On its medial aspect the nucleus accumbens abuts the ependymal floor of the lateral ventricle and is bordered anteriorly by subcortical white matter. At more posterior levels of this nucleus, it is bordered medially and ventrally by the olfactory tubercle (see above). The most posterior portion of the nucleus accumbens is found at the anterior limit of the anterior commissure. Cholinergic neurons are distributed throughout the nucleus, and are seen as patchy aggregations, with a density similar to that found in the caudate-putamen complex. The somas of the cholinergic cells formed elongated polygonal shapes, with one or two primary dendrites emanating from each end (fig. 4B). The dendrites formed a local plexus.

Caudate-Putamen Complex. The caudate-putamen complex occupies a significant volume of the telencephalon in both species of monotreme. This complex essentially forms a sheet with an anterior swelling that wraps around the globus pallidus and dorsal thalamus, and underlies almost the entire subcortical white matter (fig. 1: 2–18; 3: 1–12). At the anterior portion of the telencephalon, the medial border of the caudate-putamen complex forms the inferior bed of the lateral ventricle. It is bordered laterally and dorsally by the subcortical white matter. It is found dorsal to the anterior commissure and at this level encapsulates the globus pallidus. Further posterior, the caudate-putamen complex surrounds the dorsal thalamus, but is separated from this structure by the internal capsule. At this level the complex thins and posterior to the thalamus is seen as a 0.5 mm sheet, part of which forms the floor of the lateral ventricle. It is bordered superiorly by the hippocampus along its entire antero-posterior length, and in the echidna has its posterior border formed by the hippocampus, whereas in the platypus this is formed by subcortical white matter. At the level of the dorsal thalamus, the caudate-putamen complex is bordered inferiorly and medially by the amygdala. In both species the nucleus appears as a heterogeneous mass, caused by the passage of the fibers between cerebral cortex and dorsal thalamus.

Throughout the caudate-putamen complex numerous cholinergic interneurons were found. These neurons were evenly, and moderately densely, distributed throughout the entire complex. The cholinergic interneurons had elongated polygonal shaped somas and had between three and five primary dendrites emanating from either end of the cell body (fig. 4A). These dendrites could not be followed for any great distance, and it appears that they form a local plexus.

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
h. th.	hypothalamus
Hb.	habenula
hip	hippocampus
i. olive	inferior olive
ic	inferior colliculus
is. call.	Islands of Calleja
IIIn	oculomotor nucleus
IVn	trochlear nucleus
lat. sept.	lateral septal nucleus
LDT	lateral dorsal tegmental nucleus
med. sept.	medial septal nucleus
n. acc.	nucleus accumbens
n. bas.	nucleus basalis
olf. tub.	olfactory tubercle
pc	posterior commissure
PPN	pedunculopontine nucleus
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

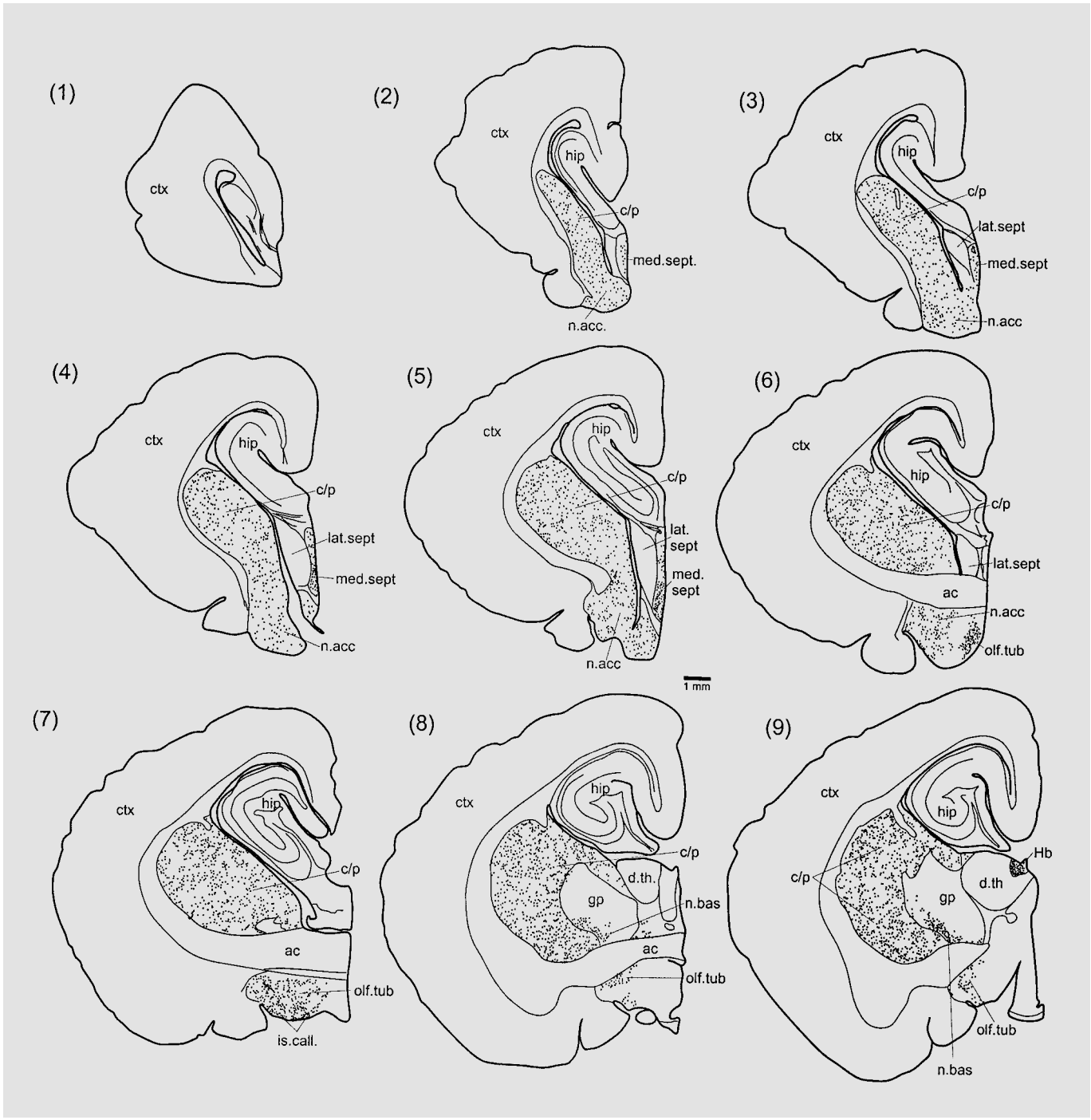
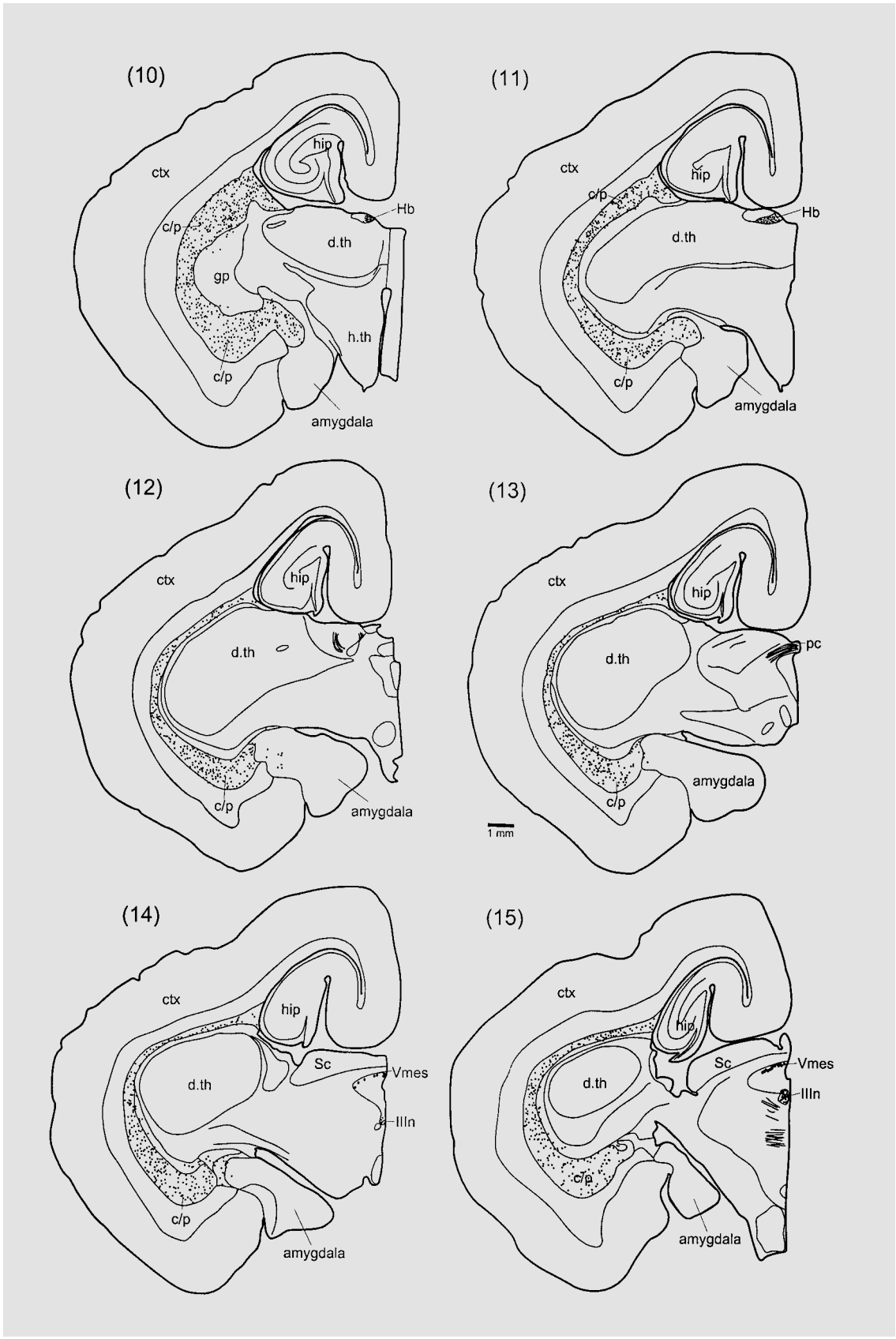


Fig. 1. Serial drawings of coronal sections through one half of the platypus brain showing the distribution of cholinergic cells (black dots). Each section is approximately 1,000 μm apart. Numbered diagrams correspond to those presented in the accompanying papers [Manger et al., 2002a, b]. See list for abbreviations.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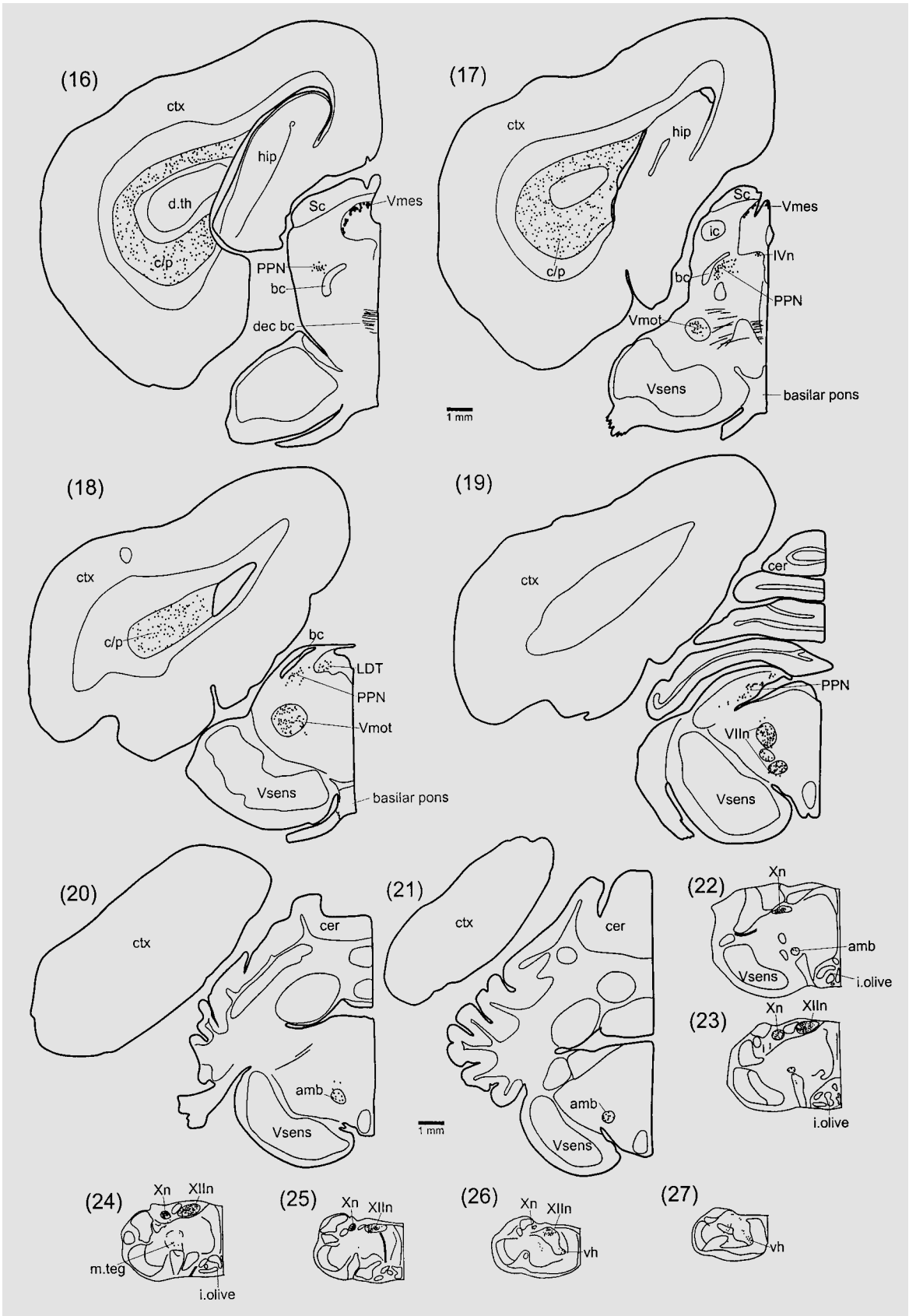
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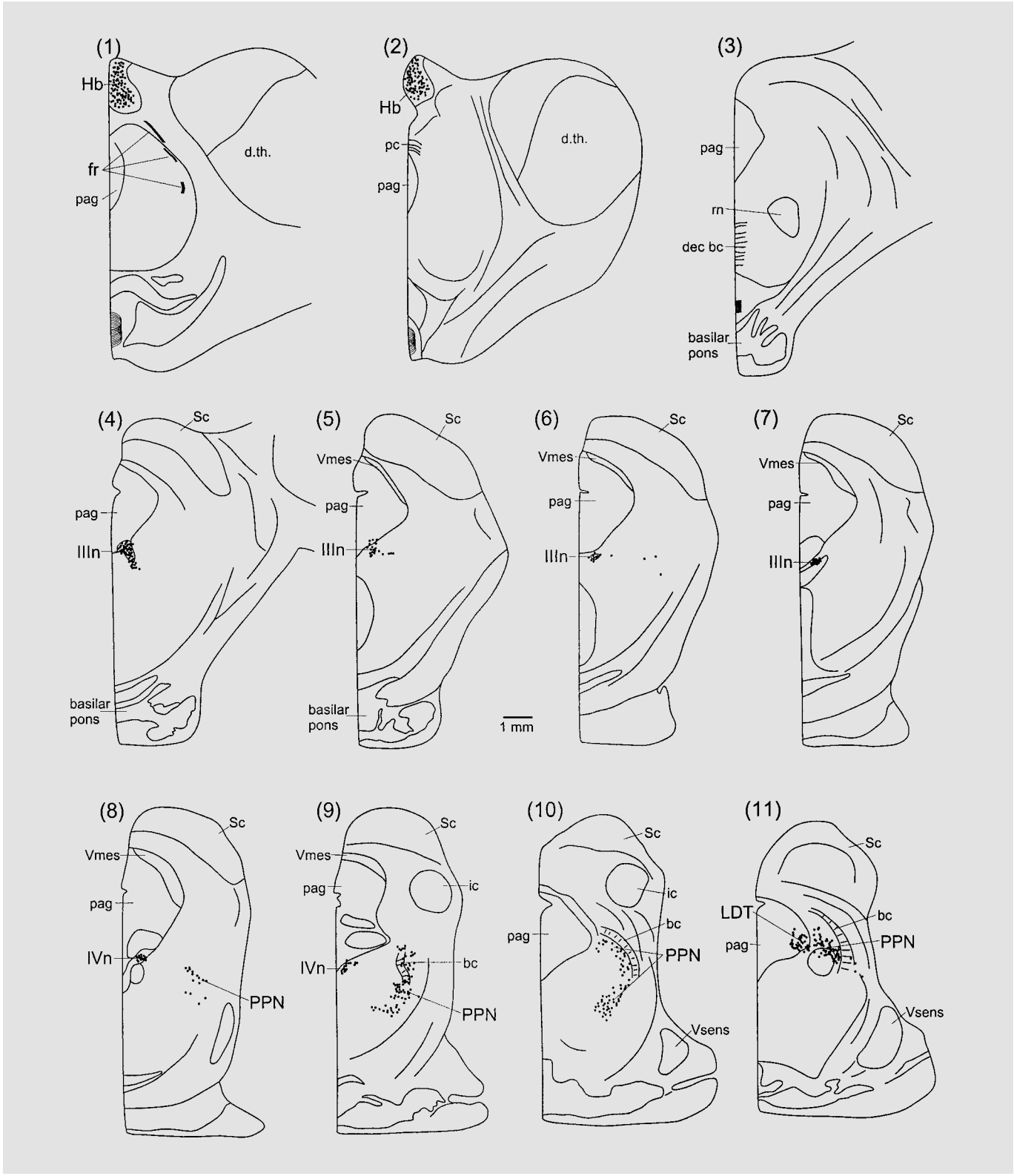
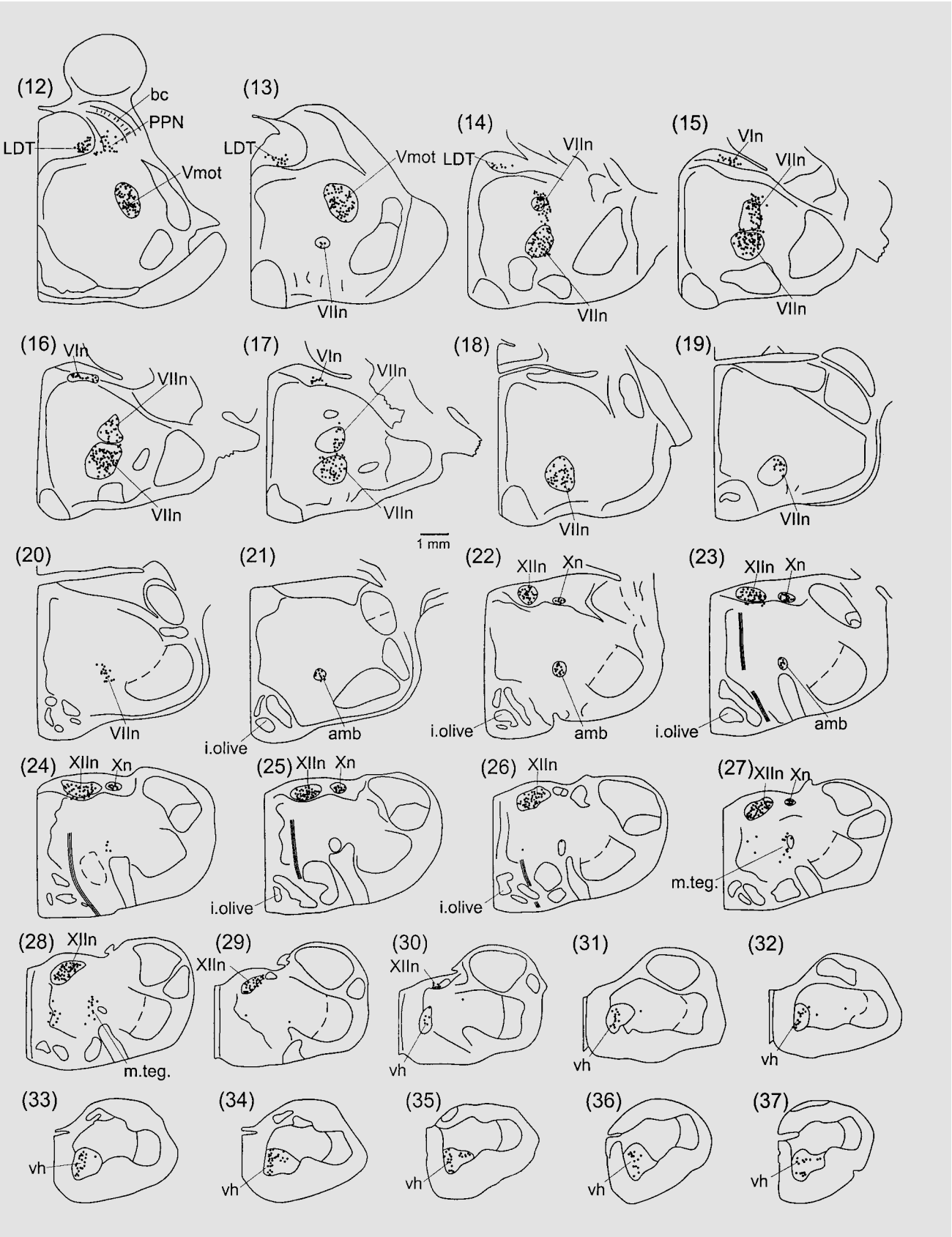


Fig. 2. Diagrammatic series of drawings through one half of the midbrain and brainstem of the echidna, showing the distribution of cholinergic neurons (black dots). Each section is approximately 500 μm apart. See list for abbreviations.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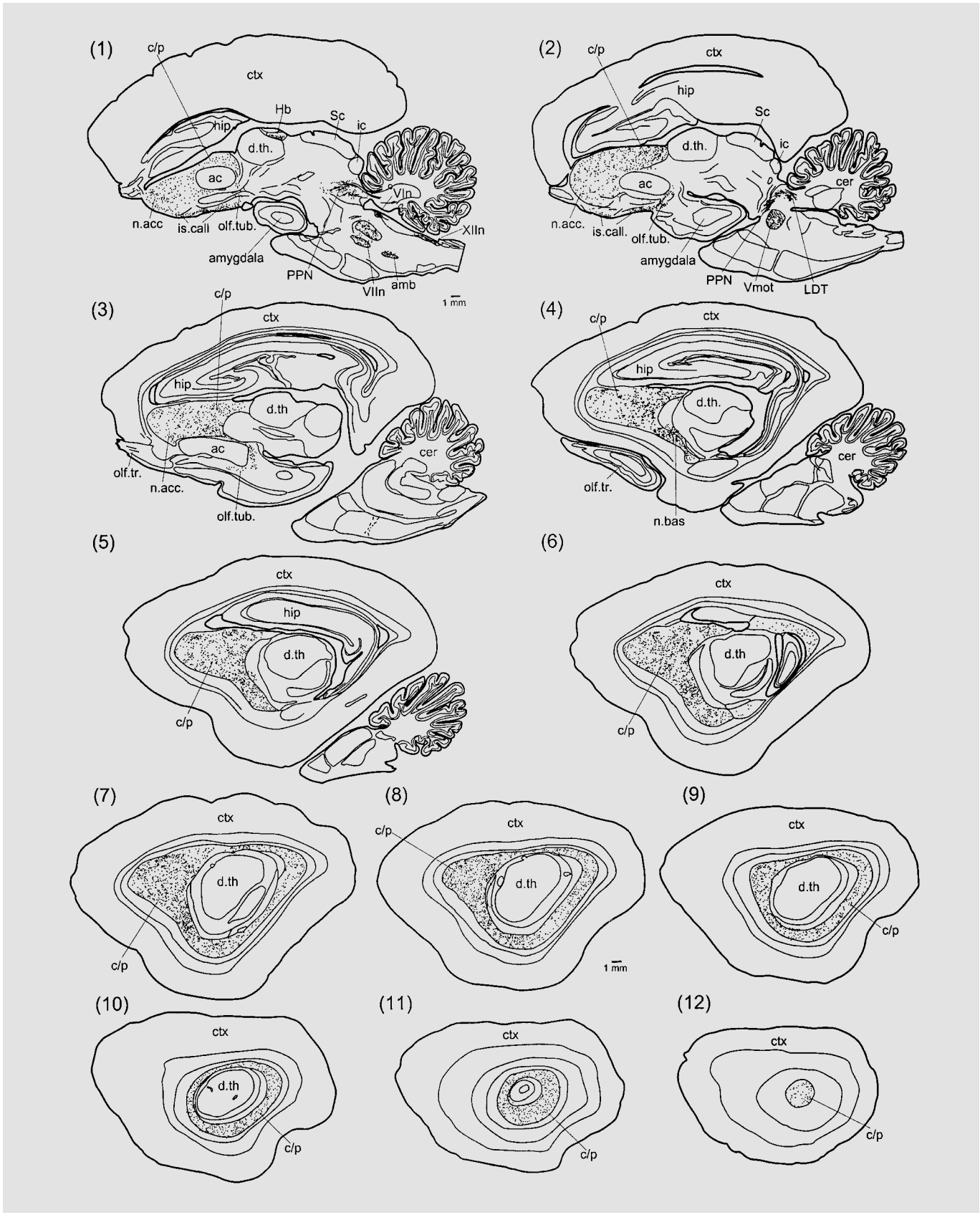
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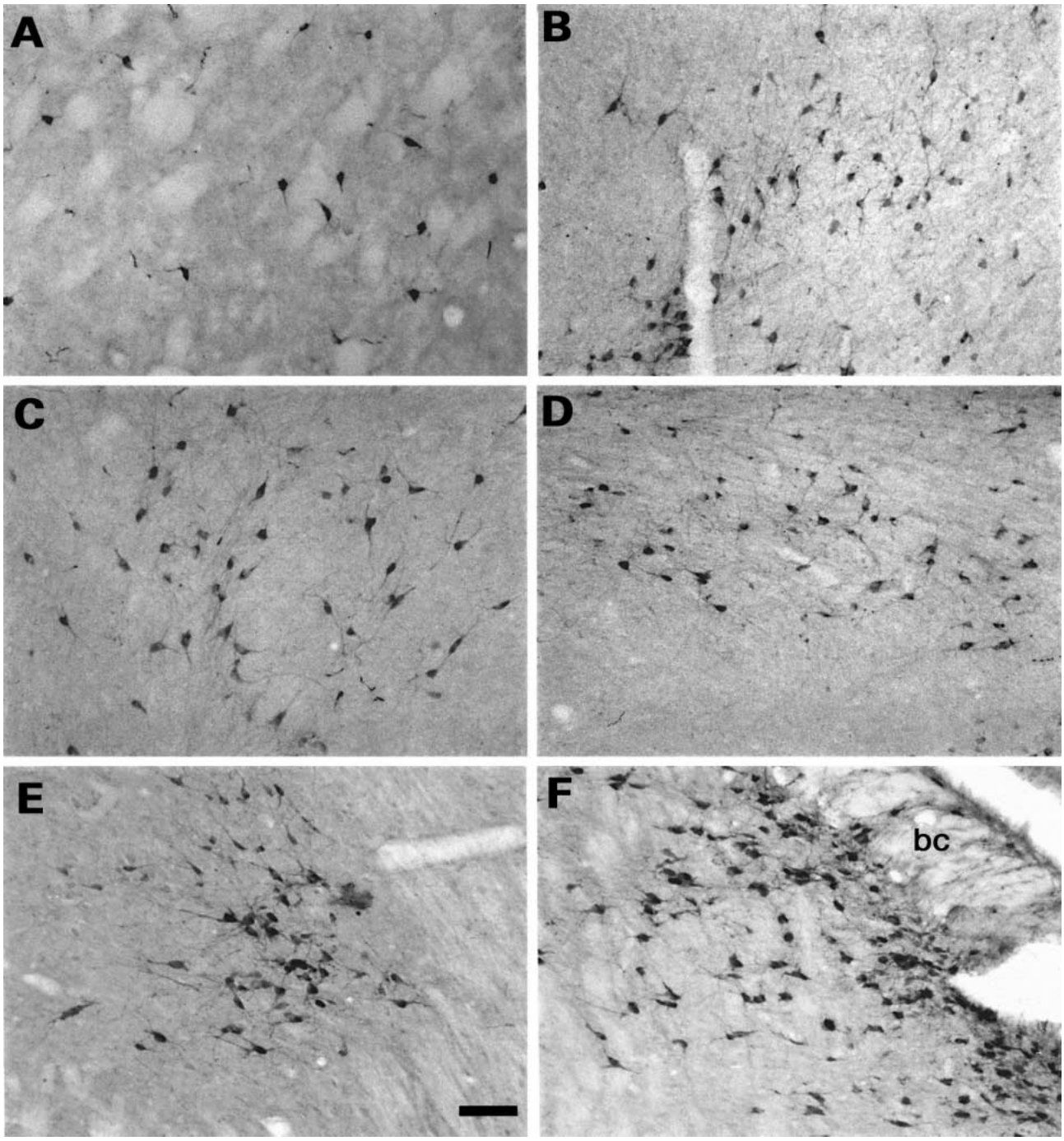

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Fig. 3. Diagrammatic series of drawings in the sagittal plane from the brain of the platypus, demonstrating the location of the cholinergic neurons. Each section is approximately 500 μm apart. The first drawing in the series is located closest to the midline. Note the hiatus created by the lack of hypothalamic cholinergic neurons. Numbered diagrams correspond to those presented in the accompanying papers [Manger et al., 2002a, b]. See list for abbreviations.

Fig. 4. Photomicrographs of selected groups of cholinergic neurons from the basal forebrain and pontomesencephalon of the platypus. A Interneurons of the caudate/putamen. B Cholinergic neurons from the nucleus accumbens. C Cholinergic neurons from the nucleus basalis, located between the globus pallidus and the anterior commissure. D Cholinergic neurons located in the medial septal nucleus by the ventricular wall. E Neurons of the lateral dorsal tegmental nucleus located in the lateral, ventral and posteriormost portion of the periaqueductal grey matter. F Neurons of the pedunculopontine nucleus in the tegmentum in close proximity to the brachium conjunctivum (bc). Scale bar = 100 μm (for all photomicrographs).

Distribution and Morphology of Basal Forebrain Cholinergic Neurons

Within the basal forebrain of mammals, three groups of cholinergic cells have been identified [Woolf, 1991]. These include: the medial septal nucleus and the vertical limb of the diagonal band nucleus; the horizontal diagonal band nucleus and magnocellular preoptic area; and nucleus basalis, substantia innominata and nucleus ansa lenticularis. Of these nuclei, only the medial septal nucleus and the nucleus basalis could be identified with any certainty in the brain of the monotremes.

Medial Septal Nucleus. The septum of the monotremes is easily delimited and has a topological location similar to that reported for all other tetrapods. The medial septal nucleus is readily identifiable within the medial and ventral half of the septum, and is separated ventrally from the nucleus accumbens by a small fiber tract (fig. 1: 2–5). The septum is bordered dorsally and posteriorly by the hippocampus and the hippocampal commissure. Cells reactive to ChAT immunohistochemistry are found throughout the medial septal nucleus and show an even and relatively dense distribution throughout the nucleus. The somatal bodies were irregularly rhomboidal shaped and had multiple dendrites which formed a plexus in the local environment (fig. 4D).

Nucleus basalis. The nucleus basalis of the monotremes is represented by a small but densely packed band of cholinergic neurons situated at the ventral border of the globus pallidus and the caudate-putamen complex, and are found lying just dorsal to the anterior commissure (fig. 1: 8–9; 3: 4). The cell bodies exhibited an elongated polygonal shape, with up to five primary dendrites (fig. 4C).

Distribution and Morphology of Diencephalic Cholinergic Neurons

Within the diencephalon of rat, cat and monkey, two sets of cholinergic neurons have been reported. These are cholinergic nuclei in the hypothalamus and the medial habenula [Woolf, 1991]. In the present study we could find no evidence of cholinergic neurons within the hypothalamus of either species of monotremes, however, a clearly definable medial habenula nucleus was identified.

Medial Habenula. The habenula of the monotremes can be divided into lateral and medial divisions, and is found close to the midline within the dorsal and caudal most portion of the thalamus, forming part of the floor of the third ventricle (fig. 1: 9–11; 2: 1–2, 3: 1). The habenula is found immediately dorsal to the medial nucleus of the dorsal thalamus. The medial habenula is closer to the midline than the lateral habenula and has an anteroposterior dimension of less than 2 mm. The ChAT-positive neurons within the medial habenula are uniformly and densely distributed throughout the entire nucleus. The soma of these cells are spherical in shape, and due to the density of cells, no clear indication of the pattern or number of dendrites could be established. Emerging from the ventral side of the habenula is the large ChAT-positive fasciculus, fasciculus retroflexus. The fasciculus retroflexus was seen to project ventrally and posteriorly from the medial habenula, coursing towards the ventral tegmentum, quite probably to terminate within the interpeduncular nucleus.

Distribution and Morphology of Pontomesencephalic Cholinergic Neurons

Cholinergic neurons were found throughout the antero-posterior extent of the pontomesencephalon of both monotremes, with an identical distribution in both species. The anterior limit of the cholinergic neurons in this region is coincident with the posterior end of the trochlear nucleus and the decussation of the brachium conjunctivum (or superior cerebellar peduncle). Cholinergic neurons are found 3.5 mm posterior to this level in the platypus and 4 mm posterior to this level in the echidna. In both species this posterior limit is coincident with the anterior limit of the trigeminal motor nucleus. The pontomesencephalic cholinergic group of neurons are usually divided into three groups, the pendunculopontine tegmental nucleus (PPN), the laterodorsal tegmental nucleus (LDT), and the parabigeminal nucleus [Woolf, 1991]. Only two of these nuclei, PPN and LDT, can be identified in both monotreme species. A distinct parabigeminal nucleus could not be identified.

Pedunculopontine Tegmental Nucleus (PPN). The cholinergic cells of PPN are associated with the brachium conjunctivum (fig. 1: 16–19; 2: 8–12; 3: 1–2). At the anterior portion of PPN the cholinergic cells are found dorsal to the decussation of the brachium conjunctivum. In more posterior sections, the brachium conjunctivum was found lateral and superior to the PPN. PPN was found at the same antero-posterior level, and intermingled with, the cells of the nucleus subcoeruleus [see Manger et al., 2002a]. The cholinergic cells in PPN exhibit a low, but consistent, density throughout the extent of the nucleus. The cholinergic cells of PPN appear to be more 'orderly' in the echidna, where they form a distinct arc that is closely associated with the medial boundary of the brachium conjunctivum. In contrast, the cholinergic cells of PPN in the platypus are more loosely associated with the bra-

chium conjunctivum and do not exhibit the distinct arc found in the echidna. The cell bodies were roughly triangular in shape and showed three primary dendrites emerging from each corner (fig. 4E).

Laterodorsal Tegmental Nucleus (LDT). The cholinergic cells of LDT are delineated ventrally and laterally by the peri-aqueductal gray matter (fig. 1: 18; 2: 11–14; 3: 2). The LDT is found in close association with the locus coeruleus. The cholinergic cells of the LDT are first observed as a continuation of the PPN cells within the periaqueductal grey matter. They then spread in their distribution to mingle with the cells of the locus coeruleus proper along its entire length. The density of distribution is comparable to that of PPN. The soma of the LDT cells were more ovoid in shape than those of PPN, and exhibited only two primary dendrites, however, there was no specific orientation of these primary dendrites (fig. 4F).

Medullary Tegmental Neurons

A loosely associated group of cholinergic neurons was located in the tegmentum of the medulla oblongata, as has been described in other mammalian species [Woolf, 1991]. These cells were few in number and varied in number and precise location among individuals. This loosely associated cell group was found approximately in the middle of the medullary tegmentum, at a level close to the middle of the hypoglossal nucleus (fig. 1: 24; 2: 27–28). The cells were similar in morphology and size to those of the nucleus ambiguus. They appear to form a continuation of the cholinergic column between the nucleus ambiguus and the ventral horn of the spinal cord.

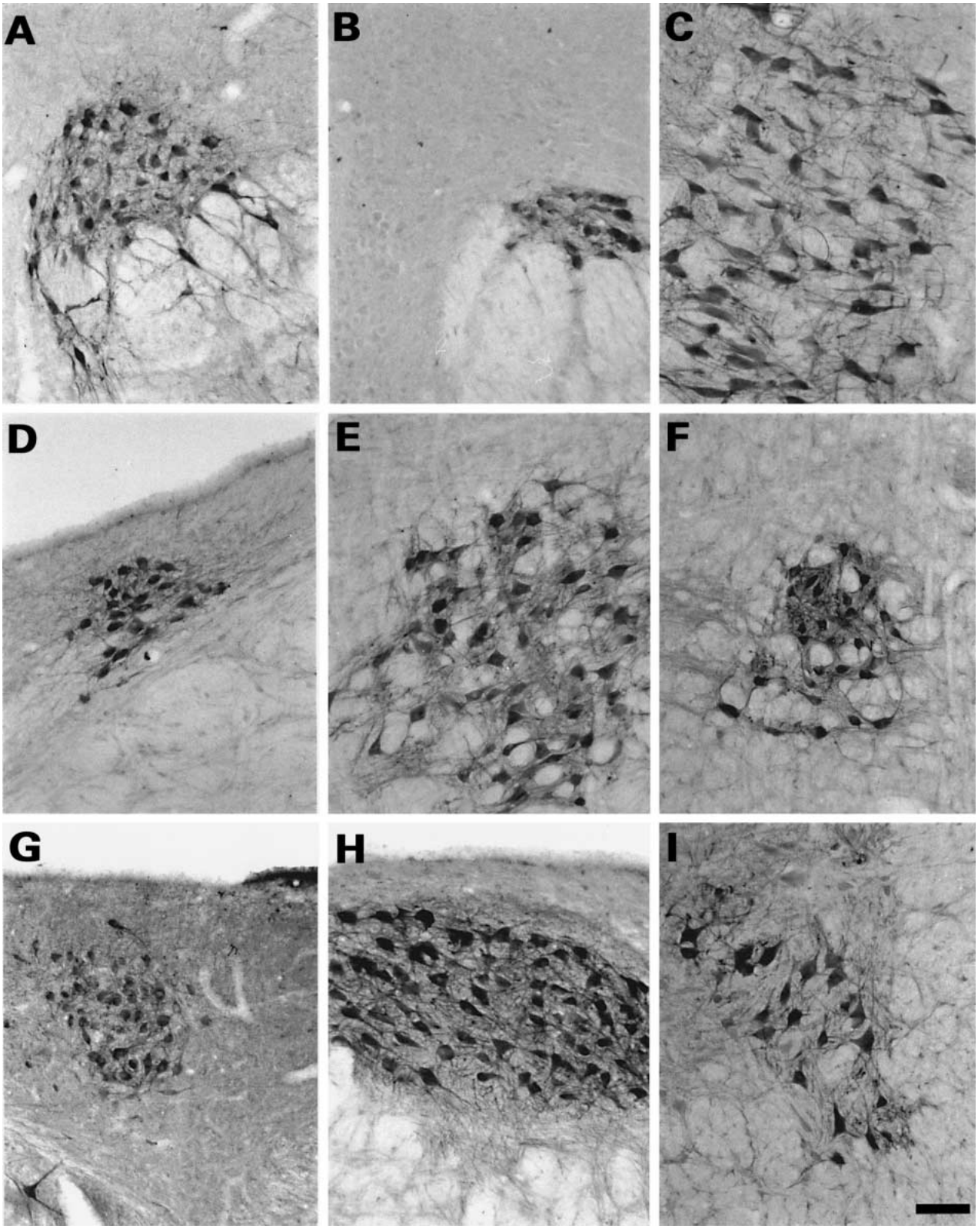
Distribution and Morphology of Cranial Nerve Nuclei and Spinal Cord Cholinergic Neurons

Many of the cranial nerve nuclei in the brainstem of both the platypus and echidna had cholinergic neurons within and closely surrounding the borders of the nuclei as defined with Nissl architecture. Nuclei that contained cholinergic motoneurons included: the oculomotor, or third cranial nerve nucleus; the trochlear, or fourth cranial nerve nucleus; the trigeminal motor, or fifth cranial nerve nucleus; the abducens, or sixth cranial nerve nucleus; the facial, or seventh cranial nerve nucleus; nucleus ambiguus; the dorsal motor vagus, or tenth cranial nerve nucleus; and the hypoglossal, or twelfth cranial nerve nucleus. The ventral horn of the spinal cord also contained cholinergic neurons.

Oculomotor Nucleus (CN III). The oculomotor nucleus of the monotremes is located within the ventral peri-aqueductal grey matter close to the midline (fig. 1: 14–15; 2: 4–7), anterior to the dorsal raphe, LDT and locus coeruleus, and has an antero-posterior dimension of approximately 2 mm. The cholinergic neurons were evenly dispersed throughout the nucleus. The posterior pole of this nucleus is coincident with the anterior pole of the trochlear nucleus. In the present description we have not subdivided the oculomotor nucleus into its various parts, as Hines [1929] has given a full description of the subdivisions of the oculomotor nucleus in the platypus, and Abbie [1934] has done so for the echidna. The somatal bodies are polygonal in shape and show two large primary dendrites, located on either side of the soma (fig. 5A). The dendrites are oriented in two directions, one away from the midline towards the third nerve, and the second oriented towards the midline.

Trochlear Nucleus (CN IV). The trochlear nucleus is located immediately posterior to the oculomotor nucleus, shows the same topological relationships, and has an antero-posterior dimension of approximately 1.5 mm (fig. 1: 17; 2: 8–9). The trochlear nucleus contained a density of cholinergic neurons similar to the oculomotor nucleus. The posterior pole of the trochlear nucleus is coincident with the anterior pole of the ventral median division of the dorsal raphe nucleus. The morphology of the cholinergic motor neurons is similar to that of the oculomotor nucleus (fig. 5B). There is a gap of around 3 mm between the posterior pole of the trochlear nucleus and the anterior pole of trigeminal motor nucleus, representing a break in the continuity of the cholinergic motor neuron group in the brainstem. This discontinuity is filled, however, by the cholinergic neurons of the pontomesencephalon (see above description).

Trigeminal Motor Nucleus (CN V). This nucleus is hypertrophied in both species of monotremes [Hines, 1929; Abbie, 1934], and is located in the lateral tegmentum of the brainstem, its anterior pole being coincident with the posterior most cells of PPN and nucleus subcoeruleus (fig. 1: 17–18; 2: 12–13; 3: 2). It has an antero-posterior dimension of approximately 1.5 mm, and its posterior pole is coincident with the anterior pole of the facial nucleus. The density of cholinergic cells is similar to that for the oculomotor and trochlear nuclei but it is lower than that of the facial nucleus. The cholinergic cells show a uniform distribution throughout the nucleus. The soma exhibited a triangular shape with three primary dendrites, one from each corner of the triangle (fig. 5C). There was some specificity in the orientation of one of the dendrites, which appeared to be directed towards the fifth motor nerve. The other two dendrites appeared to form local plexuses close to the cell of origin.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Abducens Nucleus (CN VI). In both monotremes the abducens nucleus is smaller than the other cranial nerve nuclei. It is located on the dorsal surface of the brainstem, in apposition to the floor of the fourth ventricle, dorsal to the facial nerve nucleus (fig. 2: 15–17; 3: 1). It has dimensions of less than 1 mm in all directions. The cholinergic neurons are uniformly distributed throughout the nucleus. The soma of these cells exhibited a polygonal shape, normally with three primary dendrites (fig. 5D). One dendrite appeared to be oriented toward the nerve associated with this nucleus, the others forming a plexus close to the cells of origin.

Facial Nucleus (CN VII). In the monotremes the facial nucleus is hypertrophied in comparison to other mammals. In the platypus this nucleus shows three divisions, a ventral division and two dorsal divisions, whereas the echidna has only a single dorsal division and single ventral division (fig. 1: 19; 2: 13–20; 3: 1). Cholinergic motor neurons are found uniformly distributed throughout the extent of the nucleus. The facial nucleus is located closer to the midline than the trigeminal motor nucleus and has an antero-posterior dimension of approximately 2 mm. The posterior pole of this nucleus is coincident with the anterior pole of nucleus ambiguus. The somatal shape is polygonal and there are between three and five primary dendrites (fig. 5E). These dendrites appear to form a dense plexus within the nuclear divisions. There does appear to be some dendritic communication between the subdivisions, but only from cells located closest to the boundaries of the divisions.

Nucleus ambiguus. Nucleus ambiguus appears as a caudal extension of the hypertrophied ventral division of the facial nucleus, has an antero-posterior dimension of approximately 3 mm and is found immediately dorsal to the fifth arcuate nucleus along its extent (fig. 1: 20–23; 2: 21–24; 3: 1). The shape of the soma closely approximated that of a diamond and from each corner a large primary dendrite was seen to emerge (fig. 5F). The primary dendrites were oriented away from the cell body and formed both dense plexuses within the nucleus as well as projecting dorsally and ventrally into the surrounding tegmentum.

Fig. 5. Photomicrographs of cholinergic motor neurons from the brainstem of the platypus. A Oculomotor nucleus. B Trochlear nucleus. C Motor trigeminal nucleus. D Abducens nucleus. E Facial nucleus. F Nucleus ambiguus. G Dorsal motor vagus nucleus. H Hypoglossal nucleus. I Ventral horn. Scale bar = 100 μm (for all photomicrographs).

Dorsal Motor Vagal Nucleus (CN X). The dorsal motor vagus nucleus is located on the dorsal surface of the brainstem. Its anterior pole is coincident with the posterior pole of nucleus ambiguus and has an antero-posterior dimension of 4 mm (fig. 1: 22–26; 2: 22–25). Its caudal pole is coincident with the most rostral portion of the cervical spinal cord. It is located laterally to another cholinergic nucleus, the hypoglossal nucleus (see below). The cells are uniformly distributed throughout the nucleus and are numerous. The cells of this nucleus are the smallest of the cholinergic motor neurons in the monotremes and stain slightly lighter than the cells of other cranial nerve nuclei. The somas are almost round in shape and have one or two primary dendrites emerging from the dorsalmost point (fig. 5G). These dendrites project dorsally and do not seem to form plexuses within the nucleus.

Hypoglossal Nucleus (CN XII). The hypoglossal nucleus is located medial to the dorsal motor vagus nucleus and shows a similar antero-posterior dimension. This nucleus is approximately twice the size, in its other dimensions, of the dorsal motor vagus nucleus (fig. 1: 23–26; 2: 22–30; 3: 1). The cholinergic motor neurons of this nucleus are evenly distributed throughout the nucleus. The shape of the somas was polygonal, with two or three primary dendrites emerging from corners of the somatal body (fig. 5H). The majority of these primary dendrites formed plexuses within the nucleus, but several dendrites were found to be oriented towards the descending nerve root of this nucleus and formed plexuses in the tegmentum.

Ventral Horn of the Spinal Cord. Small clusters of three to five cholinergic neurons were found in the ventral horn of the spinal cord in both monotremes. Cholinergic neurons were found in the ventral horn in each part of the spinal cord examined. However, we only examined the rostralmost portion of the upper cervical spinal cord (fig. 1: 26–27; 2: 30–37). These cholinergic motor neurons of the spinal cord were the largest of the cholinergic neurons found in the monotreme central nervous system. The somas were polygonal in shape and had four to five primary dendrites emerging from the corners of the somatal body (fig. 5I). These dendrites showed no particular specificity in their orientation and formed a moderate plexus within the ventral horn. Some of the dendrites extended into the surrounding white matter forming a matrix around ascending fiber bundles.

Somatal Areas of Cholinergic Neurons

The average somatal area of cholinergic neurons is given in table 1 and presented graphically in figure 6. The

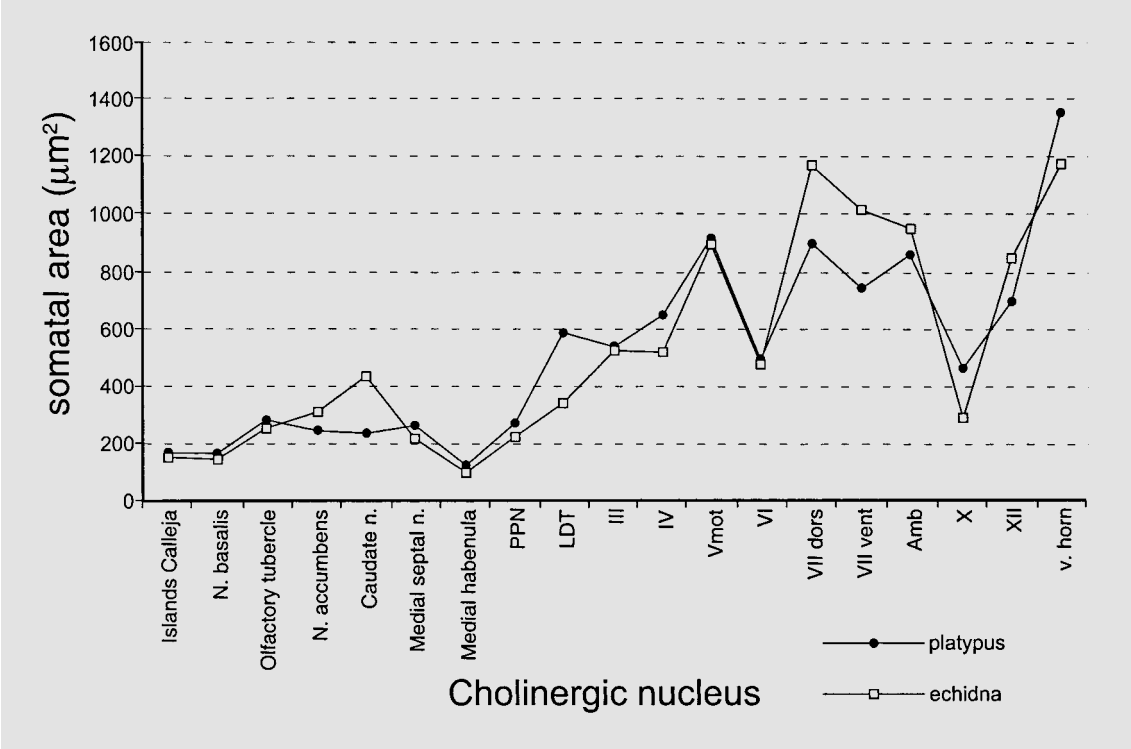

Fig. 6. Graphical representation of the data on somatal areas of monotreme cholinergic neurons given in table 1. Despite the almost threefold difference in brain size between platypus and echidna, the somatal areas of the cholinergic nuclei show a great deal of overlap. One notable difference is in the size of the neurons of the facial nucleus of the echidna which are larger than those of the platypus.

largest cholinergic neurons in both species were the motor neurons of the ventral horn. In the platypus these cells had an average area of approximately 1,360 µm^2, and in the echidna an average of 1,182 µm^2. The smallest cholinergic cells were those of the medial habenula (platypus – 122 µm^2; echidna – 98 µm^2). The graphical representation of the somatal areas of the echidna and platypus demonstrates that there is virtually no difference in the somatal areas of the cholinergic cells between these species (fig. 6), despite an almost threefold difference in brain and body weights [platypus = 9.2 g, 1,040 g; echidna = 27.5 g, 4,720 g; Pirlot and Nelson, 1978]. However, the cells of the facial nucleus of the echidna are substantially larger than those of the platypus.

Within table 1 the results from previous studies in other species providing similar measurements (with similar non-stereological techniques as that used in the present study) are given for comparison. Despite previous suggestions of a correlation between brain size and cholinergic somatal area [Woolf, 1991], it is clear that no such allometric relationship is evident.

Discussion

The present study details the distribution and morphological characteristics of cholinergic cells in the brain of two species of monotreme, the platypus *(Ornithorhynchus anatinus)* and the echidna *(Tachyglossus aculeatus)*, as determined by ChAT immunohistochemistry. The impetus for the present study, and the two accompanying studies on the serotonergic and catecholaminergic cells of the monotremes [see Manger et al., 2002a, b] is derived from observations regarding the physiological phenomenology of sleep in the monotremes. The most inexplicable finding of the studies of monotreme sleep is the presence of slow waves in the EEG whilst the animal is exhibiting the brainstem correlates of REM sleep [Siegel et al., 1996, 1998, 1999; Siegel, 1999]. The cholinergic system has been implicated as a major determinant in the cortical EEG pattern during the sleep-wake cycle [Woolf, 1991; Steriade et al., 1993; Braun et al., 1997; Siegel, 2000]. Thus, the present investigation of the cholinergic system in monotremes might provide clues regarding the unique

sleep pattern exhibited in these most unusual mammalian species.

Comparison of the Cholinergic System to Other Mammalian Species

Certain cholinergic cell groups described for typical laboratory animals [Woolf, 1991] are absent in the monotremes. These include cells in the cerebral cortex, and nuclei in the basal forebrain, diencephalon, and pontomesencephalon. The nuclei which were lacking include: the vertical diagonal band nucleus, the horizontal diagonal band nucleus, the magnocellular preoptic area, the substantia innominata, nucleus ansa lenticularis, the hypothalamic nuclei, and the parabigeminal nucleus.

In the only detailed anatomical account of the monotreme telencephalon, Hines [1929] describes the nuclei of the diagonal band as 'scattered polymorphous pyramidal cells which appear lateral to the fibers of the medial forebrain bundle ... and to the nucleus of the subcommissural region ... and anterior to the optic commissure.' Hines does not identify a magnocellular preoptic area. Despite using this description, careful observations of this area did not allow us to identify these nuclei.

Within the diencephalon of the common laboratory mammals, several groups of cholinergic cells have been identified in the hypothalamus [Woolf, 1991]. No ChAT-positive cells were found in the hypothalamus of either species of monotreme despite an intensely stained fasciculus retroflexus in this region. Within the pontomesencephalon, no clear parabigeminal nucleus could be identified. However, in all other aspects the cholinergic system of the monotremes appeared similar to that reported for eutherian mammals.

Comparison of the Monotreme Cholinergic System to Other Tetrapods

Three basic subdivisions of the striatal cholinergic interneurons are described across all tetrapods. These consist of the striatum (caudate-putamen), nucleus accumbens and the olfactory tubercle [Medina et al., 1993; Medina and Reiner, 1994; Marin et al., 1997]. In this comparison the monotremes are equivalent to other tetrapod species. Within the basal forebrain there is a degree of variability in the precise distribution of cholinergic cells across species. Briefly, those cells of the medial septal nucleus and diagonal band are similar in anuran amphibians, non-monotreme mammals, turtles and crocodiles [Mufson et al., 1984; Brauth et al., 1985; Woolf, 1991; Powers and Reiner, 1993; Marin et al., 1997]. In birds, lizards and urodele amphibians these cells are limited to the diagonal band [Medina et al., 1993; Medina and Reiner, 1994; Marin et al., 1997]. In monotremes the diagonal band nuclei are not identifiable [present study; Hines, 1929] but a clear medial septal nucleus is found. Thus, the basal forebrain nuclei of the monotremes appears different from those of other tetrapod species.

As mentioned above, one major distinction between monotremes and other mammalian species is the lack of cholinergic neurons in the hypothalamus. In all other tetrapod species cholinergic cells have been demonstrated in the supraoptic and infundibular hypothalamus [Mason et al., 1983; Ekstrom, 1987; Tago et al., 1987; Medina et al., 1993; Powers and Reiner, 1993; Medina and Reiner, 1994; Marin et al., 1997]. Thus, among the tetrapods monotremes appear to be the only species lacking hypothalamic cholinergic neurons. In common with all other tetrapods, the monotremes have habenular cholinergic neurons with the associated cholinergic fasciculus retroflexus.

The nucleus isthmus of non-mammalian vertebrates is considered homologous to the parabigeminal nucleus of mammals, due to the shared features of ChAT-positive reactivity and a projection to the optic tectum. The nucleus isthmus has been identified in amphibians [Marin et al., 1997], reptiles [Brauth et al., 1985; Medina et al., 1993] and birds [Medina and Reiner, 1994], and the parabigeminal nucleus has been identified in all mammalian species [Woolf, 1991], except for the monotremes [present study]. Thus, among the tetrapods, monotremes appear to be the only species lacking a parabigeminal nucleus (or nucleus isthmus). Within the pontomesencephalon of all tetrapods species (see above references), the lateral dorsal tegmental and pedunculopontine nuclei (or homologous cell groups) have been described. The monotremes do not differ from the rest of the tetrapods in this respect. Similarly, in all tetrapod species the motor neurons of the cranial nerve nuclei and spinal cord have been identified as cholinergic. The situation is the same for monotremes.

In summary, monotremes exhibit a majority of the features of tetrapod cholinergic systems. There are three significant exceptions: the monotremes appear to lack the cholinergic cell groups in the cerebral cortex (or its homologue), hypothalamus and also the parabigeminal nucleus (or nucleus isthmus). These differences are discussed in more detail below.

Lack of Cholinergic Cell Groups in Monotremes

The above comparison shows that some cholinergic cell groups are missing from the monotremes in comparison to those reported for all other tetrapods, in particular

those in the cerebral cortex, parabigeminal nucleus and hypothalamic nuclei. This raises an initial concern that in the present study we might have failed to identify these cells groups due to technical problems.

Cholinergic cells have been described with the use of a monoclonal antibody in the cat [Woolf, 1991], and with polyclonal antibodies in the rat, monkey, human [Tago et al., 1987], and several other species [e.g., Marin et al., 1997]. In the present study we employed the polyclonal antibody (goat anti-ChAT antibody from Chemicon International Inc., Temecula, Calif.). This particular polyclonal antibody has been used for many studies that map the distribution of cholinergic cells in vertebrate brains [e.g., Marin et al., 1997]. Thus, our selection of reagents used in the present study is in accordance with several other published reports in mammals and other vertebrates. Our protocol might have been refined by the use of colchicine treatment and monoclonal antibodies, but several previous studies have noted no significant differences in the revelation of ChAT-positive neurons using these additional treatments [e.g., Reiner, 1991]. Thus, we must assume for the present that the cell groups we failed to identify are indeed lacking in the monotremes.

The lack of cholinergic cells in the neocortex of monotremes is a feature that might be significant in the face of theories regarding the evolution of mammalian neocortex. Reiner [1991] has demonstrated that cholinergic cells are generally found in the supragranular layers of neocortex but most reports indicate a lack of these cells in the dorsal cortex of reptiles, the presumed homologue of mammalian neocortex [Brauth et al., 1985; Hoogland and Vermeulen-Vander-Zee, 1990; Reiner, 1991]. From this and many other anatomical observations, Reiner [1991] has concluded that cortical layers II–IV are a newly evolved feature with no homologue or predecessor in reptilian cortex. However, there is a single report of ChAT-positive neurons in the dorsal cortex of reptiles [Medina et al., 1993]. In the Medina et al. study, two antibodies were used, but only one revealed cholinergic neurons in the dorsal cortex. This indicates that it is possible that the results of the present study, and those of others which did not identify cortical cholinergic neurons, might in fact be false negatives. Only by re-examining monotreme cortical tissue with a variety of ChAT antibodies will the results of the present study be validated or negated. However, if the results of the present study do withstand further scrutiny, then we might conclude that cortical cholinergic neurons evolved after the divergence of monotremes from other mammals, and might play no part in the evolution of increased cortical lamination.

In most mammals the parabigeminal nucleus stains lightly for ChAT immunohistochemistry and has a strong projection to the superficial layers of the superior colliculus. Monotremes have a reduced visual system [Krubitzer et al., 1995], and the lateral geniculate nuclei of the thalamus are greatly reduced and appear to have an almost reptilian form [Hines, 1929; Jones, 1985]. In comparison, the visual system of the majority of other mammals is enhanced (the parabigeminal nucleus has not be examined in anophthalmic mammals), and in other vertebrates the superior colliculus (or optic tectum) is highly developed [Butler and Hodos, 1996]. Thus, it is not unreasonable to assume that the monotremes might have lost this cell group. Furthermore, the cholinergic projection to the superior colliculus of the monotremes may be compensated for by the pedunculopontine nucleus, which has substantial projections to the superior colliculus even in other more visually biased mammals [Woolf, 1991].

The hypothalamic cholinergic nuclei have been identified in all previously studied tetrapods [e.g., Marin et al., 1997], thus, one might presume their presence in the monotremes. In the present study we did identify strongly ChAT-positive somas in the medial habenular nucleus, and also the strongly ChAT-positive fibers of the fasciculus retroflexus. Both of these structures are found close to the region where one would have expected to find cholinergic hypothalamic cells. As these structures were readily identifiable, we must assume that there was no methodological problems precluding the visualization of other ChAT-positive structures in this region of the brain.

The Global Cholinergic System

Woolf [1991] has proposed that the cholinergic neurons of the central nervous system are organized in a global fashion, in contrast to the discrete organization of the sensory systems. This idea gains support from the dendritic interconnectedness of all the cholinergic subsystems, plus the ubiquitous cholinergic innervation of the brain, and allows for an interpretation of the overall physiological action that the cholinergic system effects on the brain. This proposal appears to hold across all tetrapod species studied [e.g., Marin et al., 1997].

The main anatomical feature of this global cholinergic system is that in the rat, cat and monkey, the cholinergic subsystems form an interconnected, continuous aggregation of neurons from the spinal cord to the anterior forebrain. A lack of a particular cholinergic subsystem would create a gap in what we might otherwise have expected to be a continuous cell aggregation (fig. 7). In the monotremes such a gap is created due to the lack of hypotha-

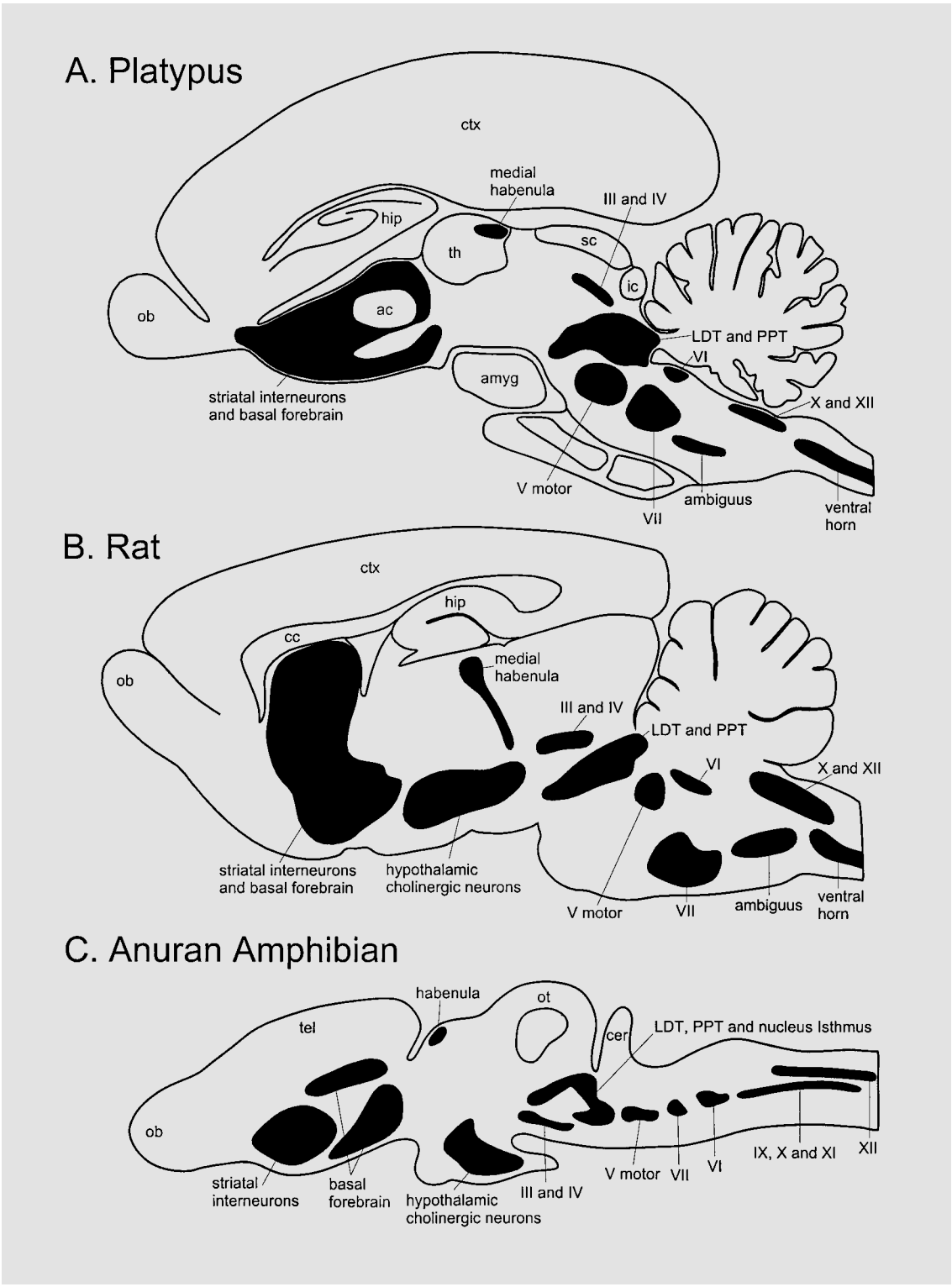

Fig. 7. Diagrammatic sagittal sections showing the cholinergic cell groups from three vertebrates, a platypus (A) (present study), a rat (B) [re-drawn from Woolf, 1991], and an anuran amphibian (C) [re-drawn from Marin et al., 1997]. In both the rat and amphibian the continuous aggregation of cholinergic cells, linked at the edges by the distal dendrites [Woolf, 1991], can be easily seen. The platypus, however, clearly shows a hiatus in this antero-posterior aggregation, due to the lack of hypothalamic cholinergic neurons.

lamic cholinergic nuclei where a substantial hiatus, approximately 5 mm, is produced between the pontomesencephalic subsystem and that of the basal forebrain. Within this gap are the cholinergic cells of the medial habenula and the fibers of the fasciculus retroflexus, but given the specific nature of the axonal projection and trajectory of the cells of this nucleus, it is unlikely that they will function to bridge this gap. It is possible that the diffuse nature of the axonal projections of the pontomesencephalic cholinergic neurons might effect a closure of this hiatus. However, this potential discontinuity in global interconnectedness of the cholinergic system dendrites is something that sets the monotremes apart from other mammals and other tetrapods studied to date.

Evolutionary Trends in the Cholinergic System

In a review of the cholinergic system of mammals, Woolf [1991] describes a relationship between somatal areas of cholinergic cells and size of the brain. Briefly, Woolf found an almost direct relationship between the average somatal area of a cholinergic neuron with the measurement of greatest brain width. The present study allows a wider comparison of this trend across a variety of species (table 1). In the majority of cases, the predicted value of cholinergic somatal area that is correlated with brain width was not found. It is possible that other measurements of the cholinergic system might show a better correlation. For example, as the pontomesencephalic neurons have been shown to globally innervate the brain, it is possible that there might be a correlation between the number of cells in this cholinergic subsystem and total brain weight. A second possibility might be that the number of cells in the basal forebrain might be related to indices of cortical surface area or volume. However, a full analysis of these allometric proposals is beyond the scope of the present study.

Of interest in the global sense of vertebrate brain evolution is the segmental approach to understanding the brain [Puelles, 1995]. This paradigm suggests that adult vertebrate brains should be considered segmented in relation to the developmental pattern of these brains. Marin et al. [1997], among others, have shown that this approach is useful in understanding the organization of cholinergic cell groups in the brain. In the series of sagittal brain sections presented in this study (fig. 3), the segmental appearance of cholinergic cell groups becomes quite visible. This is compared to another mammal and an amphibian (fig. 7), which highlights the cholinergic structures across species and makes the determination of homologies more apparent, and also shows the hypothalamic hiatus present in the monotremes. A complete discussion of the usefulness of this approach for determining brain homologies has been given elsewhere [Puelles, 1995; Marin et al., 1997]. Of particular interest to the present study and to sleep research in general is the observation that the pontomesencephalic cholinergic nuclei lie within a single developmental segment of the brain – rhombomere 1. Interestingly, this rhombomere also contains the locus and subcoeruleus and the majority of the dorsal raphe nuclei [see Manger et al., 2002a, b]. Furthermore, it is this region of the brain that has been shown to be critical for the generation of REM sleep [reviewed in Siegel, 2000].

In the present study we describe a complete absence of cholinergic cells in the hypothalamus and neocortex, which are located in the prosomeric developmental segment p5, along with the lateral and medial ganglionic eminences, anterior entopeduncular area and the tuberal region, in all of which we could not identify cholinergic neurons. Thus, two of the significant differences found in the cholinergic system of monotremes may be based in an altered developmental profile of the fifth prosomere.

The Cholinergic System and Sleep in the Monotremes

The monotremes have a sleep pattern that sets them apart from all other mammals studied so far. Recent reports on two species of monotremes, the echidna and platypus [Siegel et al., 1996, 1998, 1999; Siegel, 1999], have detailed the neural, physiological and behavioral aspects of monotreme sleep. The monotremes exhibit high voltage EEG during phasic activation of brainstem reticulo-motor systems. Thus, the forebrain of the monotremes shows the EEG of non-REM sleep, whereas the brainstem and behavioral measures of monotreme sleep appear to be consistent with REM sleep. In all other mammals studied, EEG desynchronization is exhibited during phasic activation of the brainstem in REM sleep. Nicol et al. [2000] have reported REM sleep with low voltage EEG in the echidna. However, their claim of REM sleep with low voltage EEG was not convincing because no arousal threshold measurements or behavioral observations, to distinguish sleep from waking, were carried out. Prior work using arousal measures (evoked response tests and response to deprivation) concluded that all states with low voltage EEG were waking [Allison et al., 1972]. Thus, the forebrain of monotremes shows the EEG of slow wave sleep, whereas the brainstem and behavioral measures of monotreme sleep appear to be consistent with REM sleep.

The combined elements of sleep in monotremes has no equivalent when compared to adult sleep in other mam-

malian species. Two examples that exhibit the combination of sleep elements described for adult monotremes have been reported: (1) studies of midbrain transections on sleep patterns, and (2) studies of juvenile mammal sleep. The specific transection studies that exhibit similar features to adult monotreme sleep are those that transect the brainstem just rostral to the pontomesencephalic cholinergic cell groups (at the anterior border of the first rhombomere). In these cases, during periods of sleep the forebrain generates a sleep-like state characterized by periods of high voltage EEG, independent of the brainstem, that shows periods of phasic activity similar to that seen in REM sleep [Jouvet, 1962; Siegel, 2000]. Moreover, lesions of the rostral brainstem cholinergic cell groups in adult mammals [McGinty, 1969], or blockade of all cholinergic cell groups by atropine administration [Szymusiak et al., 1994], produces periods of REM sleep with high voltage EEG [Shoham and Teitelbaum, 1982]. Secondly, juvenile mammals show a similar pattern of brain activity during sleep, i.e., high voltage EEG and phasic brainstem activity [McGinty et al., 1977; Frank and Heller, 1997; Siegel, 1999]. In the developing mammal this combination of elements disappears as the animal matures, for example in rats at 30 post-natal days [Frank and Heller, 1997].

In the present study we have shown that there is a hiatus in the continuous cell aggregation of the cholinergic system that occurs at the level of the hypothalamus. This splits the cholinergic system into two dendritically interconnected systems, one in the telencephalon and one in the brainstem, as opposed to a global system in other mammals and tetrapods. That this hiatus might be the basis for the high voltage EEG during phasic brainstem activation in monotreme REM sleep gains support from the recent PET imaging studies of human sleep which suggest that the ventral, or cholinergic, portion of the ascending reticular system is the major pathway for cortical arousal during REM sleep [Braun et al., 1997]. However, monotreme sleep phenomenology might also result from an order specific difference in the cholinergic circuitry such as a lack of innervation from PPN to the basal ganglia.

Four conclusions can be drawn from this series of observations. First, the combination of sleep elements in monotremes might be the result of the hiatus in the continuous dendritic interconnectedness of cholinergic neurons. Second, the transection studies that give the same combination of sleep elements as that seen in monotremes might be, to some extent, the result of splitting the cholinergic column into two smaller columns similar to that seen in monotremes. Third, a maturation of dendritic interconnections of the cholinergic system might be a causal factor in the changing combination of sleep elements as the juvenile mammal matures. Finally, hypothalamic cholinergic nuclei might play a role in the differentiated, two-stage pattern of sleep alternation (SWS and REM) that characterizes the cerebral hemispheres of eutherians, as monotremes lack both the hypothalamic cholinergic nuclei and two-stage sleep.

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